SMARTER

SMAll RuminanTs breeding for Efficiency and Resilience

Research and Innovation action: H2020 – 772787
Call: H2020-SFS-2017-2
Type of action: Research and Innovation Action (RIA)
Work programme topic: SFS-15-2016-2017
Duration of the project: 01 November 2018 – 30 June 2023

Report on the characterization of efficiency and resilience in lines of sheep and goat divergently selected. (M48/INRAE)

Rachel Rupp[1], Frederic Douhard[1], Beatriz Gutiérrez Gil[2], Gabriel Ciappesonni[3]

1-INRAE*

2-UNILEON

3-INIA-UY

* Deliverable leader – Contact: rachel.rupp@inrae.fr

DELIVERABLE D3.2

Workpackage N°3

Due date: M48

Actual date: 20/08/2023 (first submission 30/11/2022)

Dissemination level: Public

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 772787

About the SMARTER research project

SMARTER will develop and deploy innovative strategies to improve Resilience and Efficiency (R&E) related traits in sheep and goats. SMARTER will find these strategies by: i) generating and validating novel R&E related traits at a phenotypic and genetic level ii) improving and developing new genome-based solutions and tools relevant for the data structure and size of small ruminant populations, iii) establishing new breeding and selection strategies for various breeds and environments that consider R&E traits.

SMARTER with help from stakeholders chose several key R&E traits including feed efficiency, health (resistance to disease, survival) and welfare. Experimental populations will be used to identify and dissect new predictors of these R&E traits and the trade-off between animal ability to overcome external challenges. SMARTER will estimate the underlying genetic and genomic variability governing these R&E related traits. This variability will be related to performance in different environments including genotype-by-environment interactions (conventional, agro-ecological and organic systems) in commercial populations. The outcome will be accurate genomic predictions for R&E traits in different environments across different breeds and populations. SMARTER will also create a new cooperative European and international initiative that will use genomic selection across countries. This initiative will make selection for R&E traits faster and more efficient. SMARTER will also characterize the phenotype and genome of traditional and underutilized breeds. Finally, SMARTER will propose new breeding strategies that utilise R&E traits and trade-offs and balance economic, social and environmental challenges.

The overall impact of the multi-actor SMARTER project will be ready-to-use effective and efficient tools to make small ruminant production resilient through improved profitability and efficiency.

1. Summary

The challenge for livestock breeding is to improve the resilience traits simultaneous with feed efficiency and other traits important for a sustainable livestock sector (i.e., growth, production, product quality and reproduction). Simultaneous breeding for multiple traits can fall foul of trade-offs between traits. The objective of the study reported here was to better understand the biological mechanisms underlying trade-offs and synergies between resilience and efficiency (R&E) traits and how they affect resilience and efficiency.
To achieve this objective, we created 4 SMARTER experiments (INRAE and UNILEON) in genetically selected sheep and goat under both nutritional and infectious challenges and we characterised the effect of genetic background and environmental stress (nutrition, infection) on a comprehensive set of efficiency- and resilience- related phenotypes. In addition, we added supplemental phenotyping to a fifth experiment provided by INIA-UY. Results of the five experiments are reported hereafter.
These original experiments first demonstrated that selection can be made on new resilience and efficiency (R&E) traits (such as feed efficiency, longevity and parasite resistance) as expected response were produced despite low to moderate heritability. New challenge and phenotyping protocols were implemented that allowed to derive new resilience phenotypes. The main finding of the report from the five experiments is that there was little evidence of major trade-offs between selection for resilience and response to efficiency and also conversely between selection for efficiency and response to resilience.

2. Introduction

Genetic selection focused purely on production traits has proven very successful in improving the productive performance of livestock. Besides, selection on some functional traits (adaptation and heath traits) has been progressively implemented as well. However, heightened environmental and infectious disease challenges have raised the need to also improve the resilience of animals to such external stressors, as well as their efficiency in utilizing available feed resources. A better understanding of the relationship between efficiency and production and health traits is needed to properly account for it in breeding programs and to produce animals that can maintain high production performance in a range of environmental conditions with minimal environmental footprint.

The aim of the study reported here was to better understand the biological mechanisms underlying trade-offs and synergies between R&E traits and how they affect resilience and efficiency. To do so we created and analysed five experimental data in genetically selected sheep and goats which underwent nutritional and infection challenges.

3. Material and methods

Experimental designs

We created five experimental data in genetically selected sheep and goats which underwent nutritional and infection challenges. Animals belong to divergently selected lines either for production traits (milk yield, feed efficiency) or for resilience traits (parasite resistance, functional longevity). Animals were monitored for growth trajectories, feed efficiency, production (milk or growth), health

status and temperature, and resilience to challenges to determine trade-off/synergy between biological functions, and their genetic control. The model for infectious challenge was lipopolysaccharides (LPS) (Salvesen et al., 2016) for dairy sheep and goat and gastrointestinal parasites in meat sheep. The experiments are summarised in Table 1 and detailed further below.

Table1. Summary of the five trade-off experiments created in Smarter (Several phenotypes, marked with *, were funded by other projects to complement the datasets and were made available for smarter with restriction on the public opening of data)

	Breed & Species (Partner)	**Genetic group**	**Challenge**	**Phenotypes**[1]
1	ASSAF Dairy sheep (UNILEON) N=76	Milk production	Nutritional: low versus high protein growing period (N=40)[2] Infectious: LPS[3] in L1 (N=30)	Milk traits, Feed intake, body weight, SCS, gene expression, Fatty acids, metabolomics SCS, temperature, 14 Cytokines (N=24) *, Gene expression* (N=20) from milk RNA& blood RNA
2	ALPINE Dairy goat (INRAE) N= 199	Longevity	Nutritional: 2 days on hay in L1 (N=95) Infectious: LPS[3] (N=87)	Milk traits, SCS, 5 blood metabolites, 14 milk metabolites** temperature, health status (diarrhea), 14 Cytokines
3	ROMANE Meat sheep (INRAE) N=192	Parasite resistance	Nutritional: low versus high protein at lambing (N = 48) Infectious: parasite *H. Contortus* (N = 48 to 91)	FEC, blood hematocrit, body weight, backfat and muscle thickness, voluntary concentrate intake
4	CORRIEDALE Meat sheep (INIA-UY) N = 67	Parasite resistance	Nutritional: None (feed intake records) Infectious: parasite *H. contortus* (N= 67)	FEC, blood hematocrit, body weight, backfat and muscle thickness, haylage intake
5	ROMANE Meat sheep (INRAE) N = 90	Feed efficiency	Nutritional: None (feed intake records) Infectious: parasite *H. Contortus* (N= 90)	FEC, blood hematocrit, body weight, backfat and muscle thickness, voluntary concentrate intake

[1] **SCS**= milk somatic cell count; **SCS** = somatic cell score (log transformed SCC) **; FEC**= Faecal egg count

[2] nutritional challenge in ASSAF sheep previously described in Deliverable D1.1

[3] inflammatory challenge with E. coli lipopolysaccharide (LPS) mimicking mastitis (half of number funded by a French funding body APISGENE)

*, **: these phenotypes were funded by other projects and made available for smarter with restriction on the public opening of data

* Analyses funded by the National Spanish project EpiMilkSheep (RTI2018-093535-B-100) funded by the Spanish Ministry of Science and Innovation)

** Analyses funded by a French-funded project (RESILAIT, Apisgene)

Milk production ASSAF sheep lines

Animals. Animals from the Assaf breed were selected from one flock according to estimated breeding values (EBV) for milk yield (MY). Based on the production and pedigree information provided by the Spanish Association of Breeders of the Assaf Ovine Breed (ASSAFE), a total of 76 new-born Assaf female lambs, showing very high or medium-low EBV for MY (38 with high EBV, 38 with low EBV), were selected to be included in our experiment. Animals aged 2.5 months at purchase, when they were moved to the experimental farm of the Instituto de Ganadería de Montaña (CSIC, Unileon).

Nutritional challenge (N = 40). Once they passed the veterinary control and adapted to the new facilities, they were separated into Control group (C) and Nutritional Challenge group (NC), with a uniform distribution of EBV for MY in both groups. The lambs in the C group received the same diet as during the first month (standard feed for growth lambs), whereas the animals in the NC group received a diet with a 42% protein restriction compared with the C group. The nutritional challenge lasted approximately two months, between 3.5 and 5.5 months of age of the animals. Then, females were mated at around ten months of age (AI), and parturition occurred five months later. Forty primiparous lactating Assaf ewes (BW = 63.6 ± 1.23 kg; DIM = 33 ± 1.3 at the beginning of the experiment; MY = 2.40 ± 0.098 kg/d and VF_150 62.1 ± 4.57) were used in this assay. Estrus had been synchronized and lambing concentrated in a few days to avoid differences due to the lactation stage.
Measures collated during the nutritional challenge were the following: individual feed intake, body weight, and total milk produced by each animal, at morning and evening milking (beginning and the end of the 4-week period). Individual milk samples were analyzed to determine the fatty acid composition following the procedure detailed by Hervás et al. (2021). Furthermore, two milk samples per animal were collected for RNA extraction from milk somatic cells on weeks 6-8 of lactation (gene expression). Blood samples were collected for extraction of plasma on week 6 of lactation for metabolomic analyses. As the nutritional challenge of those 40 sheep were also used in WP1 to investigate feed efficiency, the experimental design, phenotyping and analyses of the nutritional challenge were previously described in Deliverable D1.1.

Infectious challenge (N = 30). From the total of 40 ewes, 30 ewes were selected to be included in the inflammatory challenge of the mammary gland at the end of their first lactation. The distribution of the 30 animals included in the experimental challenge reported here for the two effects mentioned was as follows: 16 NC vs. 14 C, and 15 High_EBV & 15 Low_EBV. Following Bouvier-Muller et al. (2016), the inflammatory challenge consisted on a mammary gland infusion of a 10 µg/ml solution of an E. coli lipopolysaccharide (LPS) performed on the 30 selected ewes. Based on measurements of the somatic cell count (SCC) from milk samples collected in the morning milking of 2-3 days before the challenge day, the healthiest half udder of each animal was chosen to be inoculated with LPS. The LPS solution was prepared as a sterile solution of Pam3CSK4 (10 µg/mL, In-Vivogen, Toulouse, France) and MDP (10 µg/mL, InVivogen) in PBS (Life Technologies SAS, Saint Aubin, France) containing 0.1% ovalbumin (Sigma-Aldrich, Saint-Quentin Fallavier, France). After preparation, the solution was stored overnight at 4°C until inoculation the next morning. Before the inoculation, both glands were manually emptied.

The tips of the teats were disinfected with a 70% alcohol compress. The teat canal of the treatment gland was catheterized before injecting 1 mL of the LPS solution. Immediately after inoculation, the cannula was removed, and the udder was massaged to facilitate diffusion of the inoculated solution. Measures collated during the inflammatory challenge (-72/-96h to +144h) were the following.

- somatic cell score as a local response phenotype, the somatic cell count (SCC) was measured from milk samples collected at 7 out of the 11 time points defined (-72/-96h; 6h, 24h, 48h, 72h, 144h from both udders, the LPS-inoculated udder (SCC_i) and the control udder (SCC_c). In addition, milk samples obtained at -72/-96h were subjected to analysis of the metagenome.
- For the analysis of the systemic response, we considered
  - rectal temperature at 11 time points (-24h, 0h, 2h, 4, 6, 8, 12, 24, 48, 72, 144h).
  - the analysis of 14 cytokines/chemokines in plasma related to the immune response (IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MIP-1α, MIP-1β, IL-36RA, IP-10, TNF-α, VEGF-A) which were analysed with the MILLIPLEX® MAP Ovine Cytokine/Chemokine Panel 1. For that, the plasma samples obtained from blood samples collected at each of the 11 time points considered and kept at -80ºC were sent to the laboratory of Dr. Foucras (ENVT, Toulouse, France) for analysis. These analyses were funded by a Spanish-funded project (RTI2018-093535-B-100 funded by the Spanish Ministry of Science and Innovation) and were only performed for 24 of the ewes included in the intramammary LPS-challenge (**N =24**; 13 NC and 11 C).
- gene expression data.: RNA was extracted at time points 6h and 24h after the LPS-inoculation (**N=20**):
  - from milk somatic cells (MSC)
  - from blood samples

  Sample collection and preparation was funded by smarter and analyses (RNA sequencing) was funded by the Spanish-funded project (RTI2018-093535-B-100 funded by the Spanish Ministry of Science and Innovation).

Longevity ALPINE goat lines

Animals. A total of 199 dairy goats belonging to the high and low functional longevity lines with phenotyping of longevity were produced within Smarter (year of birth 2019 and 2020). The production of the lines is fully described in Ithurbide et al. (2022). Briefly, following the method developed by Palhière et al. (2018), a genetic evaluation for functional longevity of 8,787 Alpine AI bucks was carried out. Briefly, length of productive life was computed for 84,454 Alpine goats as the time interval (in days) between first kidding (first milk recording) and the last milk recording registered in the national performance-recording database. Estimated breeding values for length of functional life were then estimated for AI bucks using BLUP based on phenotypic information, pedigree information, and variance component estimates. Sires of the 2 lines were also required to show similar and favorable EBV for milk production traits to avoid confounding effects from an indirect response to selection for production traits. In 2020, the EBV of the bucks used were +85.1 d of functional life for the bucks of high longevity (high_LGV) lines and -108.7 d for the bucks of low longevity (low_LGV) lines (Ithurbide et al., 2021). Using AI, we produced goats belonging to the high_LGV and low_LGV lines at the

experimental INRAE Farm (La Sapinière, Osmoy, France). Birth year of daughters in the full experiment ranged from 2017 to 2021, but only 199 goats belonging to year of birth 2019 and 2020 were funded by smarter. The smarter goats were sired by 23 AI bucks: 12 high_LGV and 11 low_LGV bucks. The average EBV were +104.7d (±95.5) and -99.2 (±119.4) in EBVs high_LGV and low_LGV bucks, respectively.

Nutritional challenge (N = 95). Out of the 199 dairy goats created, 95 underwent a nutritional challenge in early lactation (about one month after kidding) in 2020 (N = 49) and 2021 (n= 46). Each challenge consisted of a 7-d control period), 2-d of straw only feeding, and a 4-d recovery period. During the pre- and post- challenge periods, the goats received a ration based on Lucerne hay offered in collective troughs, complemented with concentrate that was dispensed in the milking parlour. All feeds were offered ad libitum, as was water.

Measures were collated during the nutritional challenge from day-7 to day +6, Day 0 being the last morning milking before the underfeeding challenge that started this day.

- Weight, as well repeated milk samples for further assessment of milk quantity and composition
- Blood samples were collected (at -7, -4, -1, 0, 1, 2, 3, 4, 5 and 6) were analysed for four metabolites : urea, glucose, beta-hydroxybutyrate (BHB), bilirubine and non-esterified fatty acids (NEFA) using a Cobas Mira-Analyzer (Roche, Mannheim, Germany) with commercial kits for urea (urea/BUN, Roche), glucose (Gluco-quant, Glucose/ HK, Roche), NEFA [NEFA-HR(2), Wako Chemical GmBH, Neuss, Germany], and BHB.
- Milk samples were collected (-7, -4, -1, 0, 1, 2, 3, 4, 5 and 6) during morning milking and concentrations of 14 milk metabolites were measured: glucose-6-Phosphate (Glu6P), glucose (Glu), galactose (Gal), beta-hydroxy-butyrate (BOHB), isocitrate, glutamate, NH2, lactate deshydrogenase (LDH), Urea, Cholin, Malate, urate, triacylglycerol (TAG), cholesterol (Chol). Each goat had 14 milk metabolite trajectories with 10 data points. These analyses were funded by a French-funded project (RESILAIT, Apisgene)

Infectious challenge (N = 87). From the total of 98 ewes submitted to the nutritional challenge only 46 underwent the infection challenge in 2021. Indeed, the experiment on the 49 goats in 2020 was aborted because it fell in the first COVID lockdown. As a remediation plan, a new experiment was carried out in RP4 on 41 additional primiparous goats with the financial support of a French funding body (APIS-GENE). As for UELEON, the inflammatory trial was adapted from the procedure described by Salvesen et al. (2016). Accordingly, we propose a general inflammatory challenge based on Lipopolysaccharide (LPS), a non-infectious compound of bacterial origin for mimicking mastitis-like inflammation. A pilot study was implemented on 5 goats (Autor. APAFiS #20629) carried out at INRAE, with intravenous injection of LPS. The protocol made it possible to trigger an inflammatory reaction of the goats which was accompanied by a rise in rectal temperature reaching on average +1.9°C (±0.77) degree between +3 and +6 hours after the LPS injection. The return to the normal state (temperature and respiratory rate) is observed between 12 and 48 hours after the start of the experiment. Accordingly, we challenged 46 goats in April 2021 and 41 goats in April 2023 with an intravenous injection (0.5 µg/kg live weight) at T0. The trial was performed under specific authorization number APAFIS#2019120512104160_v13

Measures collated during the inflammatory challenge (-72/-96h to +144h) were the following:

- Rectal temperature: 8 time points (T4, T6, T8, T10, T12, T24, T48 and T72)
- Diarrhoea (presence/absence): 6 time points (T4, T6, T8, T10, T12 andT24)
- 14 cytokines/chemokines plasma markers related to the immune response (IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MIP-1α, MIP-1β, IL-36RA, IP-10, TNF-α, VEGF-A) which were analysed with the MILLIPLEX® MAP Ovine Cytokine/Chemokine Panel 1. For that, the plasma samples obtained from blood samples collected at each of the 11 time points considered and kept at -80ºC were sent to the laboratory of Dr. Foucras (ENVT, Toulouse, France) for analysis. These measures were similar to the immune measures implemented in the ASSAF milk production sheep lines.

In addition, milk bacteriology during lactation and blood metabolites around kidding were measured (by ENVT and INRAE, respectively)

- milk bacteriology: primiparous goats were sampled between 2018 and 2021 (total equal to 1469 qPCR results for 216 primiparous goats after quality check)
- blood metabolites: A total of 174 primiparous goats were studied between 2018 and 2021. Jugular plasma collected on wk -4, -3, -2, -1 relative to expected parturition, and wk 1, 2, 4, 13, 24, 33 of lactation was analyzed for NEFA, BHB, glucose, urea and bilirubin.

Parasite resistance ROMANE sheep lines

Animals (N = 192). We used ewes from a divergent selection experiment for resistance to gastro intestinal parasites. Data came from 91 females from the second generation (G2) of divergent selection and from 91 of the fourth generation (G4). The divergent selection experiment is detailed in Sallé et al. (2021). At each generation, we collected Faecal egg count (**FEC**) as a measure of parasite resistance following a protocol of artificial infection with third-stage larvae (L3) of *H. contortus* from the strain 'Humeau'. The protocol was made up of two successive infections of 10,000 L3/sheep and lasted 11 weeks: first, naïve lambs were infected to stimulate a primary immune response; 4 weeks later they were treated (0.2 mg/kg of live weight of ivermectin; Oramec, Boerhinger Ingelheim, Lyon, France); after 2 weeks of recovery they were re-infected to simulate a secondary immune response and finally treated 5 weeks later. At the end of first and second infection, FEC was recorded just before treatment. EBVs of these two FECs were computed using a model including fixed effects (lamb age, group pen, body weight, litter size, and sex) and an individual random effect estimated from the pedigree relationship matrix. The two EBVs per animal were then combined and used to select in each line 2–5% of males with most extreme EBVs as fathers for the next generation (Sallé et al., 2021). Mating was planned to limit inbreeding. In G2 sheep, the divergence in FEC between resistant (R) and susceptible (S) sheep reached 1.9 phenotypic SD and 3.8 genetic SD calculated from the initial population (G0) (Sallé et al., 2021). The 91 G2 females were born between August 29th and October 10th 2017. The 91 G4 females were born between March 5th and March 15th 2021.

Infectious challenge (n = 48 to 91). Responses to an infectious challenge were at the basis of the lines selection. In addition to the infection protocol previously described, we assessed lines responses to infectious challenges with *H. contortus* under other infection conditions.
For G2 females, the different infection conditions that we used are described in details in Douhard et al. (2022). Briefly, our overall experimental design included artificial infections with the Humeau strain of *H. contortus* in four stages over the first two years of life (between February 2018 and March 2020):
(1) at 4-5 months of age, all **91** female lambs went through the infection protocol used to phenotype parasite resistance (as previously described)
(2) at about one year of age, **48** of those females (24 R + 24 S) went through a second phase of infection. This phase lasted 15 weeks, started about 4 weeks before lambing (peripartum) and was based on a trickle infection (about 1000– 2000 L3/ewe/week during the first 9 weeks)
(3) at about 21 months of age, **81** females (out of the 91 initially included) received a single-dose of L3 in early pregnancy. This infection lasted 5 weeks before treatment.
(4) at about two years of age, **55** females were infected around lambing. This was similar to the second phase of infection during the periparturient period at 1 year of age, except that instead of a trickle infection, a single-dose of 10,000 L3 was given to initiate the infection.

For G4 females, the infection protocol used to phenotype parasite resistance at 4-5 months of age was set up except that a different strain of *H. contortus* was used (susceptible isolate, Weybridge, UK; Roos et al., 1990). In addition, 2/3 of the female lambs were infected in each line whereas 1/3 was kept uninfected and made up the control groups. This phase of infection took place in July and August 2021. At about two years of age (in December 2022), 48 of the G4 females (24 R and 24 S) will be infected in peripartum as in the fourth phase of infection of the G2 females. During this phase, ewes from lines divergently selected on feed efficiency will be infected simultaneously and in the same conditions than R and S lines (see part 1.1.5).

During each phase of infection, two infection traits (FEC and blood haematocrit) and three condition/production traits (body weight, backfat and muscle thickness) were measured at least weekly. FEC was measured using the modified McMaster technique. On each day of faecal sampling, blood was also taken from the jugular vein to measure blood haematocrit by the micro-haematocrit centrifugation technique. We performed dorsal ultrasound scans to measure back fat and muscle thickness on the left and the right at the 12th– 13rd lumbar vertebra (Easi- Scan™, IMV imaging).

In addition, during the infection protocol used to phenotype parasite resistance at 4-5 months of age, voluntary feed intake (kg/day) was recorded individually as feed concentrate was distributed *ad libitum* by automatic feeders.

Nutritional challenge (N = 48). When R and S ewes were infected, they were generally well fed: energy-rich diet provided *ad libitum* during ewe lambs infection and protein-rich diet during adult ewes infection. However, we tested the effect of a protein restriction in the second phase of infection associated with the periparturient period of G2 females. Indeed, it is well known that the periparturient relaxation of immunity usually occurring in ewes can be stronger when the amount of dietary protein is restricted. We then assessed if this nutritional effect is different between the R and S lines. In each line, half of the ewes (n = 24) were fed a low-protein concentrate to meet 70% of ewes

protein requirements. The other half (n = 24) were fed a high-protein concentrate to meet 120% of ewes protein requirements.

Parasite resistance CORRIEDALE sheep lines

Animals (N = 120/year). The animals from the study come from two genetically divergent selection lines for gastro intestinal parasites resistance (resistant R, susceptible S). The lines are based on animal selection by FEC Expected Progeny Difference (EPD) after natural mixed infection and grazing native pastures at Dr. Alejandro Gallinal Experimental Research Centre of SUL since 1998 (Castells and Gimeno, 2011). After screening for low or high FEC EPD of 3,545 progeny lambs, the nucleus flock was established. More than 150 ewes were annually mated in the R-Line and 120 ewes in the S-Line, and at least five sires were used in each line per mating. Three FEC measurements (FEC1, FEC2, and FEC3) were recorded post weaning and after natural nematode challenge of different cycles: summer (December 21 to March 20), autumn (March 20 to June 21) and winter (June 21 to September 22). Post-weaning and production traits (body weight and wool) and complete pedigree were also recorded each year. The average number of lambs registered per year were 40 and 80 for the S and R lines respectively. Additionally, for R-Line some rams from stud-flocks were used.

Infectious challenge (N = 67) The experiment is fully described in Ferreira et al. (2021). This study aimed to determine whether genetic selection for resistance to gastrointestinal nematodes would alter the feed intake and feed efficiency of sheep with or without an infection of *Haemonchus contortus*. *Methods*. Sixty-seven Corriedale lambs (357±14 days old) derived from flocks genetically selected to be resistant (n = 29) or susceptible (n = 38) to gastrointestinal nematodes were evaluated for individual dry-matter intake, feed conversion ratio and residual feed intake). Considering body weight, genetic lines and sires, males were allotted to one of three outdoor pens and females to one of two, each pen being equipped with five automated feeding systems and two automatic weighing platforms to record individual feed intake and BW. Feed (lucerne haylage, crude protein 20.5%, metabolisable energy 9.2 MJ/kg DM) and water were offered ad libitum. The experiment was conducted in two periods. First, animals were maintained worm-free (14 days of acclimatisation and 44 days of records) and then, in Period 2 (42 days), animals were artificially infected with 6000 L3 of *Haemonchus contortus*. Worm egg counts were recorded on Days 9, 23, 27, 30, 42 post-infection.

Feed efficiency ROMANE sheep lines

Animals (N = 90). We used Romane sheep lines divergently selected on residual feed intake (**RFI**). The divergent selection is described in details in Tortereau et al. (2020). Briefly, RFI was measured during a 6-week period, starting at 60-90 days of age. Lambs had *ad libitum* access to low-energy concentrated pellets containing 15.7% protein, 12% cellulose, 15% starch, 2.5% fat and 3.9% sugar (same diet than for the R and S lines during the infection protocol at 4-5 months of age). Daily feed intake was averaged over the 6-week testing period to estimate the average daily feed intake (ADFI). The individual RFI was estimated as the residual of the multiple linear regression of ADFI on average daily gain of body weight, backfat and muscle thickness at the end of the testing period to account for production requirements, and on the metabolic body weight ($BW^{0.75}$) at the end of the test to account for maintenance requirements. EBVs of RFI were calculated and used to select in each line ~10% of males with most

extreme EBVs as fathers for the next generation (Sallé et al., 2021). The 90 females used in the experimental design here (46 high-RFI and 44 low-RFI) were from the 4th generation of selection and were born between November 23rd, 2020 and January 6th, 2021.

Infectious challenge (N= 90) (authorizationnumber APAFIS#2020081909587718_v1)

The experimental design was the same than for the parasite resistance Romane sheep. At about 4-5 months of age, 2/3 of the ewe lambs of each line went through the infection protocol previously described to phenotype parasite resistance in lambs whereas 1/3 was kept uninfected and made up the control groups. This took place in May and June 2021

At about two years of age (in December 2022), 50 females (25 high-RFI and 25 low-RFI) were infected during the periparturient period simultaneously and in the same conditions than R and S lines (see part 1.1.3).

Data analysis

Mixed model analyses

Classical mixed model analyses were used to quantify the extent of genetic control (line effect) and nutritional challenge affecting efficiency and resilience phenotypes in the five experiments.

Sparse partial least squares-discriminant analysis (sPLS-DA)

Complementary methods were applied to multivariate and multitrait datasets generated upon challenge (UELEON and INRAE).
For the analysis of the 14 cytokines/chemokine upon the inflammatory challenge in Assaf sheep lines (UELEON), we also performed a sparse partial least squares-discriminant analysis (sPLS-DA) with the "mixOmics" package (Rohart et al., 2017). sPLS-DA seeks the components that best separate the sample groups, and also selects variables that best discriminate between groups using lasso penalization.
Similarly sPLS-DA combined with Functionnal Principal Component Analysis, was used to analyze the responses to the nutritional challenge in the Longevity Goat lines (INRAE) (95 goats* 14 metabolites * 10 time points). First, we fitted the individual milk metabolite curves using the functional data analysis smoothing method (with natural cubic splines) described by Ramsay and Silverman (2005). The batch effect that we observed between the two years experiments was then corrected by running a functional regression analysis. Using functional PCA (R package "FDA"), each individual milk metabolite curve was then characterized by two to four PCscores. We then used Sparse PLS-Discriminant Analysis (sPLS-DA) to evaluate the ability of milk metabolites PCscores to distinguish the longevity lines with the "mixOmics" package (Rohart et al., 2017). A preliminary analysis with all goat (N=95) showed high variability in response according to the age of the animal. Therefore, we ran a second analyses with the reduced set of goats of one year of age (N=69).

RNA-seq analyses

Gene expression upon LPS challenge (UELEON) in sampling of milk cells and blood samples were analysed. First, to assess time changes upon LPS challenge, pair-wise comparison analyses have been done with the three time point available for the Control animals (not subjected to the diet restriction). Different pair-wise comparisons were performed with DESeq2 software (0h vs 6h; 0h vs 24 h; 6 vs 24h). The same contrasts were subjected to an analysis based on the Wilcoxon test using R software. The DEGs commonly identified by these two analyses were considered in further enrichment analyses. Then, to assess the effect of the nutritional challenge group on the transcriptome after the LPS, we focused on the RNA samples extracted at time point 6h from milk cells, which corresponded to the peak of SCC. Differential expression analysis for detection of DEGs between the NC and C groups of the nutritional challenge experiment (12 NC *vs* 8 C) were then performed using the DESeq2 software (Love et al., 2014).

4. Results

Divergence of lines

Milk production ASSAF sheep lines

Of the 76 animals initially purchased, six did not reach mating age, and 21 ewe-lambs did not become pregnant with AI flock and therefore could not be included in the intensive sampling period as they lambed one month after the main group. Finally, 49 animals were available for the intensive sampling period. We thus sampled 40 animals, to created two groups with extreme EBVs for milk production. The EBV distribution for milk production allowed us to make the following differences: high_EBV with an average EBV of 86.5 ± 3.4, and low_EBV with an average EBV of 37.4±3.5. The Controls and nutritional challenge (NC) sheep were balance in the two extreme EBV groups with EBVs equal to 58.7 ± 7.1 and 62.6±6.1, respectively. A description of the basic statistics of the EVBs is provided in **Table 2** below.

Table 2. Average, standard deviation (SD), standard error of the mean (SEM), maximum (Max), and minimum (Min) EBV values in each of the groups considered. The distribution of control (C) and nutritional challenge (NC) animals are equal in the two groups (high vs. low) of estimated breeding values (EBV) for milk production.

Group	N	Average Milk EBV	SD	SEM	Min	Max
High_EBV	20	86.45	15.19	3.40	60.5	109.5
Low_EBV	20	37.38	15.45	3.46	5.5	57.5
C	20	58.66	31.68	7.08	5.5	109.5
NC	20	62.63	27.07	6.05	9.0	109.5

NB. The nutritional challenge experiment of those 40 sheep was also used in WP1 to investigate feed efficiency, so the experimental design was also previously described in Deliverable D1.1.

Longevity ALPINE goat lines

The production of the lines is fully described in Ithurbide et al. (2022). The analysis of survival in the full design (including also previously created goats) showed that the overall survival of high_longevity goats (**Figure 1**) was significantly better than low_longevity goats (hazard ratio of culling/death = 0.63, confidence interval = 0.47; 0.86).
The 95 challenged goats in Smarter were selected from the 199 longevity line goats born in 2019 and 2020. These smarter goats were sired by 23 AI bucks: 12 high_LGV and 11 low_LGV bucks. The average EBV were +104.7d (±95.5) and -99.2 (±119.4) in EBVs high_LGV and low_LGV bucks, respectively. Therefore the average difference between sires (203 days) was similar than in the full design described in Iturbide et al. (2022), i.e. 194 days (about 1.6 genetic standard deviation). In goats, despite the limited number of animals, the difference in survival was significantly different (**Figure 1**) and represented about 0.4 genetic SD.

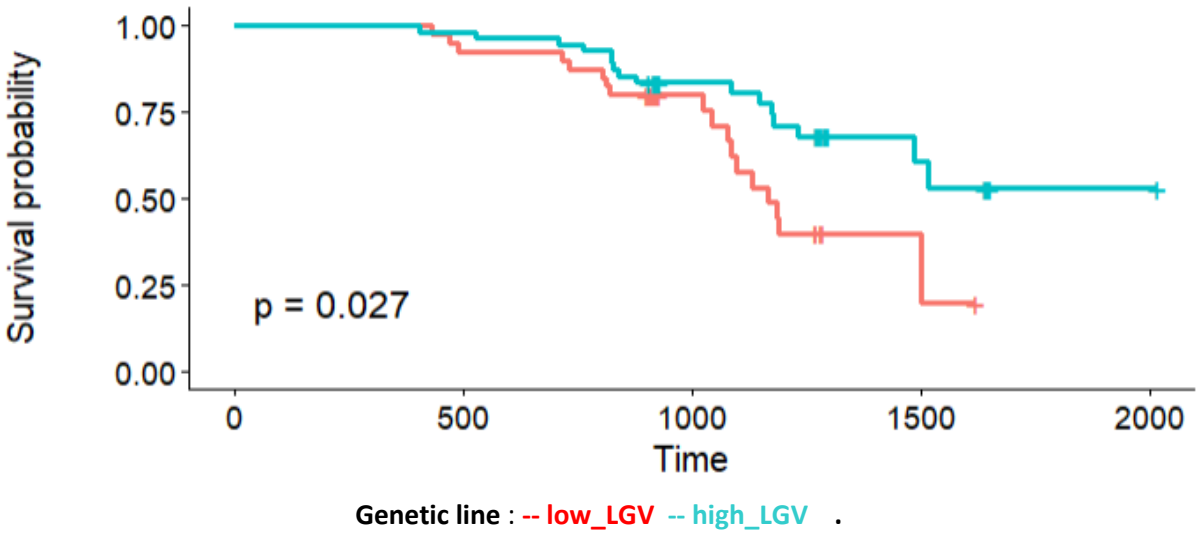


Figure 1. Plot of Kaplan Meier curve showing survival against time for low longevity line (low _LGV) and high longevity line (high_LGV) groups.

As expected, analysis of milk bacteriology with mixed models confirmed an increase in the quantity of bacteria (Staphylococci) between the beginning and end of lactation (+0.36 in log). Differences between years were also significant. However, no significant differences were found between longevity genetic lines. These results may seem contradictory with the difference in inflammation (Milk SCS) between the lines (Ithurbide et al.,2022). One hypothesis could be that longevity lines differ in inflammatory response and resilience to infection, but not in resistance to intramammary staphylococci.

Additionally, we analysed blood metabolic adaptations peripartum (Pires et al., conference EAAP 2023). Data were analyzed using SAS mixed models with repeated measures, including strain, litter size (LS), wk, and interactions as fixed effects, and year and goat (year) as random effects. Significant wk

effects were observed for all metabolites. Low_LGV goats had greater plasma NEFA on wk-3 (181 vs 123 µM; strain x wk prepartum: P= 0.05), and greater BHB prepartum (0.45 vs 0.41 mM; strain effect: P = 0.04) than high_LGV which denotes greater fat mobilization and partial oxidation in late gestation in LGV-. 35% of goats carried multiple fetus (LS2+) and LS not differ with LGV. Prepartum plasma NEFA, BHB and bilirubin were greater for LS2+ compared to single (LS1; P < 0.001; 260 vs 174 µM; 0.51 vs 0.39mM; 0.069 vs 0.056 mg/dL, respectively), whereas glucose was lower for LS2+ (P < 0.001; 49.8 vs 54.7 mg/dL). Conversely, plasma NEFA was greater for LS1 during wk 1 and wk 2 postpartum (P < 0.05; 558 vs 442 on wk 1, and 421 vs 330 µM on wk 2, respectively), reflecting greater availability of body reserves to support lactation in LS1. Prepartum incidence of BHB > 0.80 mM was significantly greater for LS2+ than LS1 (30 vs 3.7%), and for low_LGV carrying LS2+ than high_LGV carrying LS2+ (42 vs 28%). Marked LS effects were observed in plasma metabolite profiles in primiparous goats. In conclusion we showed that longevity goat lines differ in their metabolic adaptations peripartum.

Parasite resistance ROMANE sheep lines

We used data of 91 females from the second generation (G2) of divergent selection and of 91 of the fourth generation (G4). The divergence between the Resistant (R) and Susceptible (S) lines was estimated from the 91 females in G2, as described in the published paper by Douhard et al., 2022. The 91 females were produced from seven G1 sires (3R and 4 S), and mated with 37 G0 rams (22 R and 15 S) and 32 G1 rams (14 R and 18 S). At G2, the divergence in FEC between R and S sheep reached 1.9 phenotypic SD and 3.8 genetic SD calculated from the initial population (G0) (Sallé et al., 2021). Achieved genetic divergence between sheep lines across generations is shown in the **Figure 2** below.

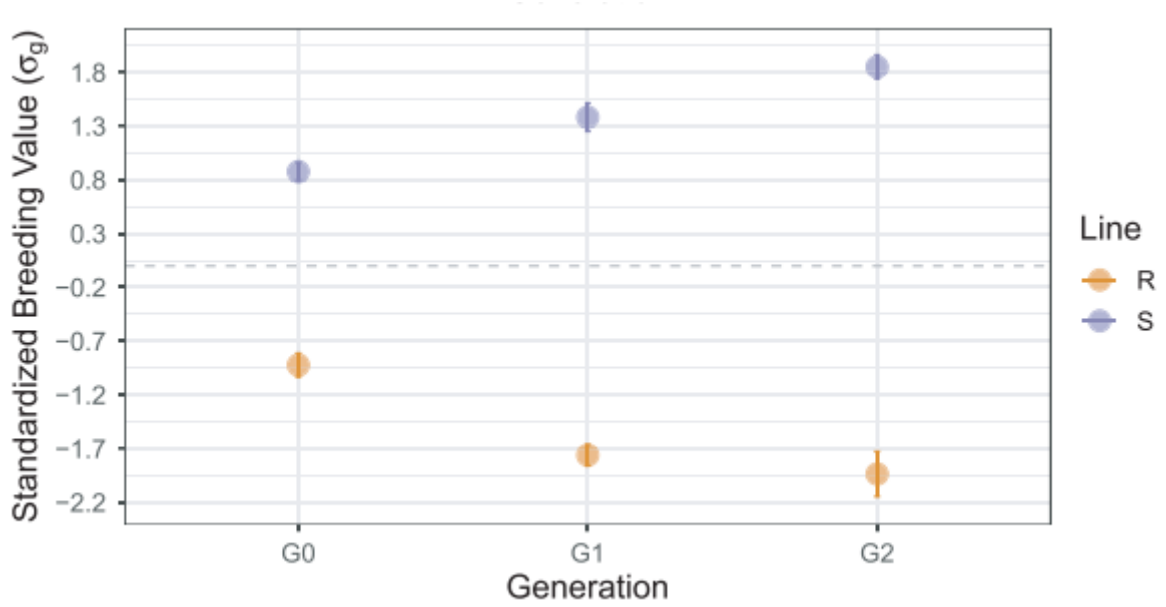


Figure 2. Achieved genetic divergence between sheep lines across generations. Estimated breeding values (eBVs) (using the pedigree information only) were calculated for corrected average faecal egg counts (mean ± SE) observed across two infections for parental (G0), first (G1) and second (G2) generations. Grey dashed line indicates G0 mean value. Reproduced from Sallé et al. 2021.

Parasite resistance CORRIEDALE sheep lines

In the parasite resistance Corriedale sheep lines, the difference in average EBVs between the first (2000) and last generation (2019) of the R-line represented genetic progress of 15% (**Figure 3**), indicating annual genetic progress of -0.71% per year. Average difference between R and S lines for the SMARTER sheep (N =67; born in 2017) was equivalent to 0.75 genetic SD (**Figure** 3).

When analyzing R-line FEC average breeding values of the progeny 2019 in comparison with the rest of the Corriedale population in 2019, a genetic difference of 11% was observed. This distance between the R-line and the rest of the Corriedale population would be between 5 to 10 years of selection. Considering means of the three records FEC1, FEC2, and FEC3 (at 210±33, 298±43, and 391±53 days of age respectively) for the last five years (2015-2019 drop) the values for the S-Line were 1.31, 2.47, and 3.26 times higher than the R-Line, respectively (**Figure 4**). Marked differences were observed between years depending on climatic and epidemiological conditions. Additionally, those phenotypic responses were also observed on lactating ewes during the spring rise 73 days after lambing. The FEC means of R-Line ewes was 395, and 660 for the S-Line (Castells and Gimeno, 2011). It is concluded that by selecting for decreased FEC EPD it is possible to achieve considerable progress, which is reflected, in addition to the genetic trends, in lower infestations in lambs and ewes at spring rise. This experimental flock represents a valuable demonstration population where breeders can see the genetics in practice.

This results together with results from other research flocks were presented at Ciappesoni et al. (2022) and Navajas et al. (2022).

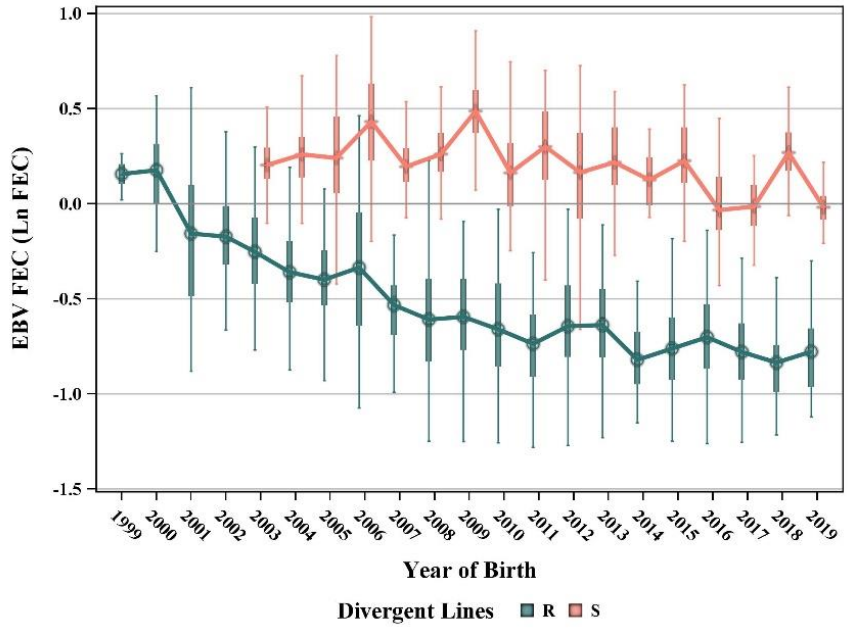


Figure 3. Genetic trends of FEC EBV in Corriedale parasite resistance divergent lines (Resistant -R and Susceptible -S) from the Uruguayan Wool Secretariat

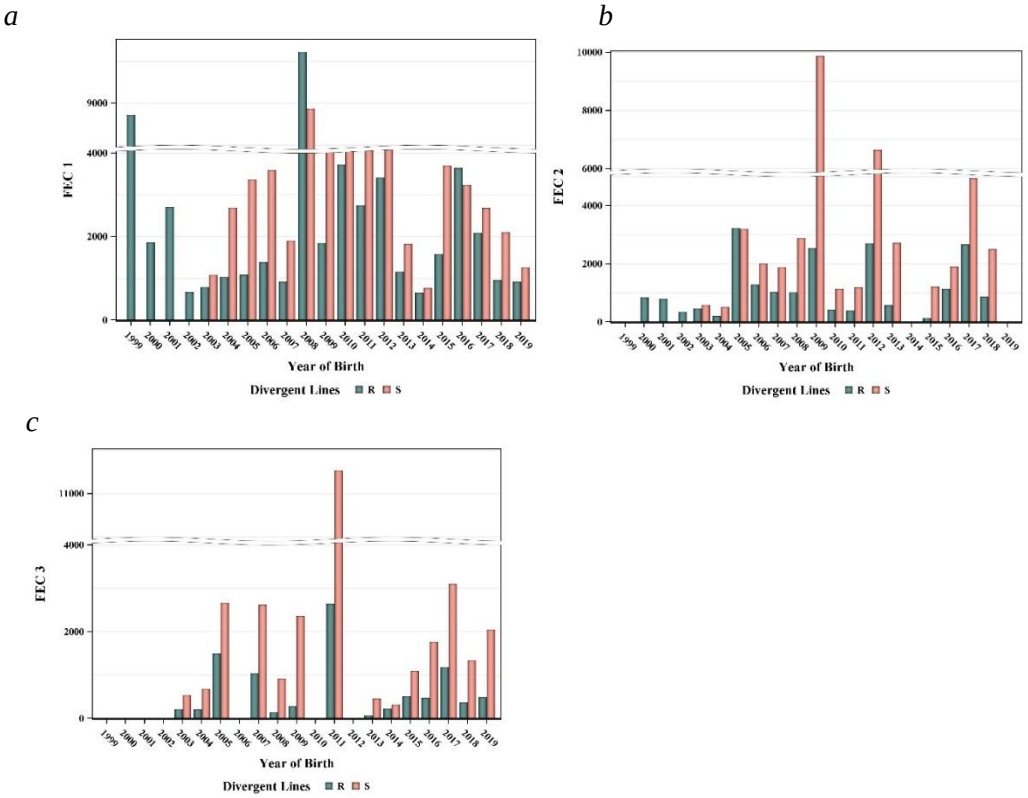


Figure 4. Phenotypic average for FEC 1 (a), 2 (b), and 3 (c) of Resistant (R) and Susceptible (S) lines from 1999 to 2019 progeny from the Uruguayan Wool Secretariat.

Feed efficiency ROMANE sheep lines

After four generations of selection, the divergence between Romane males from the low-RFI and high-RFI lines reached ~140g/d (i.e. 1.9 genetic standard deviation). This divergence in RFI results in a difference of concentrate intake, with low-RFI animals eating 130g of concentrate per day less than high-RFI animals, which is a decrease by 5.9% of feed intake.

The distribution of residual feed intake (RFI) per generation and line is given in **figure 5**.

The 85 females involved in the infectious challenge are half-sisters of males from the 4th generation of selection.

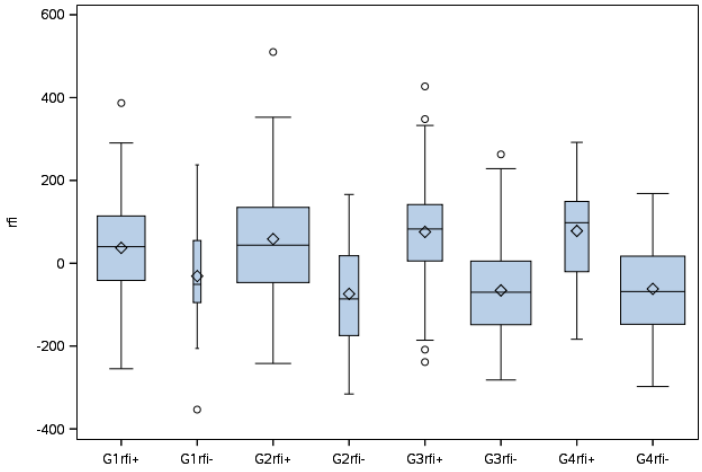


Figure 5. Distribution of phenotypic residual feed intake (RFI), in g/d, per generation of selection and divergent line. RFI+ and RFI- stand for high-RFI and low-RFI divergent line, respectively.

Effect of nutritional challenge

Two of the experiments (Parasite resistance CORRIEDALE and Feed Efficiency ROMANE sheep) had no specific nutritional challenges, but the interaction between nutritional and infection challenges was assessed by monitoring feed intake and efficiency regularly around the infection challenge.

Additionally, the nutritional challenge of the 40 ASSAF sheep were also used in WP1 to investigate feed efficiency. Therefore, the experimental design, phenotyping and analyses of the nutritional challenge were previously described in Deliverable D1.1. Here, we will focus on the impact of the nutritional challenge on the response to infection (N=20 to 30) as foreseen in the DOA.

Accordingly for these three experiments, the results will be reported in the section 1.5 “Interaction between nutritional and infection challenges”.

Below we describe the responses to nutritional challenges of the longevity ALPINE goat lines and for the parasite resistance ROMANE sheep.

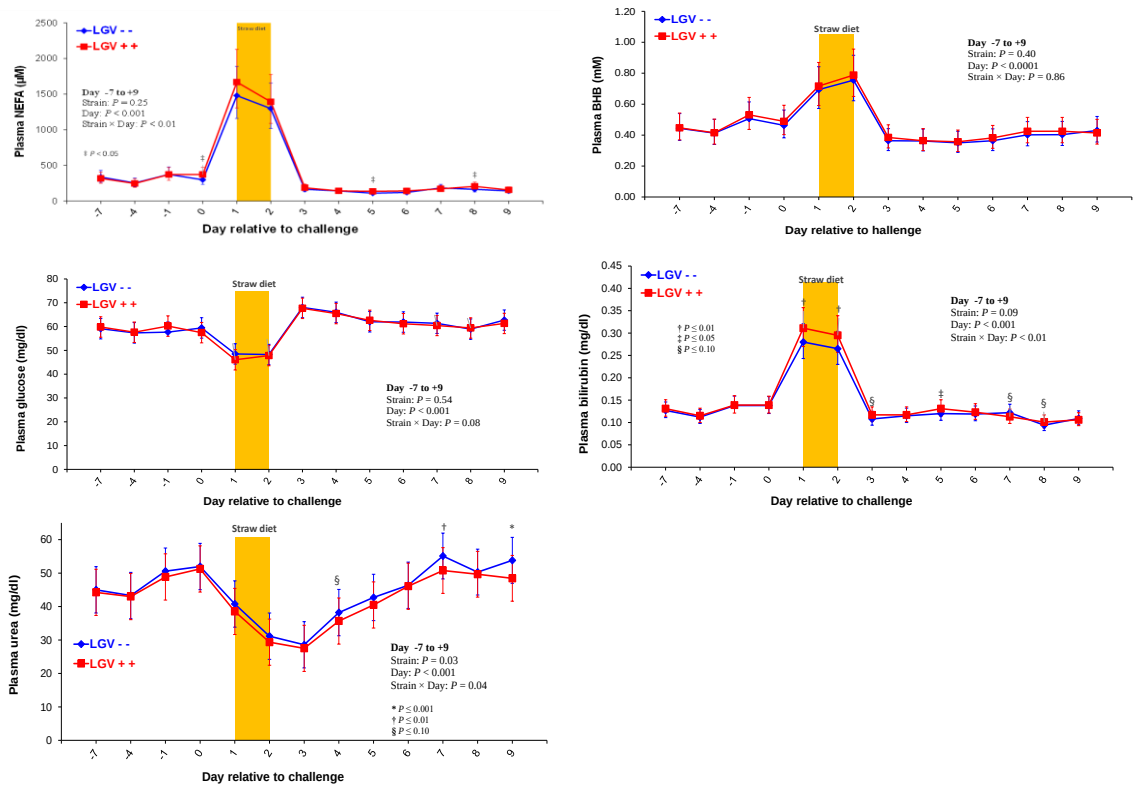
Longevity ALPINE goat lines

We characterized the response to the nutritional challenge in the two genetic goat lines on milk production traits, based on milk production phenotypes and metabolites measured in blood and milk. The study on profiling of milk metabolites was published in J. Dairy Sci (Ithurbide et *al.*, 2023).

Milk production and SCC and blood metabolites upon nutritional challenge

Using mixed model analyses, no difference ($p<0.05$) between longevity lines were observed for milk production, weight, somatic cell counts or blood metabolites, except for bilirubin. Results are presented in **Figure 6** for the five blood metabolites (urea, glucose, BHB, NEFA and bilirubin). Data

came from 496 (2020) and 626 (2021) blood samples analyzed in the frame of Smarter as well as additional samples collected in 2018 and 2019 from the same experiment design (background data). So, the total was 2367 samples from 156 goats that were analysed with a mixed model including the fixed effect of line and day relative to challenge and the random animal effect. A complete model including also the effect of year and litter size did not modify the results. Bilirubin was different between goat lines only at D+1 and D+2 after the challenge.

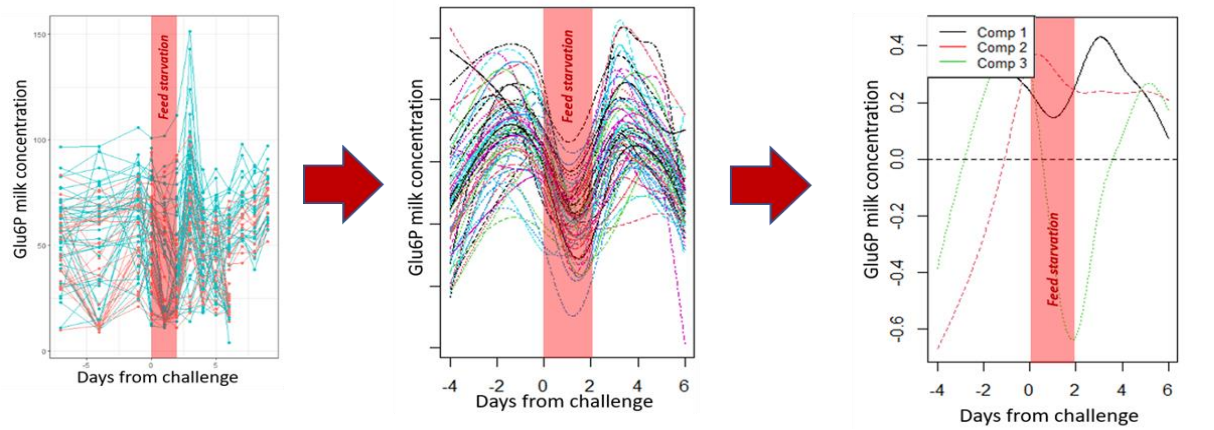


Genetic line (=strain) : -- high_LGV -- low_LGV

Figure 6. Average (LSmeans ± SE) profile for 5 blood metabolite profiles upon nutritional challenge in the two longevity ALPINE goat lines (strain).

Profiling of milk metabolites upon nutritional challenge in the longevity goat lines.

Using functional principal component analysis, it was possible to summarize the 14 milk metabolite profiles upon nutritional challenge with two to three components per metabolite (explaining at least 80% of variation), resulting in a total of 35 variables (**Figure 7**) for the Sparse PLS-DA



A B C

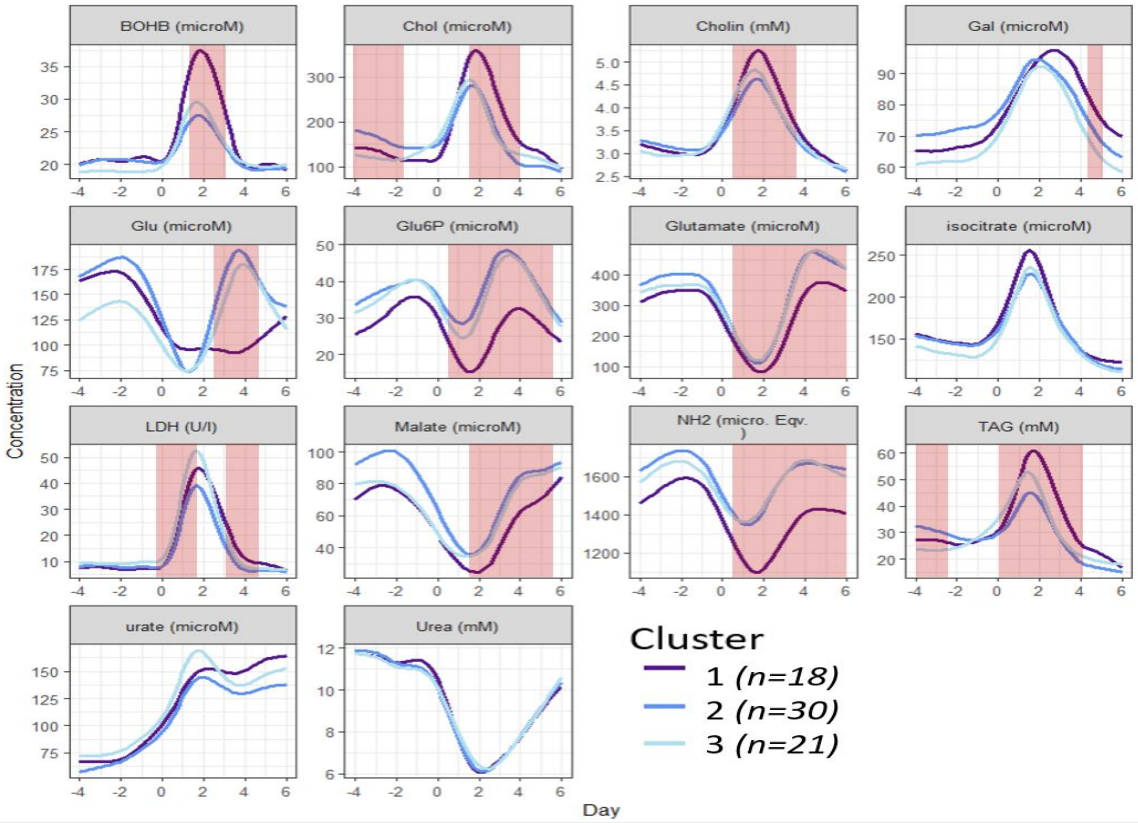
Figure 7. Functional principal component analysis results for one milk metabolite (Glu6P) through the underfeeding challenge. **A**-Individual raw data, each line represents 1 goat. Colors represent lines. **B**-Estimation of the mean curve by functional smoothing. **D**- elaboration of 3 PCscore by functional Principal Component Analysis (underlying Eigenfunctions)

We then applied the supervised prediction (sPLS-DA) to the 35 fPCscores of milk metabolites to discriminate lines of goats for functional longevity. The prediction were done using the Smarter goat lines from Bourges, and additional longevity line goats created with the same sires in another facility (Paris, outside smarter). Very little difference was found between lines This result show that the metabolite curves in response to a short challenge did hardly explain differences between the longevity lines but suggests that other biological factors are also responsible for part of the differences in longevity. So, we further decided to explore the large overall variability of milk metabolite curves with an unsupervised clustering. This resulted in 3 clusters of goats defined by different metabolic responses to underfeeding (**Figure 8A**). One of these clusters was associated with significant differences in survival (lower survival, **Figure 8B**). The metabolite curves that are different between clusters are: Glu6P, BOHB, and a few other metabolites.

These results support that a modulation of the metabolic response to nutritional challenge is associated with difference in longevity and probably resilience. Furthermore, multivariate analysis of non-invasive milk measures shows potential for deriving new resilience phenotypes. Finally, this analysis suggests that the physiological mechanisms underpinning differences in resilience are related in part to lipid metabolism.

Preliminary results were presented at WCGALP conference in July 2022 (Ithurbide et al., 2022b) and complete description of the study was published in J. of Dairy Science (Ithurbide et al., 2023).

A



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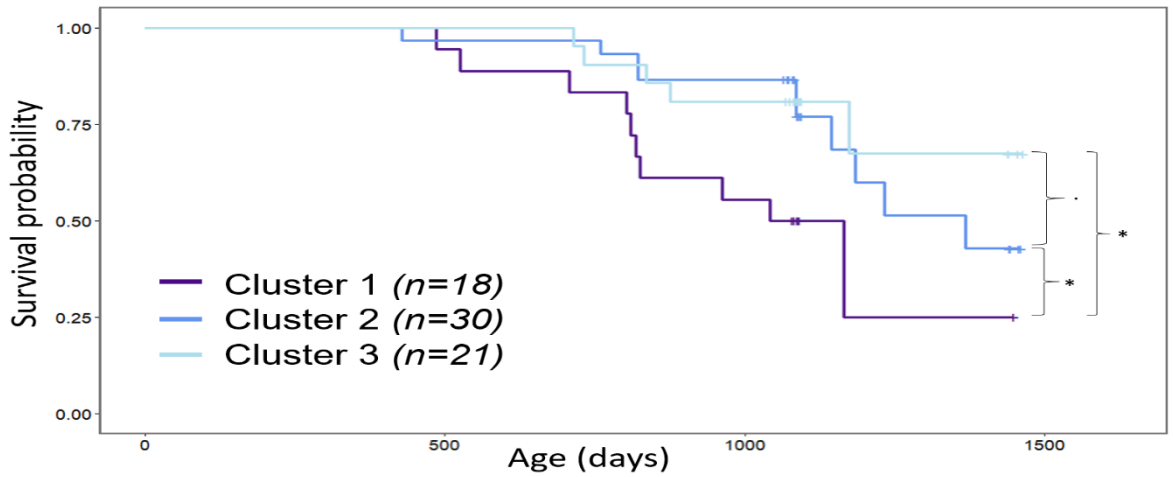


Figure 8: A) Mean curves of the milk metabolites within the 3 clusters identified by unsupervised clustering in 138 goat [Bourges (smarter) + Paris (non smarter)] through a 2-day underfeeding challenge. These curves are corrected for the year x facility effect with a functional linear regression. The red area indicate the time period during which the variables are significantly different between clusters (permutation test, 5% critical value). **B)** Plot of Kaplan–Meier curve showing survival against time for the 3 clusters of goats identified by unsupervised clustering. Data on survival were only available for P3R Bourges facility (n=69 goats). The cox model analysis showed significantly poorer survival of the cluster 1 over cluster 2 and 3 (* $p<0.05$).

Protein restriction had clear negative effects on condition traits regardless of the lines (**Figure 9**). Lactating ewes fed with the low- protein diet were 2.87 ± 1.43 kg lighter than those fed with the high-protein diet. Backfat and muscle thickness were also reduced during pregnancy (BFT: 4.71 ± 0.15 mm for high- protein vs. 4.33 ± 0.16 mm for low- protein; MT: 22.5 ± 0.26 mm for high- protein vs. 21.4 ± 0.26 mm for low- protein), but no longer after lambing. At birth, lambs born from protein-restricted ewes were about 10% lighter than those born from unrestricted ewes. During lactation, protein-restricted ewes had lower milk fat content (difference = 17.1 g/L, t_{46} = 3.89, $p < 0.001$); however, only lambs from the S ewes were growing slower.

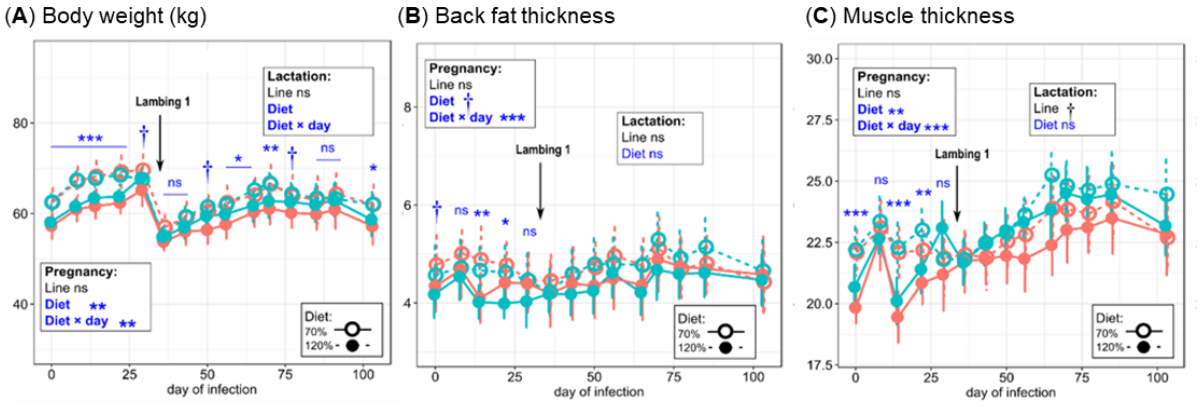


Figure 9. Effect of protein restriction on the condition traits of ewes from lines selected for parasite resistance (blue) or susceptibility (red) during artificial infection with *Haemonchus contortus* one month prior to lambing until 2 months post-lambing. Condition traits included body weight (A), backfat thickness (B) and muscle thickness (C). Circles are adjusted means with their error bars representing 95% confidence interval. Asterisks indicate statistical differences between lines (†: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Effect of infection challenge

Results from infection challenges were not presented here for the CORRIEDALE (INIA-UY) parasite resistance lines sheep as these results were already presented in the "Divergence of line" section.

LPS inoculation in milk production ASSAF sheep lines

A comprehensive analysis of the inflammatory challenge in the milk production ASSAF sheep lines (UELEON), including clinical signs, SCS and cytokines as well as RNAseq, showed differential gene expression related to immune response. The latter analysis was published in Veterinary Science in 2023 by Pelayo et al.

Evolution of clinical signs, inflammation and cytokines/chemokines after LPS inoculation.

After the LPS-inoculation, mild signs of local inflammation (redness and pain on palpation) were observed in the LPS-inoculated half-udder, whereas no signs of local inflammation were observed in the contralateral control half-udder. The graphical evolution of logSCC, as a local indicator of local inflammation and the evolution of the body temperature and the 14 plasma biomarkers analysed are given in **Figure 10AB** across the different sampling points considered in the study.

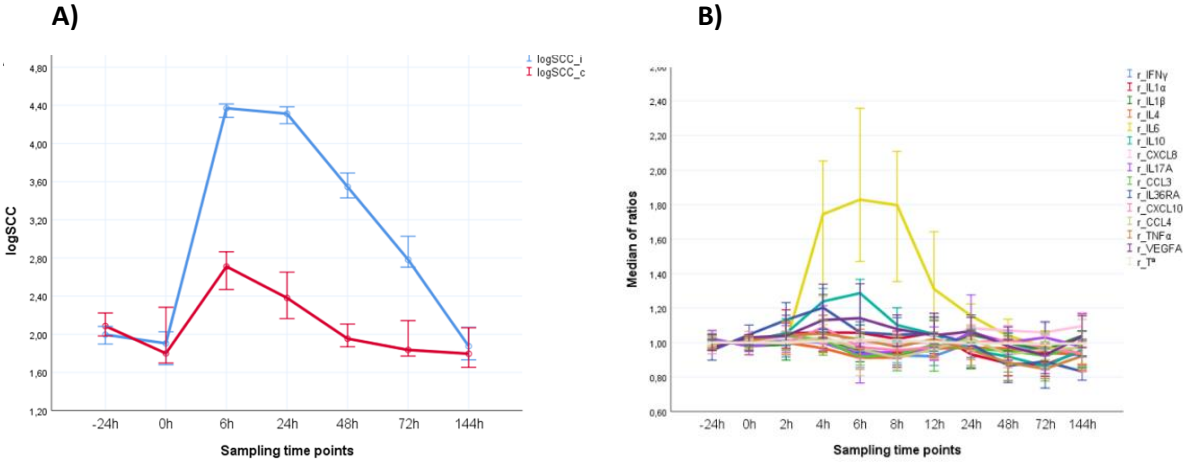


Figure 10A: *A) Graphical representation of the logarithm of somatic cell count (logSCC) measured in both the* LPS-inoculated *udder (logSCC_i) and the contralateral control udder (logSCC_c) across the seven milk sampling points. Error bars show the 95% confidence interval of the median. B) Graphical representation of the systemic ratio traits defined for the rectal temperature (r_Tª) and the 14 cytokine/chemokine across the 11 time points considered in this study. Error bars show the 95% confidence interval of the median. Note that the analysis of the cytokine/chemokine markers was funded through the Spanish National project EpiMilkSheep (RTI2018-093535-B-100).*

The GzLM analyses performed provided a statistical assessment of the relevance of the dynamic changes of those traits observed across the sampling points analysed. Trait were expressed as ratio trait (r_) referencing to the basal measure (estimated as the average between the 24h and 0h values for the corresponding trait). Briefly, time points 6 h, 24 h, 48 h, and 72 h were highly significant ($P < 0.005$) for the r_logSCC (LPS inoculated) trait, whereas for the r_logSCC(control) trait some sampling time points were also significant (0 h, 6 h, 24 h, and 144 h). For the systemic traits considered, the most significant time points for the r_Rectal_temperature, were 6 h and 8 h ($P < 0.000$). Regarding the cytokines/chemokines, the most significant time points were identified for r_IL-6 (6 h, 8h; $P < 0.000$) and r_IL-10 (4 h, 6 h for $P < 0.000$). Other significant time points were detected for r_VEGF-A (4 h, 6 h; $P = 0.002$), r_IL-36RA (2 h, 4 h; $P = 0.001$) and r_CXCL8 (24 h; $P = 0.001$). Preliminary results of these analyses have been presented in the 12th WCGALP Congress (Pelayo et al., 2022).

The genetic group (high or low EBV for milk production) showed significant effect on the r_logSCC(LPS inoculated) trait and the r_CXCL8 and r_CCL4 plasma biomarker traits. The Low-EBV class, compared with the High-EBV class, was associated with a positive estimate of the r_logSCC(LPS inoculated)trait, and negative estimates of the other two mentioned traits. Based on our time-point analysis, CXCL8 had been detected as a later biomarker of inflammatory response in the ovine mammary gland.

As a summary, in response to intramammary LPS inoculation, the SCC local indicator trait, rectal temperature, and five plasma biomarkers showed significant changes in basal levels across the dynamic analysis (IL-6, IL-10, CXCL8, IL-36RA and VEGF-A). Differences between genetic groups selected for milk production were found for SCS, and two cytokines : CXCL8 and CCL4.

Dynamic RNA-Seq changes in the MSC transcriptome after LPS inoculation

The results from all the comparisons performed for the three time points considered showed clear differences in the expression profile of the different time points (**Figure 11**). Considering all the comparisons performed (and only the commonly detected DEGs by the two methods applied) and also the direction of the gene expression (up-regulated or down-regulated), we defined three sets of DEGs: (i) General Inflammation Response-DEGs (1,686 genes); (ii) Acute Inflammatory Response-DEGs (271 genes DEGs, with higher expression at 6h), and (iii) Late Inflammatory Response (67 genes with higher expression at 24h). The significant enriched terms identified for the General Inflammation Response-DEGs were all related with the inflammatory and immune responses (*alpha-beta cell factor production, regulation of leukocyte cell-cell adhesion, regulation of adaptive immune response, positive regulation of cytokine production, tool-like receptor signalling pathway, positive regulation of defense response, neutrophil activation, neutrophil degranulation, regulation of tumor necrosis factor production*). For the Acute Inflammatory Response-DEGs, the enrichment analysis highlighted terms such as *cellular response to LPS, response to molecule of bacterial origin, response to type I interferon, type I interferon signalling pathway, regulation of type I interferon production,* whereas for the Late Inflammatory Response-DEGs some of the enriched DEGs were *regulation of endothelial cell proliferation, regulation of vascular endothelial growth factor signalling pathway, regulation of inflammation response, smooth muscle cell migration, regulation of neutrophil extravasation, positive regulation of neutrophil migration, regulation of leukocyte migration, glutathione transport, etc.*

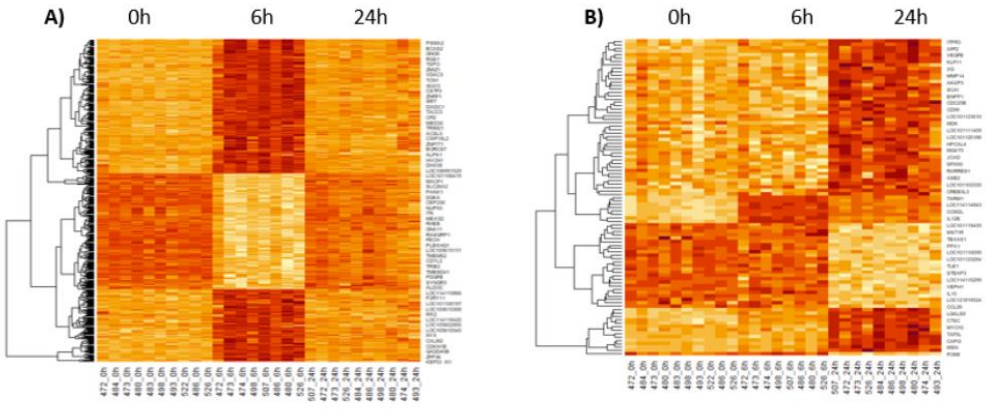


Figure 11. Examples of heatmap representing the expression profiles of some of the DEGS identified in the comparisons of the the MSC transcriptomes analysed in this project for the Control animals of the nutritional challenge experiment across the three considered time points analysed**. A**) Heatmap representing the expression profiles of the 426 DEGs commonly identified in the 0h*vs*6h and 6hvs24h transcriptome comparisons. Those genes showing a darker colour (upregulated at 6h in the two comparisons) were defined as Acute Inflammatory Response-DEGs. **B**) Heatmap representing the expression profiles of the 96 DEGs commonly identified in the 0hvs24h and 6hvs24h transcriptome

comparisons. In this case, those genes showing a darker color (upregulated at 24h in the two comparisons) were defined as Late Inflammatory Response-DEGs.

In addition, there was a group of DEGs detected through the different comparisons that highlighted the DEGs with higher expression in time point 0h compared to other two time points studied after the LPS-inoculation (so had lower expression at time points 6h and 24h compared to 0h), which were related to protein and fatty acid metabolic process, lipid biosynthetic process and gland development (e.g. *lipid catabolic process, fatty beta-oxidation, carboxylic acid catabolic process, alpha-amino acid metabolic process, cellular amino acid biosynthetic process, acyl-CoA biosynthetic process, alcohol metabolic process, epithelial tube morphogenesis, cell-cell junction organization, etc)*. Hence, these results would suggest that the genes directly related with milk components synthesis are downregulated after the LPS-challenge due to the increase in the expression of immune-related genes. Preliminary results of these analyses were presented as an oral communication in the 38th International Society for Animal Genetics Virtual Conference (Hervás-Rivero et al., 2021).

LPS inoculation in Longevity ALPINE goat lines

A comprehensive analysis of the inflammatory challenge in the longevity ALPINE goat lines (INRAE), including clinical signs, SCS and cytokines is described below

Evolution of clinical signs, inflammation and cytokines/chemokines after LPS inoculation.

After the LPS-injection, signs of general inflammation were observed with elevated temperature rising up to 41.5°C at +4hours and decreasing to normal after 3 days (**Figure 12**). Also, LPS injection gave diarrhoea in the 48h post LPS injection, with up to 40% occurrence at +8hours.

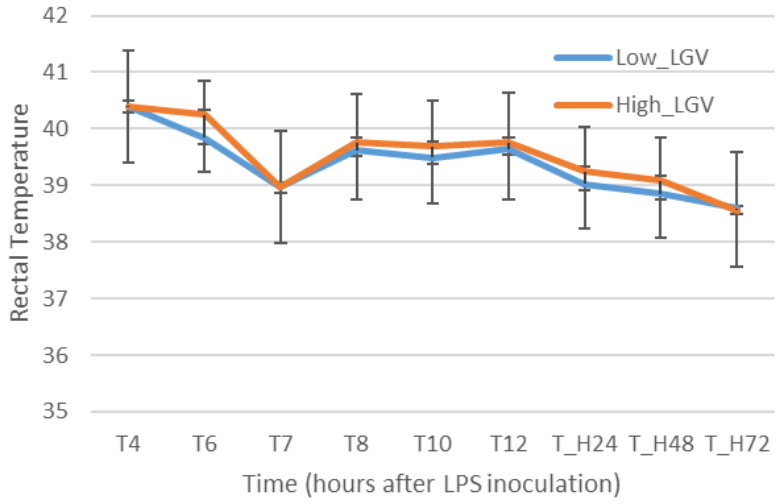


Figure 12: Profile of rectal temperature (lsmeans±SE) following LPS injection according to the longevity line (high_LGV and Low_LGV) in 46 goats challenged in 2021

Evolution of cytokines/chemokines after LPS inoculation.

The graphical evolution of 14 cytokines/chemokines plasma markers related to the immune response (IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MIP-1α, MIP-1β, IL-36RA, IP-10, TNF-α, VEGF-A) upon LPS challenge is given **in Figure 13**

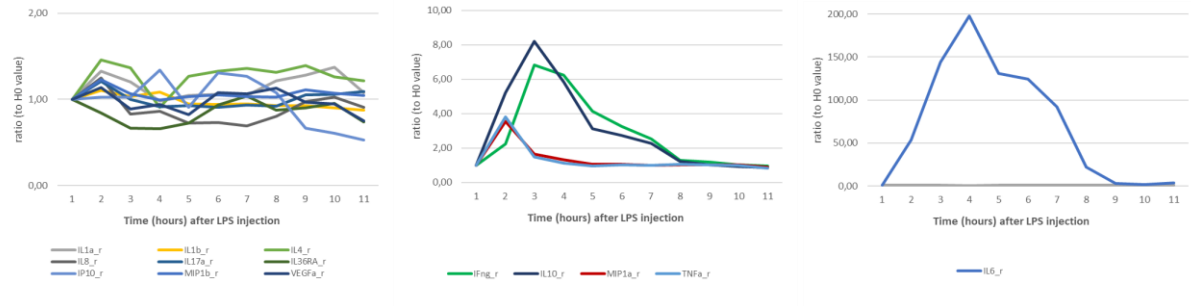


Figure 13: Average profile of 14 cytokines/chemokines (ratio to value at H0) following LPS injection in 87 goats challenged in 2021 and 2023

Mixed model analyses were run to assess

- Fixed effects of time, year, genetic line and the interaction between these two effects
- The random animal effect to account for repeated measures across the sampling points following LPS injection

Trait were expressed either in original unit (after log transformation) or as ratio trait (r_) with reference to the measure at H0 (as performed in the UELEON LPS experiment). Changes upon LPS challenge were significant ($p<0.001$) for all cytokines. Main changes were observed for IL6, IL10, IFNg and MIP1 as illustrated in **Figure 13**, between 2 and 10 hours after the LPS injection. Year effect was also significant for all cytokines.

The genetic group (high or low longevity line) showed significant effect on IL6, IL4, TNFa, MIP1b and MIP2b (Line and/or LineXtime) as illustrated in **Figure 14**. The low longevity line showed higher response to challenge than the high longevity line for IL6.

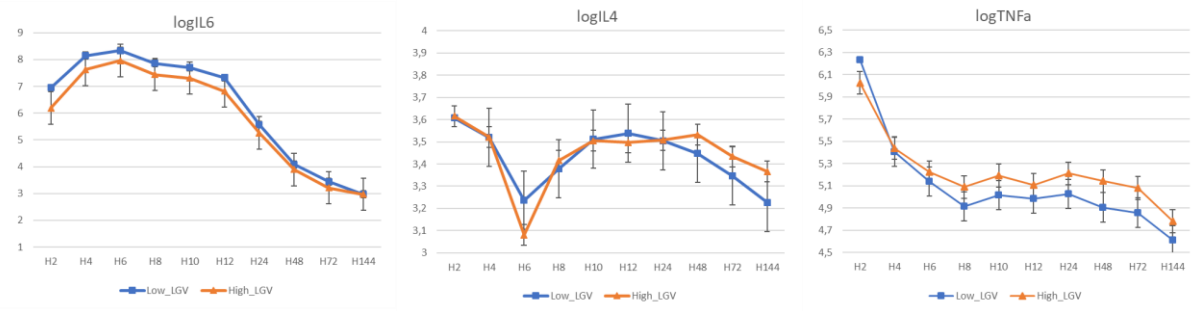
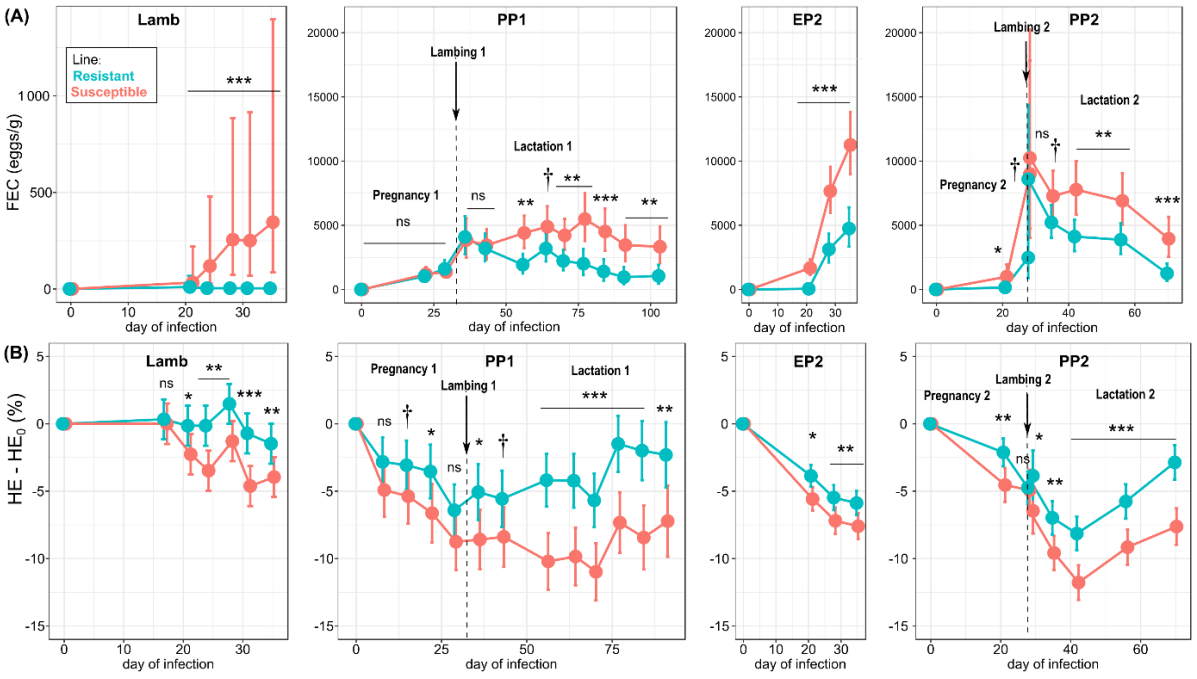


Figure 14. Average (LSmeans ± SE) profile for 3 cytokines upon LPS injection that were significantly different according to the longevity genetic line IL-6 , IL-4 and TNFa.

As a summary, in response to systemic LPS injection, rectal temperature, and all plasma biomarkers showed significant changes in basal levels across the dynamic analysis. Differences between genetic groups selected for longevity were found for several cytokines including : IL6, IL4 and TNFa . The high longevity line showed lower response to challenge than the low longevity line in IL6. As the High longevity line is associated with increased length of life and lower incidence of mastitis (Ithurbide et al., 2022) we can hypothesise that genetic selection has improved resilience mechanisms related to immune response involving TNFa and IL6 pathways.

Parasite infestation in Parasite Resistance ROMANE sheep lines

During the successive infections in four stages over their first 2 years of life, sheep responses in terms of fecal egg count (FEC) and blood haematocrit (HE) were consistent with the genetic divergence that has been selected in lambs (**Figure 15**). However, the divergence in FEC was strongly attenuated shortly before lambing and for about 1 month in lactation, both during first peripartum (PP1; Figure 12) and the second peripartum (PP2, **Figure 15**). Interestingly, the peripartum reduction in the FEC difference between lines followed a similar pattern during PP1 and PP2, although those two phases of infection had a different mode. In the S line, FEC continued to increase about 7 weeks after lambing during PP1 (as trickle infection still occurred 3 weeks after lambing and was bringing novel cohorts of worms), whereas it declined after lambing during the single-dose infection of PP2 (reflecting the decline in the single cohort of worms whose mean life expectancy is about 50 days). In contrast, the R line always reached maximum FEC around lambing and decreased afterwards. Consistently with those changes in FEC, the R line started to recover blood haematocrit sooner than the S line (**Figure 15B**).



EP=Early Pregnancy **PP**=peri partum

Figure 15. Faecal egg count (FEC; upper panels) and the change in blood haematocrit (HE) compared to the individual initial level (HE_0) (bottom panels), in response to successive infections in female sheep divergently selected on resistance to *Haemonchus contortus*. Circles are adjusted means with their

error bars representing 95% confidence interval. Asterisks indicate statistical differences between lines (†: p < 0.1; *: p < 0.05; **: p < 0.01; ***: p < 0.001). Note the different y-axis scale for FEC during the lamb phase compared to the three adult reproductive phases to enhance visibility

To evaluate the effect of parasite infection challenge on feed efficiency in the parasite resistance lines, at about 4-5 month of age, 60 ewe lambs of each line (30 R and 30 S) went through the infection protocol whereas 31 (15 R and 15 S) were kept uninfected (**Figure 16**). Residual feed intake (RFI) was calculated during the second infection period (5 weeks) and was based on ad libitum concentrates intake.

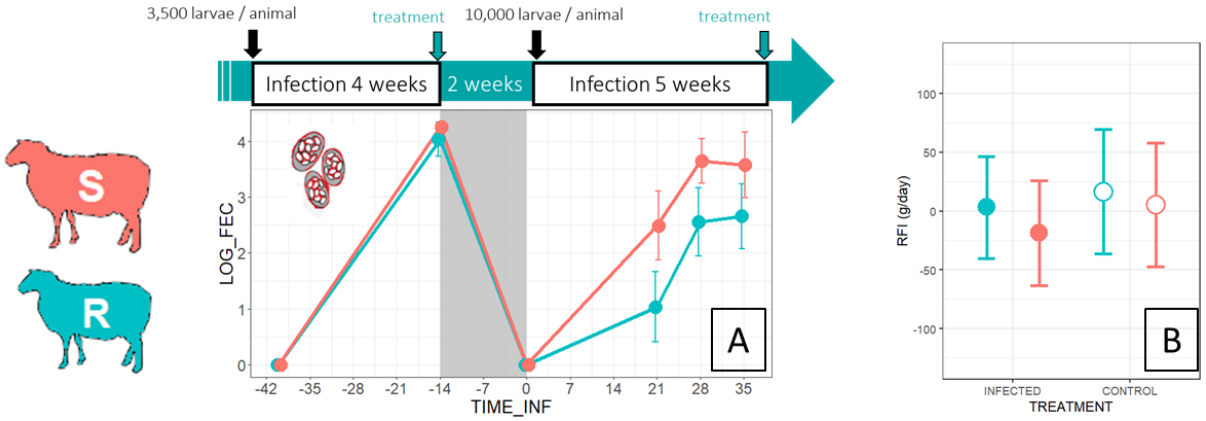


Figure 16. FEC responses between parasite resistance lines (A), and residual feed intake (RFI) in twe two lines according to the infestation statut (B)

Although the FEC responses were consistent with expected difference between lines (**Figure 16A**), line had no effect on the RFI of infected ewe lambs (**Figure 16B**). No difference in RFI was observed between lines in control sheep either. Overall the infection did not affect the residual feed intake at all (**Figure 16B**).

Parasite infestation in Feed Efficiency ROMANE sheep lines

At about 4-5 month of age, 59 ewe lambs of each line (30 high-RFI and 29 low-RFI) went through the infection protocol whereas 31 (16 high-RFI and 15 low-RFI) were kept uninfected.

No effect of the infestation was observed on the residual feed intake of ewes (**figure 17A**). The difference between lines remained significant despite the infectious challenge. A first analysis of the FEC highlighted no difference between lines: high-RFI and low-RFI ewe lambs have the same level of egg excretion (**figure 17B**). However, low-RFI ewe lambs had a greater haematocrit loss during infestation than high-RFI ewe lambs (**figure 17C**). This suggest that efficient ewes may have less ability to recover from parasite infection, thus highlighting a trade-off, but results must be confirmed.

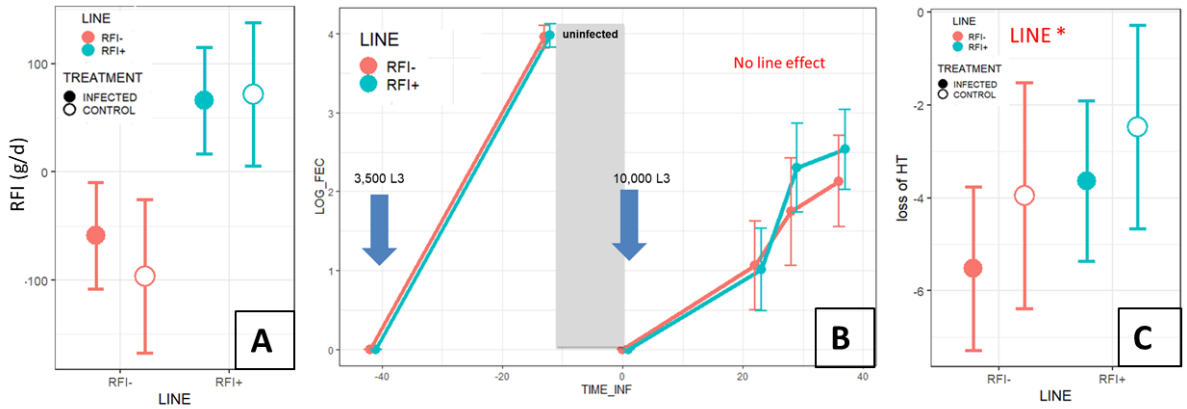


Figure 17. Results of the infestation of Romane ewe lambs: **A**- on their RFI phenotype, **B**- Faecal Egg Count and **C**- Haematocrit loss

At 4-5 weeks before their first lambing, 50 females (out of the 90 included in the experiment as lambs) were orally infested with a single dose of 10,000 larvae of *H. contortus* and drenched at the end of February 2023 (105 days of infestation). Those 50 females (25 in each line) were bearing at least two lambs at the start of the experiment. Out of those 50 ewes, 37 remained at the end of the infection period. Five high RFI and eight low RFI ewes were excluded during the experiment, mainly due to the total loss of the litter. Patterns of parasite egg excretion and blood haematocrit were consistent with the parasite dynamic and reflected a high level of infection (**Figure 18**). However no difference was observed between lines in terms of FEC or HE.

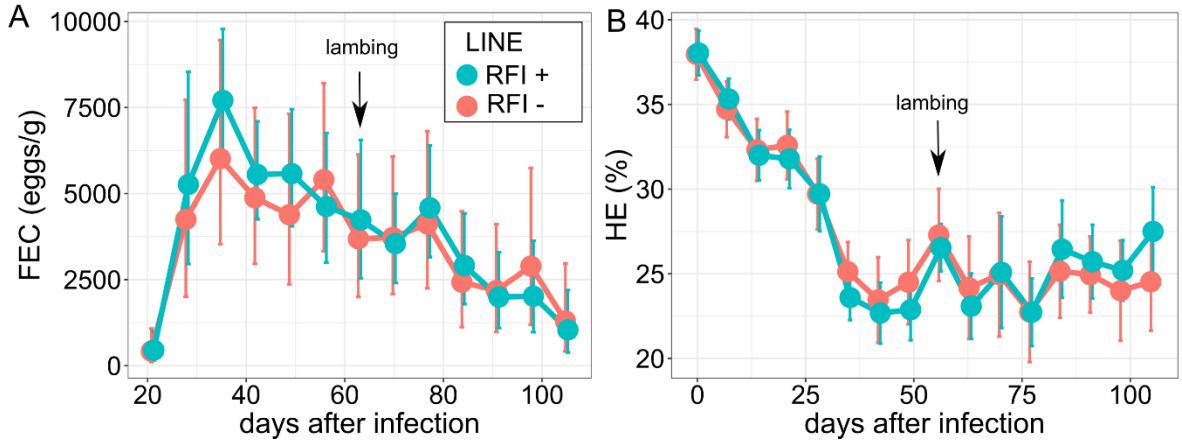


Figure 18. Faecal egg count (FEC) (**A**) and blood haematocrit (HE) (**B**) in response to a single-dose infection 4-5 weeks before lambing in female sheep divergently selected on feed efficiency.

Interaction between nutritional and infection challenges

Interactions in Milk production ASSAF sheep lines

To study the interaction between nutritional and infection challenges, we observed the response to LPS inoculation in the two groups that were previously submitted to nutritional challenge: 20 control (C) and Challenged sheep (NC) which received a diet with a 42% protein restriction.

The GzLM did not identify a significant effect of the nutritional challenge group (N; NC) on the considered local indicator traits, the r_logSCC (LPS-incolated) or r_logSCC(control) traits (**Figure 19**). For the r_Rectal_Temperature trait, the NC group effect was not significant, whereas 8 out of 14 plasma markers were significantly affected by the NC group ($P < 0.005$): r_IFN-γ, r_IL-1α, r_IL-1β, r_IL-4, r_IL-10, r_IL-17A, r_TNF-α, r_VEGF-A. For all these significant results, we observed that the effect of the diet restriction showed a lower estimate for the NC group than for the C group, suggesting that the C group had higher relative concentration values than the NC group for the corresponding relative ratio traits. However, from these 8 significant plasma biomarkers, only two of them had shown significant changes related to the basal levels across the sampling time points considered: IL-10 and VEGF-A (Figure XB). For these markers, which appear to be regulatory modulators of the inflammation response, previous studies have reported links with metabolic traits (Cintra et al., 2018, doi: 10.1016/j.jhep.2007.12.017; Elias et al., 2013; doi: 10.4161/adip.22880.), which support the findings here described. Overall, these results suggest that the nutritional challenge performed at the prepuberal age in ewe lambs did not influence the local inflammatory response, after inducing an experimental inflammatory challenge of the mammary gland later in their productive life, whereas at the systemic level, the results suggest that a potential influence could affect some regulatory biomarkers of inflammation such as IL-10 and VEGF-A. Preliminary results of these analyses have been presented in the 12th WCGALP Congress (Pelayo et al., 2022).

The sPLS-DA analysis performed for the 14 plasma biomarkers ratio traits at each sampling time-point, and in relation to the two groups of the nutritional challenge (C and NC) selected, through the cross-validation process, one component for the sPLS-DA discriminating groups, whereas the optimal number of selected variables for component 1 was 20. Among the 154 variables tested (i.e., the 14 ratios of the cytokines for each of the 11 time-points), 20 were the most discriminant variables between the NC and C groups for discrimination (VIP > 1.45). The variable with the highest VIP value was r_VEGFA_2h (2.17). Regarding the time-points associated with these discriminant variables, 2 h was associated with seven of the 20 variables with VIP > 1.45. Results therefore support the above reported GzLM analyses.

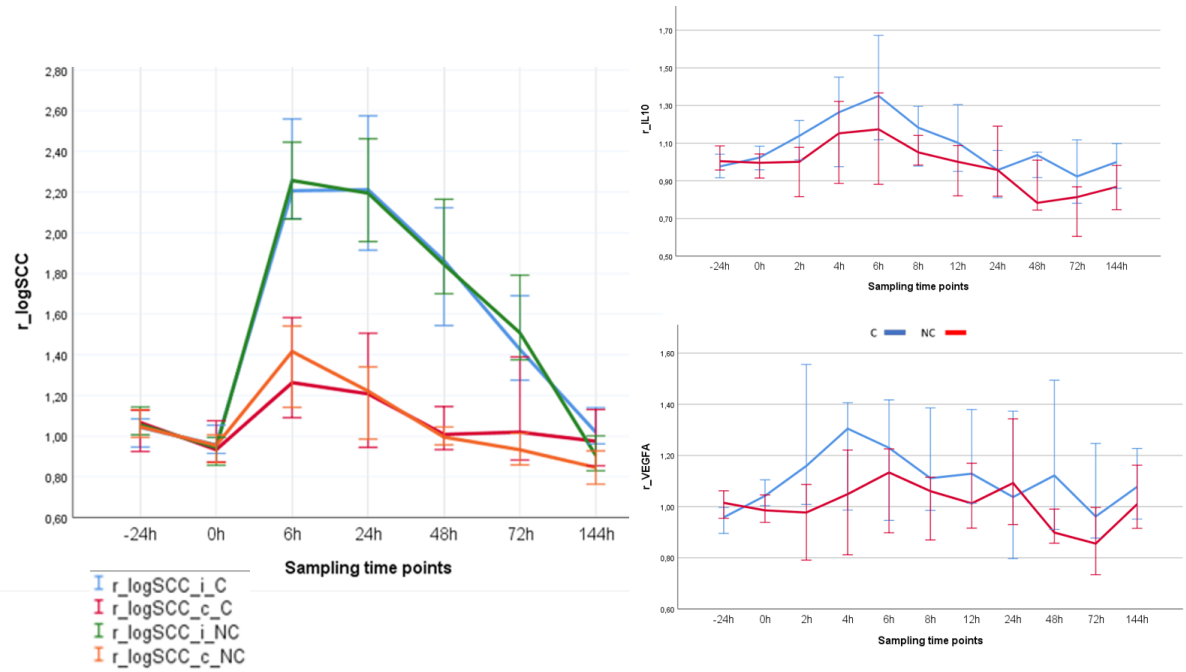


Figure 19. A) Graphical representation of the logarithm of SCC ratio (r_logSCC) measured in both the LPS-inoculated udder (r_logSCC_i) and the contralateral udder (r_logSCC_c), considering the nutritional challenge group (13 NC and 11 C). **B**) Graphical representation of two cytokines after intramammary LPS-inoculation) considering the nutritional challenge group Note: The ratio traits were calculated considering the measurement corresponding to each specific time point regarding the average of the -24h and 0h trait measurement, which was considered the basal time point. Error bars show the 95% confidence interval of the median.

Additional information was provided by the analysis of the nutritional challenge group effect on the MCS transcriptome at 6h time-points post-LPS inoculation. The comparison between the top 10 expressed genes in the MSC transcriptome of control and NC animals is shown in **Figure 20**. As it can be seen the majority of the genes are the same and in general terms are related to the inflammatory and immune responses. So the nutritional challenge group did not appear to influence the profile of the most expressed genes in the MSC. The most expressed genes in the MSCs collected at 6 h after the LPS-inoculation is *CXCL8*, for both groups, being the expression higher in the controls. This gene induces chemotaxis in target genes, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. This top 10 profile is very different to the one reported for the healthy mammary gland in sheep (Suarez-Vega et al., 2015), where the most abundant genes are caseins and alfa lactoalbumin and beta lactoglobulin. The results of the differential expression analysis performed between the C and NC group, identified a total of 585 DEGs, 495 of them with decreased expression in the "NC" group and 90 with increased expression in the "NC" group. The enrichment analysis performed for these two groups of DEGs showed that the down-regulated genes in the NC group (e.g. *LALBA, CSN3, SPP1)* were enriched for terms related to the transcriptional and translational regulation and the synthesis of proteins, whereas the up-regulated genes in the NC group were enriched for terms related to the inflammatory response such as *immune effector process, JNK cascade, cell activation, stress-activated protein kinase signaling cascade* and *leukocyte activation*

involved in immune response. Hence, the NC challenge performed in the ewe lambs appear to determine, later in life, in an inflammatory challenge of the mammary gand, a reduction of the milk protein synthesis processes and an increase in immune or inflammatory processes in response to the LPS-challenge. Further research will be needed to interpret the implications of these results, considering that the protein challenge restriction did not show a significant effect on the SCC trait. Preliminary results of these analyses were presented as an oral communication in the 38th International Society for Animal Genetics Virtual Conference (Hervás-Rivero et al., 2021).

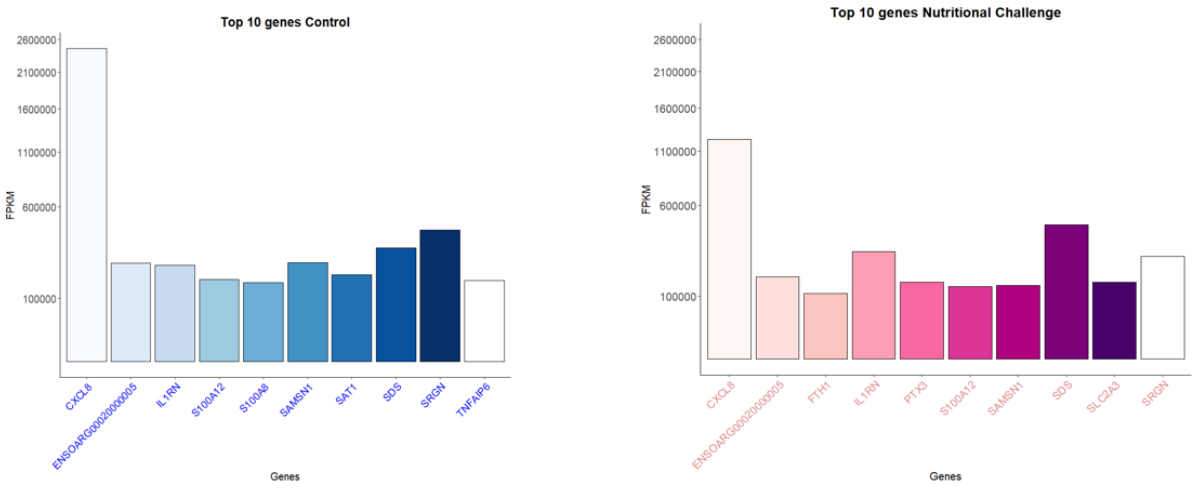


Figure 20. Top-ten genes expressed in the MSC transcriptomes obtained at 6h time point of the LPS inflammatory challenge in the C and NC groups of animals from the nutritional challenge performed at the prepuberal age. *Results funded by the National Spanish project EpiMilkSheep (RTI2018-093535-B-100).*

Interactions in Longevity ALPINE goat lines

As reported previously (nutritional challenge effect) the responses to nutritional challenge in the longevity lines highlighted differences in the dynamics of the metabolic response which suggest some links between resilience and efficiency. Whether these links are positive or antagonistic (trade off) for the animal on the long term must be clarified.

Interactions in Parasite resistance ROMANE sheep lines

During the parasite infection of G2 ewes around their first lambing (PP1), protein diet restriction affected condition traits in both lines (cf. Effect of nutritional challenge) but this had no effect on FEC. In contrast, it exacerbated the loss in haematocrit, especially in the S line (differential in HE between low-protein and high-protein during pregnancy = 11.27 points, $t_{47} = 5.76$, $p < 0.001$). Under the low-protein diet, the loss in HE was almost twice as large in the S line as in the R line (R: 6.76 ± 1.26% vs. S: 12.1 ± 1.42%). In contrast, lines had similar loss in haematocrit when they consumed the high-protein diet (R: 1.16 ± 1.26% vs. S: 0.79 ± 1.36%). This interaction between line and diet was maintained during lactation of PP1.

Overall, it seemed thus that the protein content of the diet was only limiting for the expression of certain benefits of parasite resistance such as ewe haematocrit and growth of their lambs.

Interactions in Parasite resistance CORRIEDALE sheep lines

In both periods (without and with infection challenges), parasite resistance CORRIEDALE line did not have a significant ($P > 0.05$) effect on dry matter intake, feed conversion rate, residual feed Intake, average daily gain or body weight. However, with H. contortus infection, R animals tended to have a lower feed conversion rate than did S ones, 8.0 and 11.1 respectively ($P = 0.074$), and REA was larger in S lambs than in the R group ($P < 0.05$). Worm egg count was different ($P < 0.05$) on Day 23 post-infection (infection challenge with H. contortus), being higher in susceptible line (back-transformed values were 976 and 1772 for the R and S, respectively).

The most important finding of this study is that breeding parasite-resistant animals would not have a negative effect on feed conversion efficiency when evaluated as feed conversion rate or residual feed Intake in 1-year old lambs fed ad libitum with a high-protein diet.

Interactions in Feed efficiency ROMANE sheep lines

As shown previously (**Figure 17A**), no effect of the infestation was observed on the residual feed intake of ewes from the two divergent lines selected on feed efficiency. The difference between lines remained significant despite the infectious challenge.

However, low-RFI ewe lambs had a greater haematocrit loss during infestation than high-RFI ewe lambs (**Figure 17C**). This suggest that efficient ewes may have less ability to recover from parasite infection, thus highlighting a trade-off.

This first result suggests a possible trade off for selecting for improved efficiency on resilience, but results must be confirmed. Further infection in the peri partum period (early 2023) will give additional support to this preliminary study

5. Conclusions

We manage to create and analyze five foreseen SMARTER experiments. Despite low numbers, the divergent selection experiments were made with an important selection intensity (selection very extreme funders) so that the divergence between the extreme genetic groups were significant.

- **Milk production ASSAF sheep lines** (N= 20 to 30 ewes in both nutritional and infection challenge). The divergence between lines is about 3.3 genetic standard deviation
- **Longevity ALPINE goat lines** (N= 98 goats in nutritional challenge and N= 87 in infection challenge). The divergence between lines is about 0.4 genetic standard deviation
- **Parasite resistance ROMANE sheep lines** (N= 48 to 91 ewes in both nutritional and infection challenge). The divergence between lines is about 3.8 genetic standard deviation

- **Parasite resistance CORRIEDALE sheep lines** (N= 67 sheep in infection challenge with feed intake and feed efficiency recording). The divergence between lines is about 0.75 genetic standard deviation
- **Feed efficiency ROMANE sheep lines** (N= 90 sheep in infection challenge with feed intake and feed efficiency recording). The divergence between Romane males from the low-RFI and high-RFI lines reached ~140g/d (i.e. 1.9 genetic standard deviation).

These original experiments thus demonstrated that selection can be made on new resilience and efficiency (R&E) traits (such as feed efficiency, longevity and parasite resistance) as expected response were produced despite low to moderate heritabilities. Furthermore, we expect sufficient difference and power in these smarter designs to highlight possible major interactions between genetic selection for R&E traits and the response to various challenges.

Various challenges were implemented in these lines to better explore resilience. With this regard, the original LPS challenge performed in the ASSAF and ALPINE dairy experiments allowed to test and validate a new protocol to investigate immune response and derive new resilience phenotypes. Indeed, the LPS challenge performed in both designs, showed significant response in immune response markers, with variability among animals that were associated, for some part, with genetic lines. In the dairy goat longevity lines, variability in these immune markers suggested that selection for longevity is associated with improved resilience mechanisms related to immune response involving TNFa and IL6 pathways.

The main finding of the report from the five experiments is that there was little evidence of major trade-offs between selection for resilience and response to efficiency and also conversely between selection for resilience and response to efficiency.

- In CORRIEDALE, the most important finding of this study is that breeding parasite-resistant animals would not have a negative effect on feed conversion efficiency when evaluated as feed conversion rate or residual feed Intake in 1-year old lambs fed ad libitum with a high-protein diet.
- In ALPINE, selection for longevity did not have any significant impact on milk production loss upon nutritional challenges
- In ROMANE, selection for feed efficiency did not have any significant impact on parasite load (Feacal egg counts) upon parasite infestation.

However, when exploring deeper the phenotypic responses down to fine phenotyping or mechanisms, some links (but not straightforward trade-offs) were discovered.

- In ASSAF sheep, differences between genetic groups selected for milk production were found for inflammatory response (milk SCC) SCS, and two cytokines: CXCL8 and CCL4.
- In ROMANE sheep selected for parasite resistance, we showed an interaction between diet restriction and response to infection challenge. Overall, it seemed thus that the protein content of the diet was limiting for the expression of certain benefits of parasite resistance such as ewe haematocrit and growth of their lambs.

- In ALPINE goats, the results support that selecting for longevity is associated with a modulation of the metabolic response to nutritional challenge and their blood metabolic adaptations peripartum.

Results were published in five peer-reviewed papers (Ferreira et al., 2021 ; Douhard et al., 2022; Ithurbide et al., 2020 & 2023; Pelayo et al., 2023) and presented in several conferences.

6. Deviations or delays

We succeeded in creating the four SMARTER experiments. A first version of deliverable D3.2 was submitted M48 and then updated at M58 on the REA request.
In this updated version, corrective measures having been taken, so that only minor deviations from what was planned in the DOA remain.

- Because of COVID, there was a delay of about 1 year in the Romane feed efficiency experiment. The infection challenge in primiparous sheep was done in RP4 and reported in this updated Deliverable (M58).
- Because of COVID, the infection challenge in Alpine goat lines was aborted in 2020 and we only had 46 goats (instead of 80 foreseen) in first version of D3.2. The deviation in number of goats upon LPS challenge was resolved, as a new experiment was carried out in RP4 with the financial support of a French funding body (APIS-GENE). In addition, we finally delivered more animal for both nutritional challenge (98 instead of 80) and infectious challenge (87 instead of 80).
- The analysis of a total of 12 ASSAF sheep following LPS inoculation was planned in the DOA. We over achieved this number with a total of 30 sheep with health scoring (temperature), 24 with cytokine measures and 20 with RNAseq measures. Because of costs, the fine phenotyping using cytokine measurements (not foreseen in the DOA) and RNA sequencing were funded by other project.
- Two of the experiments (Coriedale GIN lines; Romane RFI lines) had no specific nutritional challenges, but the interaction between nutritional and infection challenges was assessed by monitoring feed intake and efficiency regularly around the infection challenge.

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