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Short- and mid-term effects on performance, health and qualitative behavioural assessment of Romane lambs in different milk feeding conditions

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ABSTRACT

The common practice of artificially rearing lambs from prolific meat breeds of sheep constitutes a welfare issue due to increased mortality rates and negative health issues. In this multidisciplinary study, we investigated the possible short- and mid-term advantages of artificially feeding fresh ewe's milk instead of commercial milk replacer on lambs' growth, health and welfare. Romane lambs were either separated from their mothers on D3 and fed with Lacaune ewes' milk (**LAC**, $n = 13$) or milk replacer (**REP**, n = 15), or they were reared by their mothers (**MOT**, $n = 15$). On D45, they were weaned, gathered in single-sex groups until the end of the study on D150. Lamb performance and biomarkers of overall health were assessed by measuring: growth, dirtiness of the perianal area, enteric pathogens in the faeces, total antioxidant status and redox status assessed by plasma reduced glutathione/oxidised glutathione ratio, and immune response after vaccination against chlamydiosis. As an exploratory approach, blood cell transcriptomic profiles were also investigated. Last, qualitative behaviour assessment (**QBA**) was performed as an integrated welfare criterion. Lacaune ewes' milk and REP never differed in their average daily gain but grew less than MOT lambs in the early suckling period and just after weaning. No effect was detected afterwards. On D30, LAC and REP lambs had lower total antioxidant and higher redox status than MOT lambs but did not differ among themselves. Lacaune ewes' milk and MOT had a cleaner perianal area than REP lambs on D21, while faecal pathogen infection did not vary between the treatment groups. After vaccination, LAC also had a stronger immune response on D90 compared to REP lambs. Transcriptome analysis performed on D150 showed differential gene expression, mainly in relation to inflammatory, immune and cell cycle response, between male lambs of the LAC group and those of the MOT and REP groups. Based on QBA, LAC lambs never differed from MOT lambs in their general activity and varied from REP only on D21; REP lambs were always more agitated than MOT lambs. In conclusion, artificial milk feeding impaired early growth rate, health and emotional state mainly during the milk feeding period and at weaning. Feeding artificially reared lambs with fresh ewe's milk partly mitigated some of the negative effects induced by milk replacer but without achieving the full benefit of being reared by the mother.

Implications

In sheep production, artificial rearing is often associated with welfare and health issues and increased mortality during the suckling period. The use of dairy ewes' milk, instead of milk replacer, was tested as a way to limit these problems. We found that feeding lambs with ewes' milk partly mitigated the negative effects of milk replacer without equaling the beneficial effects of nursing. Providing waste milk from dairy breeds that is unsuitable for human consumption could be a beneficial practice in regions where dairy and meat breeds are reared in proximity.

Introduction

Reproductive efficiency in sheep has an important economic impact on meat farming systems and leads to the development of prolific breeds. Selection on of reproductive traits was successful because the

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ovulation rate and litter size are affected by fecundity genes (Abdoli et al., 2016). For instance, successive crosses between Romanov and 'Berrichon du Cher' breeds gave birth to the Romane breed with an average prolificacy of 2 to 2.3 lambs/ewe (Ricordeau et al., 1990). The main drawback is that with large litters ewes do not have enough milk to nurse all their lambs until weaning. Separating new-borns from their mothers and feeding them with a milk replacer is a common procedure. A growing concern with artificial feeding is that the mortality rate in lambs is higher compared to those fed by their mothers and may reach up to 50% (Boivin et al., 2017). This is unlikely to reflect the failure of passive immunity since lambs are usually removed from their mothers within 1–3 days after birth once they have ingested maternal colostrum (David et al., 2014).

Maternal deprivation produces detrimental psychological effects on young lambs (Napolitano et al., 2008; Gaudin et al., 2018) and a nutritional challenge due to artificial feeding. Artificially reared lambs receive a markedly differing diet from maternal milk since the milk replacer contains various non-dairy products (e.g. palm, copra or rapeseed oil, but also maze starch and wheat gluten) in addition to whey and low-fat milk powder. Compared to maternal nursing, artificial rearing leads to poorer growing performance as well as to altered health and gut microbial colonisation (Napolitano et al., 2008; Belanche et al., 2019b). Gradual maternal separation (Sevi et al., 2003) or smooth transition from maternal to artificial milk by mixing them progressively (Sevi et al., 1999) reduces the impact of artificial rearing on behavioural, endocrine and immune responses in Comisana lambs, a dairy breed. Such studies have never been performed on lambs from meat breeds, with one reason being the difficulty to get fresh milk from such breeds. These studies have only focused on the pre-weaning period, known to be critical in terms of health (Napolitano et al., 2008), with the longer-term consequences being largely unexplored. Only recently did Belanche et al. (2019b) investigated the long-term effects of artificial rearing practices in Aberdale sheep, a British meat breed. They found that lambs reared by their mothers had a larger average daily gain (**ADG**) and carcass weight at slaughter than artificially fed lambs. The authors concluded that 'direct contact with the ewe provided a competitive advantage in naturally reared lamb' but at no moment did they question the role of milk replacer composition. However, it is important to also investigate the influence of milk replacer on artificially fed lambs.

Improving artificially fed lambs milk-based diet can be achieved through two strategies. First is to identify which nutritional and bioactive components of ewe's milk are necessary for the lamb's development and health. This knowledge could then be used to improve the milk replacer composition. However, such a strategy would be very time-consuming. Second is to feed artificially lambs with ewe's milk. This is actually recommended by the Ontario Ministry of Agriculture, Food and Rural Affairs (Ontario Ministry of Agriculture, 2010). They recommend using waste milk from dairy breeds that is not suitable for human consumption. This could become a practice in areas where dairy and meat breeds are both reared. While the gradual transition from ewe's milk to commercial replacer has short-term favourable effects (Sevi et al., 1999), the postweaning consequences remain unknown.

The perinatal period is critical for lambs, which are very sensitive to external challenges and can result in enteritis, respiratory disorders and metabolic diseases (Dwyer et al., 2016). Recent studies have also shown, in humans, that early 'programming' events are responsible for increased risk of getting ill in later life due to the simultaneous existence of low-grade chronic inflammation and oxidative stress (Lupien et al., 2009). We can hypothesise that appropriate health management of the young lamb would improve its ability to cope with various challenges encountered not only during the early postnatal period but also throughout its entire life. The definition of health is a 'state of complete physical, mental and social well-being and not merely the absence of disease or infirmity' (WHO Constitution, 1948). Therefore, the development of health and welfare biomarkers is a main topic of research to determine the overall animal health state, including specific disease diagnosis, biomarkers of unbalanced homeostasis or altered welfare before the appearance of any clinical signs. Growth is an indirect indicator of health related to optimal physiological functions. In sheep, fleece dirtiness can reflect the status of intestinal physiology reflecting either digestive disorders or enteric diseases that can be confirmed by detecting faecal enteric pathogens (Jorgensen et al., 2017). The blood redox status and the immune response induced by vaccination are potential indicators for assessing the ability of the organism to self-balance cellular oxidative stress and to develop immunity against pathogens. In addition, as an integrated part of mental health, tools like qualitative behaviour assessment (**QBA**) are available in sheep for welfare assessment. Based on body language, QBA reflects the way the animal interacts more or less positively with the environment (AWIN; Richmond et al., 2017). Lastly, 'omic' technologies used to study long-term consequences of milk quality on human babies (Bardanzellu et al., 2017) and goat kids (Dong et al., 2013) provide complementary clues to understanding the biological pathways involved in these processes.

In the present exploratory study, we investigated the advantage of providing dairy ewe's milk (e.g. the French Lacaune breed) to artificially reared Romane lambs, a meat breed. Our objectives were i) to confirm the unfavourable short- and long-term effects of artificial feeding on performance and biomarkers of overall lamb health and their welfare state and ii) to investigate in what way the use of milk from dairy ewes could mitigate these unfavourable effects.

Material and methods

Animals, housing and experimental design

The study was conducted at the INRAE experimental facility (doi: 10.15454/1.5483259352597417E12, UE 0332, Bourges, France). A flock of 32 multiparous Romane ewes gave birth to pure breed lambs, over 7-day period under constant full-day supervision. Just prior to parturition, ewes were placed in individual pens (2 × 1 m) and human intervention was provided only in the case of prolonged labour. The experimental design is summarised in Fig. 1.

On day 3 (D3) after birth, lambs were randomly allocated by sex, vigour and birth weight into three treatments, the composition of which was completed day after day over a period of 1 week. Lambs below 2.5 kg at birth or born from ewes showing inappropriate maternal care were excluded from the experimental design. Due to an unbalanced sex ratio at lambing and a limited number of pregnant ewes, an unequal sample size exists between treatments. In two treatments, the Romane lambs were separated from their mothers and artificially fed with either a milk replacer (**REP**, 6 males and 9 females) or Lacaune ewe's milk (**LAC**, 6 males and 9 females). In the third treatment, one lamb per litter remained with its mother (**MOT**, 7 males and 6 females), the other sibling(s) being excluded from the experiment and reared artificially in another building. For each treatment, animals were randomly split into two replication groups in adjacent communal pens measuring 2.5 m × 4.9 m for artificially fed lambs, and 5.6 m × 5 m for lambs reared by their mothers.

In REP and LAC groups, all lambs were trained to drink from a bucket three times per day until being fully independent and then fed twice daily from two buckets per pen fitted each with rubber teats. Buckets and nipples were cleaned daily. The REP lambs were fed milk replacer following the manufacturer's recommendation (200 g/l, Agnodor Tradition plus, SOREAL NUTRITION ANIMALE, Vonnas, France). We chose this brand as it is commonly used in France, the ingredient content being given in Supplementary Table S1. The Lacaune ewes originated from another INRAE experimental farm (La Fage, 12250 Roquefort) and were transported at mid-gestation to our experimental facilities. They gave birth in an adjacent building and their lambs were artificially reared. They were at the same lactation stage

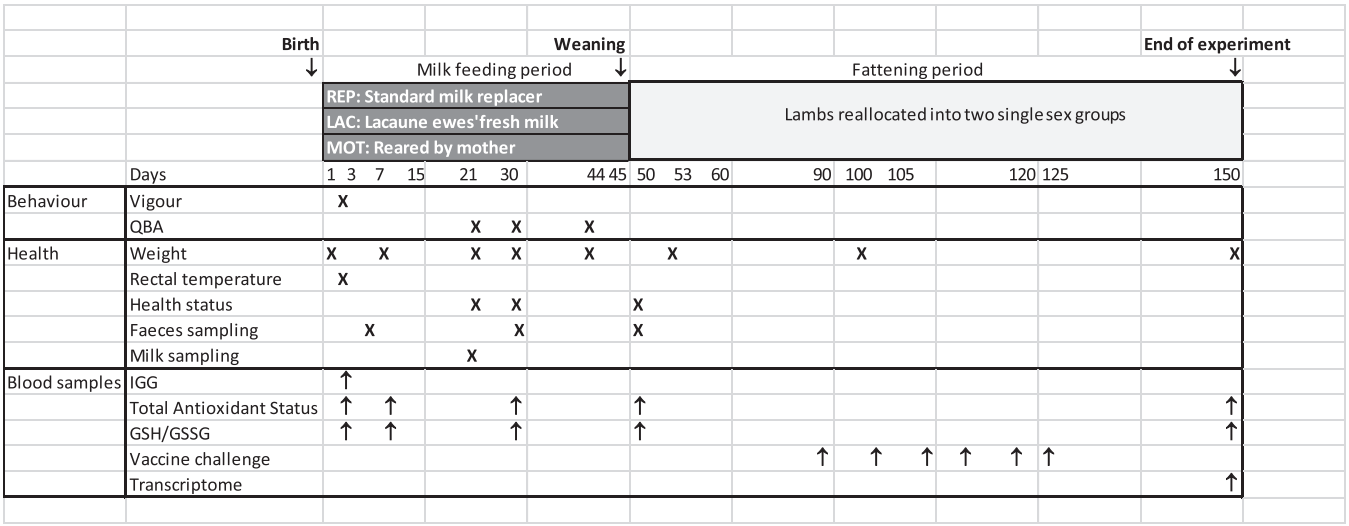


Fig. 1. Experimental design (lambs).

as the Romane ewes, machine-milked daily and a pool of milk was made for LAC lambs. Lacaune's milk and milk replacer temperature was 37 °C at each meal, the amounts provided per day and per lamb being: 1st week: 1 l; 2nd week: 1.5 l, 3rd week: 2 l, 4th–7th week: 2.5 l. These amounts were based on recommendations from the milk replacer's manufacturer. Lacaune ewes' milk lambs received the same volume of milk as REP lambs to reach a similar level of the gastric filling. In addition, concentrate pellets (net energy of 6.19 MJ/kg DM and metabolizable protein of 112 g/kg DM, according to INRA, 2007) hay, and water was accessible from D7 onwards. The MOT lambs shared water and hay with their mothers while pellets were available in a separate pen inaccessible to the ewes.

After weaning on D45, males and females were segregated and lambs of the six groups were mixed and reallocated into four pens (two per gender, randomly balanced by treatment, each pen 9 m×3 m). Specific pellets for growing lambs (net energy of 6.19 MJ/kg DM and metabolizable protein of 112 g/kg DM, according to INRA, 2007) and straw were provided until 5 months of age.

Sampling, measurements and analyses

Milk composition and lambs' growth rate

A pool of Romane and Lacaune ewes' milk, and a sample of milk replacer were collected on D21. Fat, protein, lactose and ash concentrations were measured by Galilait AgroLab's facility (Clermont-Ferrand, France). Milk fatty acid analysis was performed by a gas chromatograph equipped with a flame ionisation detector (Thermo Finnigan, Les Ulis, France) as described by Chassaing et al. (2016).

Lambs were weighed several times before and after weaning. Average daily gain was calculated for the first 3 weeks (ADG1-22) and the last 3 weeks of the suckling period (ADG22-44). In addition, ADG was also considered for the 2 weeks before (ADG30-44) and 9 days after weaning (ADG44-53), and finally for the rest of the postweaning period (ADG53-100 and ADG100-150) as there was a control weighing after every 50 days period. This allowed assessing the long-term adaptive capacities of lambs following a drastic contrast in feeding habits and social life.

Health measurements, sample collection and vaccine challenge

On D3, rectal temperature was recorded with a digital thermometer and a sample of blood was taken from the jugular vein (5 ml) to determine serum IgG concentrations using a radial immunodiffusion technique (measured by CIALSO, Auch, France).

Animal health status was monitored by a trained observer for the putative presence of nasal and ocular discharge, lameness, perianal dirtiness and diarrhoea on D21, D30 and D50. Dirtiness of the rear end was assessed visually by four trained observers on photos taken at the same age and given one of the following scores: 0 = without faeces; 1 = sporadic presence of faeces; 2 = faeces on tail, anus and legs. The perianal dirtiness score was only analysed on D21. Beyond that age, hindquarters remained soiled by dry faeces sticking to the fleece and made the interpretation of later scores inconclusive.

Individual faecal samples were taken on D7, D30 and D50 to detect pathogens. Faeces were collected in transparent plastic bags fitted on the rear-end of the lamb and attached to its neck. The bags were put on all the lambs at 9.00 am and removed as soon as they had defecated. Should lambs have not defecated by 4.00 pm, the bags were taken out. Faeces samples were stored at −80 °C until major pathogens responsible for enteric diseases (Rotavirus, *Escherichia coli* (*F4, F5, F18, F41*), *Clostridium perfringens*, *Clostridium difficile*, *Cryptosporidium parvum*) were identified using specific antigen detection by ELISA (Bio K326/2, BioX Diagnostics, Rochefort, Belgique). After thawing, faeces were diluted 1/5 in buffer, homogenised and centrifuged. Enzyme-linked immunosorbent assay was performed on supernatants according to manufacturer's instructions and results were expressed as percent positivity S/P = (sample absorbance/positive control absorbance) × 100. Samples were considered positive when the values were > 6%.

Blood samples (5 ml) were taken to assess oxidative stress indicators (D3, D7, D30, D50 and D150). The total antioxidant status (**TAS**) was measured by spectrophotometric method as described by Miller et al. (1993) and Scislowski et al. (2005) and expressed as Trolox equivalent antioxidant capacity (mmol TEAC/L). The redox status was estimated by reduced glutathione (**GSH**)/oxidised glutathione (**GSSG**) ratio, GSH and GSSG being measured by HPLC method as described by (Martin and White, 1991).

On D90, lambs were blood sampled (5 ml) and vaccinated subcutaneously against *Chlamydophila abortus* (vaccin OVILIS® Chlamydia; INTERVET, Beaucouzé, France), then sampled again from week 2 to 5 after vaccination. The immune response to the vaccine was measured in serum and antibodies were detected by indirect ELISA (ID Screen → Chlamydophila abortus Indirect, IDvet, Grabels, France). Antibody titres were expressed as percent positivity S/P = (sample absorbance/positive control absorbance) × 100 and S/P ≥ 60% were considered positive.

Lamb's vigour and qualitative behaviour assessment

On D3, a vigour test was performed. The lamb was gently put on its four legs and observed for 10–15 s by a trained observer. Lambs received one of the following scores: 1 = very weak (no tonus); 2 = weak (standing steadily, immobile); 3 = lively but with an empty stomach (standing steadily, mobile, flat belly); 4 = lively, strong and with filled stomach (standing steadily, moving, bleating, round belly).

On D21, D28 and D42, 5-minute video recordings were made in each rearing pen to use the QBA method (AWIN, 2015). Qualitative behaviour assessment was performed as an integrated welfare assessment tool (Fleming et al., 2015). Recording started in the morning (1000 h) immediately after spreading fresh straw to stimulate the lambs' activity. Two phases were then selected: the first 45 s (Phase 1) and the last 45 s (Phase 2). Six observers familiar with sheep farming practices but not involved in the experiment were trained first to perform the assessment. Twelve videos of 45 s were randomly selected and presented twice to each observer in random order. Once a 45-s-video session had been watched, the observers assessed the group using 21 descriptors (AWIN, 2015) with a positive (e.g. 'content', 'bright'…) or negative expressive connotation (e.g. 'apathetic' 'fearful'). The intensity of each adjective had to be scored between a minimal and a maximal value. The intra-observer consistency in QBA assessment was estimated by Spearman correlations calculated for each descriptor on the 12 repeated videos. The three most consistent observers (Spearman correlation >0.5, $P<0.1$) using most of the scoring scale were retained. After training, observers watched the videos in a random order so that pens, treatments, repetitions and phases were disconnected. Principal component analysis with Varimax rotation (Proc Factor SAS) was performed using all evaluation of the six experimental pens with two phases within each age. The factor scores obtained on PC1 and PC2 were used as new variables.

Transcriptomics

An exploratory integrative approach through blood transcriptomics was investigated to reveal which pathways were impacted by early life rearing conditions. AgilentSurePrint G3 Sheep GE was used as a first approach as it covered one-third of the ovine genome and no other microarray tools were available for this species. To integrate the effects of all the rearing period the analysis was only performed on D150 (MOT: 6 males and 4 females; LAC: 4 males and 7 females and REP: 6 males and 5 females). All animals were blood sampled at the jugular vein (5 ml) using vacuum tubes which contained a reagent that immediately stabilised intracellular RNA (PAXgene blood RNA system, PreAnalytiX GmbH, Hombrechtikon, Switzerland). The samples were kept at room temperature for 8 h, as required for stabilising RNA, and then stored at −20 °C until RNA extraction. Total RNA was extracted using the PAXgene Blood RNA kit (Qiagen, Courtaboeuf, France). Quality of the total RNA was assessed using RNA Nano chips on a Bioanalyser 2100 (Agilent, Boeblingen, Germany). The RNA integrity number score was higher than 8.0 in all samples. Gene expression profiles were performed at the GeT-TRiX facility (GenoToul, Génopole Toulouse Midi-Pyrénées) using AgilentSurePrint G3 Sheep GE (8x60K, design 019921). For each sample, Cyanine-3 labelled RNA (cRNA) was prepared from 200 ng of total RNA using the One-Colour Quick Amp Labeling kit (Agilent), followed by RNA clean-up using Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). Six hundred ng of Cyanine-3-labelled cRNA were hybridised on the microarray slides. Immediately after washing, the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and fluorescence signal extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters. Microarray data and experimental details are available in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE131763 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131763).

Statistical analysis of results

Data relating to health, performance and behaviour were analysed using SAS Analytics pro 9.0. All repeated data on lambs were analysed for variance with a mixed model (proc MIXED, or proc. GLIMMIX SAS according to the type of variable). As animals were gathered in different sex groups after weaning, separate analyses were performed before weaning, around weaning and during the postweaning period. We checked the group effect as a random factor for all the analyses but as it was never significant, we removed it to simplify models. The fixed effects are treatment, sex, age and two-way interactions with treatment. For the analysis of QBA, factor scores for PC1 and PC2, phase in the video editing and observer were added as fixed effects. For the analysis of dirtiness score, we combined the scores 0 and 1 and the observer was added as a fixed effect. A covariate was integrated into the model: for vaccinal antibody response (pre-vaccination antibody titres), and the level on D3 for GSH/GSSG or for TAS. Possible non-normality and heterogeneity of variance were checked. Pathogen infection rates, antibody titres and oxidative stress data needed to be log-transformed. Comparisons between three treatments of lambs displaying signs of diarrhoea were performed using the exact Fisher test. $P<0.05$ was considered significant and $0.05<P<0.1$ was considered as a tendency.

Microarray data were analysed using R and Bioconductor packages (www.r-project.org, R v. 3.1.2, Gentleman et al., 2004) as described in GEO accession GSE131763. Briefly, raw data (median signal intensity) were filtered, log2 transformed, corrected for bath effects (washing and labelling serials) and normalised using the quantile method (Bolstad et al., 2003). A model was fitted using the limma lmFit function (Smyth, 2004) considering sex as a blocking factor. A correction for multiple testing was then applied using Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995) for the false discovery rate (**FDR**). Probes with FDR ≤ 0.05 were considered to be differentially expressed between conditions. For bioinformatic analyses, we selected probes that displayed a non-adjusted *P*-value of $P<0.005$ as no probes were found with an FDR ≤ 0.05 and a fold change in expression >20%.

Enrichr software (Chen et al., 2013; Kuleshov et al., 2016) was used for gene list enrichment analysis. Transcription factors with an adjusted *P*-value<0.10 were retained. Student *t*-test was used to compare PCR results of gene expression between two groups with $P<0.10$ considered as significant.

Results

Lamb features

At birth, the lambs weighed on average 3.8 ± 0.7 kg. Vigour scores were satisfactory on D3 as 34/43 lambs had the maximum score of 4, the remaining nine lambs with a score of 3 had been allocated to each group. Rectal temperature and serum IgG concentrations on D3 did not differ between treatments ($P>0.1$).

Milk composition

Milk composition for each sample is shown in Table 1. Commercial milk replacer contained higher lactose and ash residues than Romane or Lacaune's milk and lower protein content. Lacaune's milk was richer in fat than the two other milk diets. The differences in composition led to a similar gross energy level per litre for milk replacer and Romane milk whereas Lacaune's milk values were higher. Fatty acid (FA) composition was similar in Romane or Lacaune's milk. Milk replacer differed from sheep milk mostly by a higher *trans*-MUFA, a higher *n*-6 PUFA contents and consequently a higher *n*-6:*n*-3 ratio. The *n*-3 PUFA content

Table 1
Chemical and fatty acids composition of the three types of milk consumed by the lambs.

Component	Milk replacer	Romane's milk	Lacaune's milk
Crude protein g/l	33.6	50.76	56.1
Crude fat g/l	42.9	47.55	80
Ash %	1.4	1	1
Lactose, g/l	65.1	41	42.49
Gross energy, kJ/l	3268	3326	4661
Fatty acids group type g/100 of total FA			
Σ SFA	55.99	67.32	66.79
Σ MUFA	37.27	26.46	26.94
Σ *trans*-MUFA	10.49	4.03	3.07
Σ PUFA	6.60	4.45	4.52
Σ PUFA *n*-6	6.08	2.33	1.93
Σ PUFA *n*-3	0.24	0.98	1.25
n-6:*n*-3	24.8	2.39	1.54

FA = fatty acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acids.

was low in all three diets (<1.5 g/100 g of total FA) particularly in milk replacer.

Growth rate

Compared to MOT lambs, REP lambs had a lower ADG1-22 ($P < 0.01$; Fig. 2a). Lacaune ewes' milk lambs were intermediate between REP and MOT. There was no difference in ADG22-44 between treatments. The mean weaning weight was 17.9 ± 2.9 kg and did not differ significantly between treatments ($P > 0.1$). To analyse the impact of weaning on lamb growth in the different treatments, we compared the ADG between the few days preceding and following it, i.e. ADG30-44 and ADG44-53. Weaning resulted in a sharp drop in ADG in REP and LAC lambs ($P < 0.001$, Fig. 2b) but not in MOT lambs. Growth rate during the postweaning periods (ADG53-100 and ADG100-156, Fig. 2c) and final weights were not different between treatments (mean weight: 51.1 ± 6.4 kg at D156). There was no sex difference or interaction between sex and treatment for growth rate during the milk-feeding period and around weaning ($P > 0.1$). ADG was significantly higher in males than in females in both postweaning sub-periods (ADG53-100 and ADG100-156, $P < 0.001$) but no interaction with the treatment was observed ($P > 0.1$).

Health status

During the experiment, two lambs died due to enterotoxaemia at 1 and 5 months (respectively in LAC and REP groups). In the REP treatment, there was a significantly higher proportion of dirty lambs with faeces on tail, anus and legs than in the MOT treatment (42.5% vs 3.2%, SEM = 11%, $P < 0.05$). Lacaune ewes' milk lambs, on the other hand, did not differ significantly from MOT lambs (3.8%, $P > 0.1$) but differed from REP ($P < 0.05$). Four lambs displayed clear signs of diarrhoea, all in the REP treatment ($P < 0.05$). The search for pathogens in the faeces showed that lambs were mainly positive for *C. perfringens*. The number of positive lambs for *C. perfringens* decreased with time ($P < 0.001$), 85% of them were detected positive on D7, 42% on D30 and 12% on D50, but no significant difference was found between treatments ($P > 0.1$). The percentage of positivity decreased with time ($P < 0.001$) from 24.13% on D7 to 1.72% on D50, but did not differ between treatments ($P > 0.1$).

The initial blood TAS was higher on D3 in lambs that were subsequently allocated to the LAC group than in the other groups ($P < 0.001$). No differences between treatments were detected on D7 ($P > 0.1$). On D30, REP lambs had a significantly lower TAS than MOT lambs (Fig. 3A; $P < 0.05$). It was the same for LAC lambs that did not differ from REP lambs. The GSH/GSSG ratio did not differ between treatments on D3 ($P > 0.1$). On D7, the ratio in LAC was higher than in MOT lambs ($P < 0.05$), REP and MOT lambs not being different ($P > 0.1$). On D30, both REP and LAC ratio were higher than in MOT lambs ($P < 0.001$ and $P < 0.05$, respectively; Fig. 3B) without differing between them ($P > 0.1$). On D50 and D150, TAS and GSH/GSSG ratio did not differ between treatments ($P > 0.1$).

Immune response to vaccine challenge

On D90, 3/42 lambs were excluded from the analysis: two because they were already positive at the time of injection (week 0) and one with excessively high values from week 2 onwards (between 882 and 1327 S/P %). Among the 39 remaining lambs, 31 responded to the vaccine with 79.5% of them being positive at least once between 2 and 5 weeks after vaccination. Eight lambs never developed a detectable positive immune response to the vaccine, four in REP and four in MOT treatments. In LAC treatment, each lamb was positive at least once. The antibody titres (percent of positivity) did not differ between REP and MOT lambs in the 5 weeks after vaccination ($P > 0.1$). They were higher ($P < 0.001$) in LAC lambs than in REP lambs (Fig. 4a, Supplementary Table S2) but not different between LAC and MOT lambs ($P > 0.1$) during this period. There was a trend for a treatment effect on the percentage of positive lambs ($P = 0.09$; Fig. 4b, Supplementary Table S2). This percentage did not differ significantly between REP and MOT lambs ($P > 0.1$). A higher percentage of positive lambs was found in LAC treatment (74.8%) compared to MOT (53.9%, $P < 0.05$) and REP (55.5%, $P = 0.07$). The percentage of positive lambs was significantly higher in females than in males (74.9% vs 47.1%, respectively, $P < 0.01$) and the antibody titres were twice as important (91.7% vs 48.5%, respectively, in females and males, $P < 0.001$). There was no interaction between sex and treatment ($P > 0.1$).

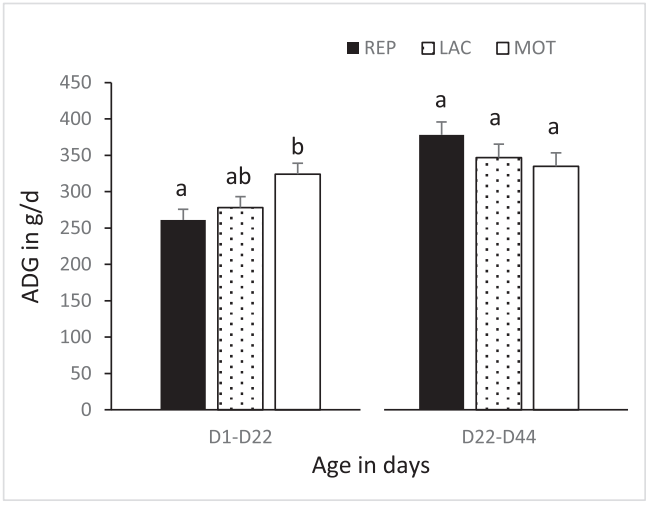
Qualitative behaviour assessment

Principal component analysis revealed two dimensions of lamb behaviour explained by the first two principal components (PC1 and PC2) corresponding to 39.9 and 21.3% of the variance, respectively. The Kaiser–Meyer–Olkin value was 0.88. The PC1 was positively associated with 'active', 'agitated', 'vigorous', and 'inquisitive' and negatively associated with 'calm'; this can be viewed as an 'activity' axis (Supplementary Table S3). The PC2 was positively associated with 'tense', 'apathetic', 'defensive', 'frustrated' and 'subdued' and can be viewed as a 'perturbation' axis. Analysis of PC1 values revealed that there was a significant treatment*age interaction ($P < 0.05$, Fig. 5). Milk replacer lambs were always reported to be more active than MOT lambs but the difference decreased with time. By contrast, LAC lambs were never found different from MOT lambs ($P > 0.1$). Lacaune ewes' milk lambs were less active and calmer ($P < 0.001$) than REP lambs but only on D21 ($P < 0.05$). No significant interaction between observer and treatment was found ($P > 0.1$). For PC2 no treatment effect was detected alone or in interaction with the other factors ($P > 0.1$).

Transcriptome analysis

We analysed 6529 genes for differential expression in blood cells on D150. Using an FDR of 5%, no genes were found differentially expressed between treatments. A non-adjusted *P*-value of $P < 0.005$ was then used which revealed a strong sex effect. The list of differentially expressed (**DE**) genes per treatment and per sex is presented in Supplementary Table S4. The Venn diagrams (Fig. 6) show the number of DE genes in each comparison of lamb treatments and DE genes common to several comparisons. There were no genes in common between males and females. Females were found much less affected by milk replacer or Lacaune's milk since only a few DE genes (less than 20 genes) were found in each comparison. Most of the DE genes were found in REP/MOT and REP/LAC comparisons. There were no DE genes in common

A



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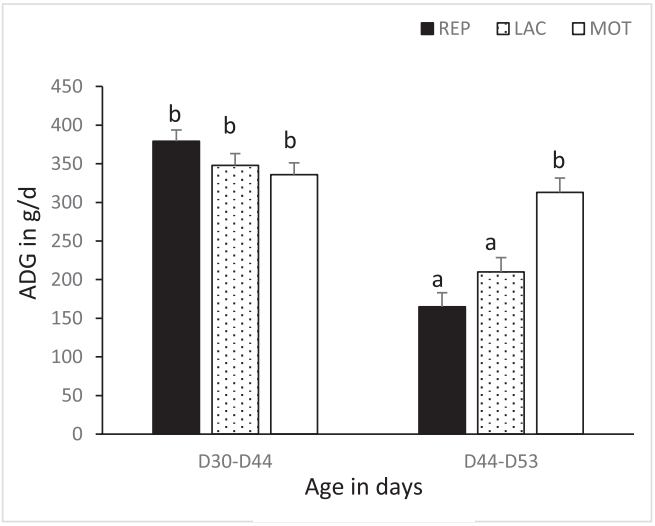

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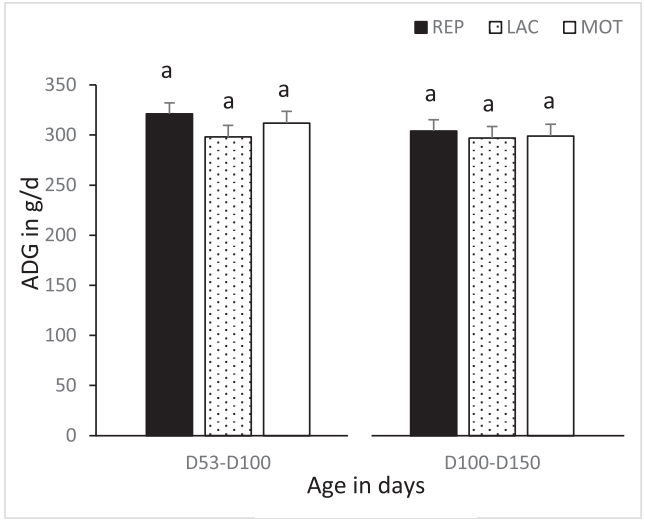

between female lamb treatments. In males, the most contrasted treatment was LAC vs both MOT ($n = 88$ DE genes) and REP ($n = 78$ DE genes) while a low number of DE genes were identified between REP and MOT groups ($n = 21$ DE genes).

To get some functional insight into the DE genes, the bioinformatics tool *Enrichr* was used. In females, no upstream regulator could be identified neither between REP and MOT nor between REP and LAC. The results found for males are summarised in Table 2. The REP vs MOT comparison showed a small enrichment for Fos and RelA as transcriptional drivers in REP lambs. In the 88 DE genes identified between LAC and MOT lambs, RelA activity was found increased in the LAC group. For the LAC vs REP comparison, RelA was again found increased in the LAC lambs as well as IRF1 and POU2AF1. Quantitative PCR analysis was performed on selected genes to confirm gene expression data (see Supplementary Figure S1).

Discussion

The study aimed to confirm the negative effects of artificial feeding on lamb's performance and on biomarkers of overall animal health and welfare state and to mitigate these effects by providing dairy ewe's milk instead of milk replacer. While the outcome did not reach the benefits of being reared by the mother, dairy ewe's milk had definite positive consequences.

Short- and mid-term effects of milk replacer compared to mothering

Drinking milk replacer from a bucket instead of suckling the mother impaired early growth rate. This is in agreement with Sevi et al. (1999) and Belanche et al. (2019b) in dairy and meat lambs, respectively, and suggests that the replacer does not cover the lamb's needs for optimal growth. One explanation could be in the biochemical composition itself. In our study, the replacer is less rich in protein and contains more lactose than Romane's milk. The lower level in proteins, and their sources (e.g. whey, skimmed milk powder or vegetal), might partly explain the impaired growth rate in young growing animals. In addition, while the mother's milk changes constantly over the lactation period (Mitoulas et al., 2002), the milk replacer has a standard composition that is the same for the entire suckling period. Therefore, there is a risk that the intake of nutrients might not be in line with the lamb's needs (Black et al., 1973).

Feeding lambs with the replacer also led to dirtier perianal areas compared to lambs reared by their mothers, which suggests altered digestive transit, unbalanced digestive function, or/and disturbed microbiota. This, however, cannot be attributed to the intestinal pathogens analysed, *C. perfringens* was the only one detected in the faeces in significant amount without any difference between the three groups of lambs. Another explanation could be in the feeding schedule. Our lambs were fed with suckling feeding buckets, a very common practice on farms, giving them two meals per day. This may be not adapted to the young lamb's natural suckling activity and digestive capacities compared with about 9 meals per day when they have free access to an automatic lamb feeder (David et al., 2014) or to the even higher suckling frequency when they are reared by their mothers. In addition, on D30, REP lambs also

Fig. 2. Average daily gain (ADG) in g/d in lambs according to treatments during three key periods: suckling (A); around weaning (B); postweaning (C). REP: lambs artificially fed with standard milk replacer; LAC: lambs artificially fed with Lacaune's milk; MOT: lambs reared by their mothers. [a,b]Values with different superscripts differ significantly at $P < 0.05$; comparisons are made between treatments within a period for 2A and 2C, and within treatments between two periods around weaning for 2B.

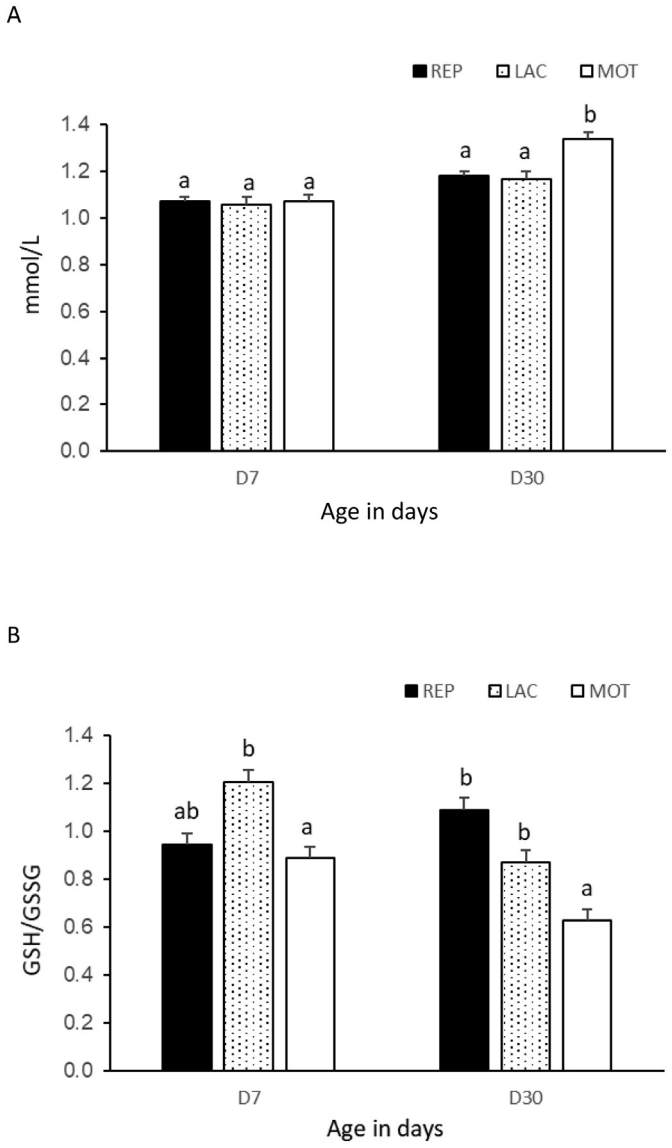

Fig. 3. Total antioxidant status (A) and reduced glutathione/oxidised glutathione ratio (GSH/GSSG) (B) in lambs during the suckling period according to treatments. REP: lambs artificially fed with standard milk replacer; LAC: lambs artificially fed with Lacaune's milk; MOT: lambs reared by their mothers. [a,b]Values with different superscripts differ significantly at $P < 0.05$. (comparisons are made between treatments within period).

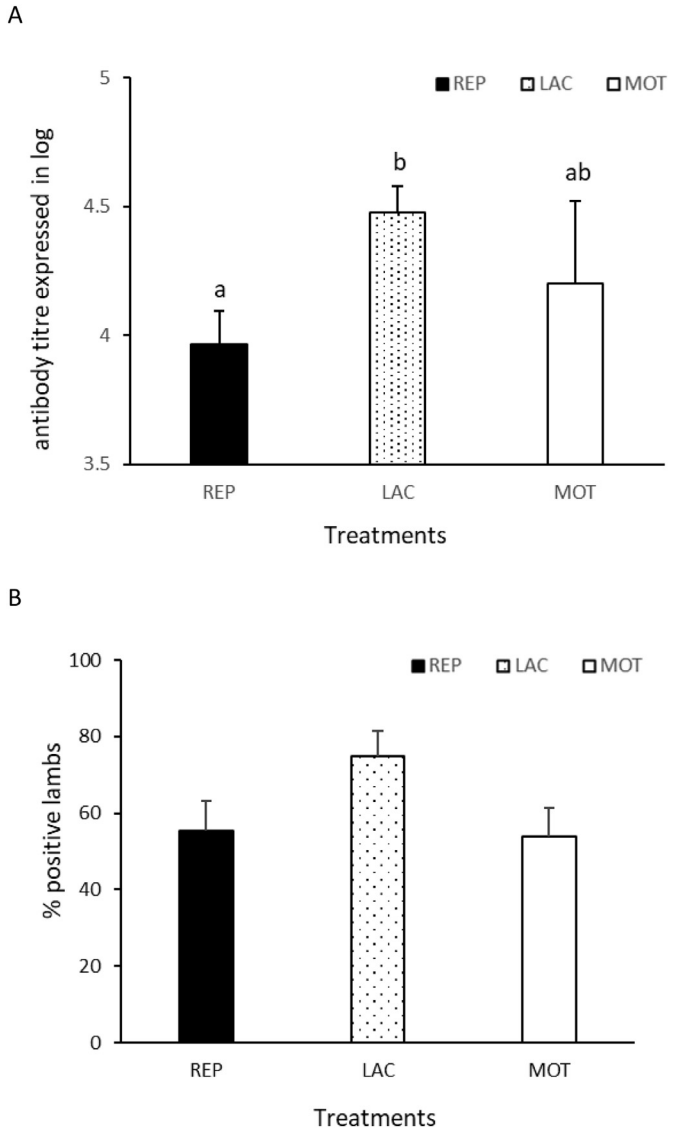

Fig. 4. Immune response after vaccination against *Chlamydophila abortus*. Antibody titres were expressed as percent positivity S/P% ((sample absorbance/positive control absorbance) × 100) for each group and transformed in log (A). Proportion of positive lambs expressed in % for each group (B). REP: lambs artificially fed with standard milk replacer; LAC: lambs artificially fed with Lacaune's milk; MOT: lambs reared by their mothers. [a,b]Values with different superscripts differ significantly at $P < 0.05$.

have a lower TAS than MOT lambs. These results are consistent with those of Abuelo et al. (2019) indicating a lower antioxidant status in calves fed milk replacer associated with an increased risk of catching a disease. In their review, these authors reported that milk replacers presented a low antioxidant capacity and they suggest that calves might benefit from additional antioxidant supplementation. Moreover, during the milk-feeding period, it is noticeable from a behaviour point of view that REP lambs were always rated as more agitated than MOT lambs. Artificially reared lambs could well be more reactive to the humans who had trained them to suckle and brought food daily. This could have triggered higher reactivity during human interventions since artificially fed lambs develop affiliative relationships and potentially a strong dependency towards their caregiver (Boivin et al., 2002).

Surprisingly, around weaning, growth rate decreased in REP lambs but not in MOT. This is in opposition to our expectation that there would be higher stress induced by the mother lamb separation than by the reallocation in the REP lamb groups. This could be explained by a better adaptation of mothered lambs to solid food after weaning, despite both having access to concentrates before weaning. Belanche et al. (2019a) suggest that mother-reared lambs have a feeding behaviour and a rumen development shaped to cope better with a fully solid diet due to a higher intake of concentrates and hay and the establishment of a balanced microbiota through the presence of adults. To confirm this, feeding behaviour, as well as individual milk and solid feed intake, should be explored in future studies. After weaning, we did not notice any mid-term consequence on growth, health or immunity to vaccine. Our results are different from Belanche et al. (2019a) who observed that lambs having been reared by their mothers had a higher growth rate after weaning once transferred onto pasture. Outdoor conditions could be more challenging, as it imposes a more pronounced change of environment and food compared to lambs kept indoors. Overall, the indicators explored in our study converge towards an imbalance at the beginning of the milk-feeding period with milk replacer that was nonetheless transitory and no longer observed at the age of 5 months.

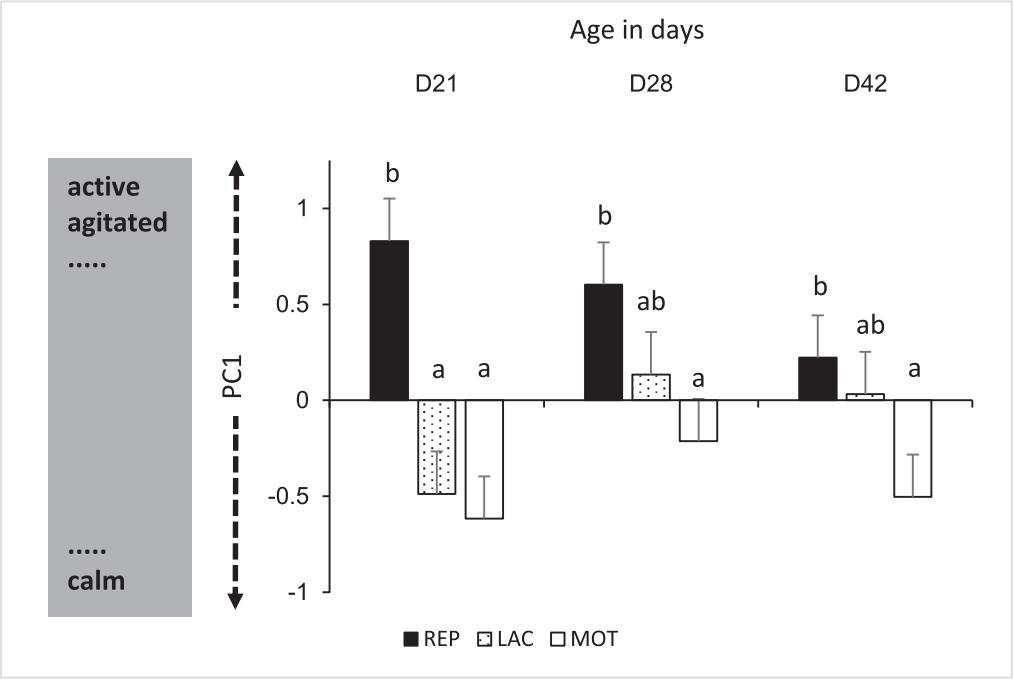

Fig. 5. Qualitative behaviour assessment of lambs during the suckling period according to age and treatment. The PC1 from principal component analysis (PCA) analysis was shown according to the descriptors used (Supplementary Table S1). REP: lambs artificially fed with standard milk replacer; LAC: lambs artificially fed with Lacaune's milk; MOT: lambs reared by their mothers. [a,b]Values with different superscripts differ significantly at $P < 0.05$ (comparisons are made between treatments within each age).

Interest of Lacaune's milk as milk replacer?

Three positive outcomes were observed from our results. Providing Lacaune's milk reduced dirtiness of the perianal region. The score in LAC was lower than in REP lambs and did not differ from MOT lambs. According to the chemical composition of the milk, one important difference between REP and LAC was a higher concentration of lactose in the milk replacer, which potentially penalises the digestion process (Glimp, 1972). In addition, with the QBA performed at a young age, LAC lambs were found calmer than REP lambs. This could be due to Lacaune's milk composition itself since soothing properties of milk have been described in rat pups and human infants through fat and β-Casomorphin, a peptide hydrolysed from casein (Blass, 1996). Lacaune's milk has higher crude fat and protein contents than the replacer. It may trigger soothing states much like those displayed by MOT lambs, the dam being a calming element on her own, in addition to her milk (Nowak and Boivin, 2015). At mid-term, a difference in the postvaccination response was detected. While no difference was

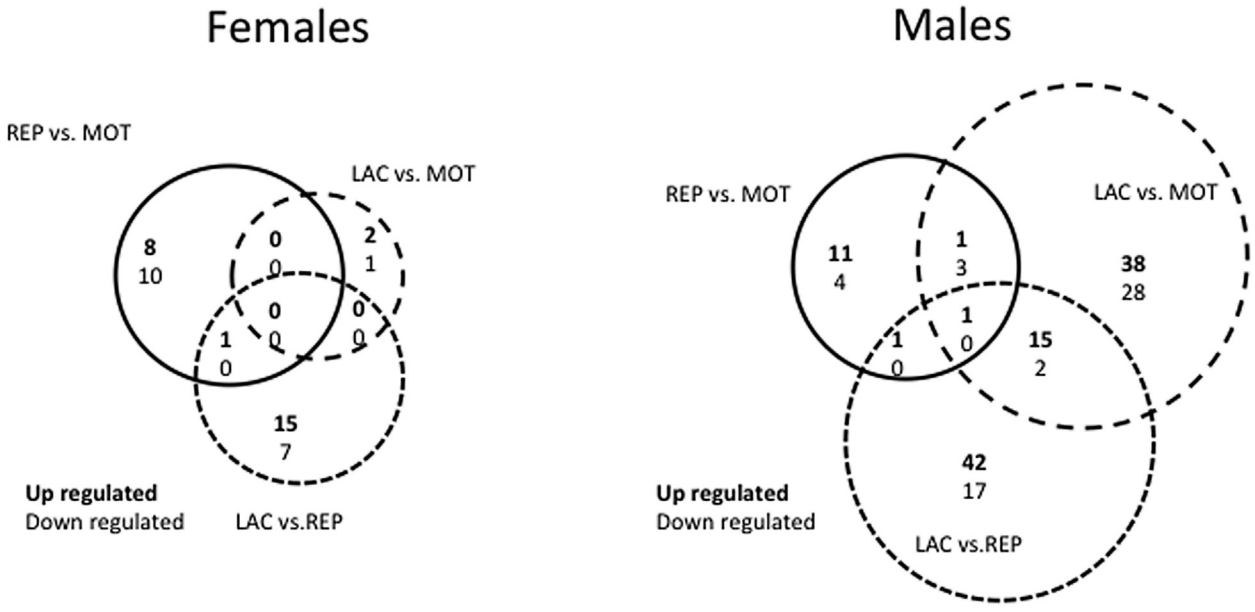

Fig. 6. Venn diagrams of the differentially expressed genes in blood cells from 150-day-old lambs reared by their mothers (MOT), or separated on day 3 and fed with milk replacer (REP) or Lacaune's milk (LAC) for 45 days.

Table 2
Enrichment analysis of the differentially expressed genes in blood cells in 150-day-old male lambs reared by their mothers (MOT), or separated on day 3 and fed with milk replacer (REP) or Lacaune's milk (LAC) for 45 days. The data are extracted from the analysis using *Enrichr* (https://amp.pharm.mssm.edu/Enrichr/). TF: Transcription Factor; ChIP (Chromatin Immuno-Precipitation) Enrichment Analysis; FOS: Fos Proto-Oncogene, AP-1 Transcription Factor Subunit, RELA: RELA Proto-Oncogene, NF-KB Subunit, IRF1: Interferon Regulatory Factor1; POU2AF1: POU Class 2 Homeobox Associating Factor 1.

Groups	Database	Term	*P*-value	Adjusted *P*-value	Odd ratio	Combined Score	Genes
REP vs MOT	Transfac & Jaspar	FOS	0.00038	0.077	4.78	37.62	SLC9A3R1;ALKBH5;INTS1;NFKBIZ;NOX5;LSM14A;VPS25
	TF perturbations followed by Expression	RELA	$4.15 \cdot 10^{-6}$	0.0028	19.84	245.88	INTS1;DMXL1;NFKBIZ;FHL3;VPS25
LAC vs MOT	ChEA	RELA	$7.13 \cdot 10^{-7}$	0.0004	3.65	51.71	TPD52;PTGER4;CR2;HGF;SWAP70;ZBTB10;IL27;PRDM1;LHFPL2;INHBA; KIAA0430;F11R;IFIH1;TSPAN14;ABI1;CHST15;JUNB;PFKP;TLR2
LAC vs REP	ENCODE & ChEA consensus	IRF1	0.00057	0.049	5.85	43.71	ATF7IP;SLC25A37;RNF149;STAT2;UBE2L6;FBXL5
		RELA	0.0028	0.09	3.71	21.80	IFIH1;MARCKS;EBI3;FAM129A;IL27;HNF1B;SAE1
	TF perturbations followed by Expression	POU2AF1	$6.96 \cdot 10^{-8}$	$3.42 \cdot 10^{-5}$		24.84	PTGER4;RABGAP1L;CYTH4;FCER1G;CADM1;CTSZ;CHST15;MCTP2;CD68;NSMAF

obtained between blood antibody titres in REP and MOT lambs, LAC lambs developed a stronger response than REP lambs. Our results are complementary to those from Sevi et al. (1999) on the cell-mediated immune response where a gradual transition from ewe's milk to reconstituted milk was shown to stimulate the lamb's immune response during the suckling period. Lacaune ewes' milk lambs might have also benefited from an enhanced *n*-3 PUFA status. While the response to *n*-3 PUFA supplementation on young animals' health is not always consistent (Lewis et al., 2008), a positive effect on B-cell activation and humoral response has been reported (Whelan et al., 2016).

In terms of performance, Lacaune's milk did not provide any significant advantage compared to milk replacer. Lacaune's milk contained similar amounts of lactose and protein as Romane's milk but more fat, which makes it more energetic. However, early growth was not improved in LAC compared to REP lambs. One explanation could be that ruminants have limited abilities to digest important amounts of fat during the first weeks of artificial feeding, as shown by the measure of hepatic enzymes (Gautier and Labussiere, 2011). After all, Lacaune ewe's milk has been selected for decades to manufacture dairy products for human consumption, and not for lambs' growth. In addition, Lacaune's milk does not prevent the fall in growth around weaning compared to MOT lambs. This suggests that more than milk composition, the preparation of lambs to cope with a fully solid diet through maternal learning is crucial.

Regarding the TAS, it was not improved in LAC compared to REP lambs, both being lower than MOT lambs. This suggests two hypotheses. The first is that the antioxidant properties of Lacaune's milk could be weaker than those of Romane's milk. Indeed (Caroprese et al., 2019) reported breed differences in antioxidant properties in milk. A second more likely hypothesis is that the TAS is reduced by periods of stress (Abuelo et al., 2019) and artificial feeding is considered to be one (Napolitano et al., 2008). Total antioxidant status results were not confirmed by the GSH/GSSG ratio that indicates more oxidative stress in MOT animals. This ratio, which indicates the use of glutathione as an antioxidant, is more sensitive to short-lasting stress. In our case, the blood sampling procedure itself with handling, restraining and venipuncture could have been more stressful in MOT lambs that were less accustomed to humans (Nowak and Boivin, 2015).

Overall, our results suggest some interest in using Lacaune ewe's milk as a replacer. However, the impaired growth around weaning and the lower antioxidant status for both groups of artificially fed lambs suggest that several aspects of this procedure deserve future attention: the absence of adults in the transmission of feeding behaviour, the stress of the weaning procedure and the development of immature microbiota.

In complement, the transcriptomic analysis performed on D150 revealed a possible signature specific to the milk-feeding condition. The most contrasted responses were observed in males and in lambs fed Lacaune's milk. Among the DE genes identified, mainly in relation to inflammatory, immune response and cell cycle regulation, transcriptional RelA activity was found increased in LAC lambs compared to the two others, as well as IRF1 and POU2AF1 in comparison to REP lambs. Interestingly POU2AF1 is more specifically activated in the response of B cells to the antigen (Zhou et al., 2016), which may explain the increased response to vaccination in LAC lambs compared to the two other groups. Therefore, the milk-feeding mode can result in lasting effects on transcriptomic profiles. In the present study, the use of fresh dairy milk instead of milk replacer or maternal nursing modified the expression of transcription pathways, with a stronger effect on males than females.

Conclusion

This exploratory and multidisciplinary approach investigated the effects of three milk-feeding conditions on lambs' performance and biomarkers of overall animal health and welfare state. Our study confirms that artificial milk feeding impaired early growth rate, health and emotional state mainly during the milk-feeding period and at weaning. Feeding artificially reared lambs with fresh ewe's milk partly mitigated some negative effects normally induced by milk replacer but without achieving the full benefit of being reared by the mother. This suggests that other factors should be explored to further improve the outcome, such as revisiting the replacer quality and the feeding schedule to the lamb's physiological needs and suckling activity, enriching the social environment by providing maternal substitutes.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2020.100157.

Ethics approval

The experiment was carried out according to the French guidelines for animal care and use. All the procedures were approved by the Val de Loire Ethics Committee, France (agreement no. 00821.03) and performed by fully trained personal.

Data and model availability statement

None of the data were deposited in an official repository.

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Declaration of interest

The authors declare no conflict of interest.

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