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Multivariate analysis of milk metabolite measures shows potential for deriving new resilience phenotypes

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ABSTRACT

In a context of growing interest in breeding more resilient animals, a non-invasive indicator of resilience would be very valuable. We hypothesized that the time-course of concentrations of several milk metabolites through a short-term underfeeding challenge could reflect the variation of resilience mechanisms to such a challenge. We submitted 138 one-year-old primiparous goats, selected for extreme functional longevity, i.e., productive longevity corrected for milk yield (60 low longevity line goats (**Low_LGV**), and 78 high longevity line goats (**High_LGV**)), to a 2-d underfeeding challenge during early lactation. We measured the concentration of 13 milk metabolites and the activity of 1 enzyme during pre-challenge, challenge and recovery periods. Functional PCA summarized the trends of milk metabolite concentration over time efficiently without preliminary assumptions concerning the shapes of the curves. We first ran a supervised prediction of the longevity line of the goats based on the milk metabolite curves. The partial least square analysis could not predict the longevity line accurately. We thus decided to explore the large overall variability of milk metabolite curves with an unsupervised clustering. The large year x facility effect on the metabolites concentrations was pre-corrected for. This resulted in 3 clusters of goats defined by different metabolic responses to underfeeding. The cluster that showed higher BOHB, cholesterol, and triacylglycerols increase during the underfeeding challenge was associated with poorer survival compared with the other 2 clusters ($P = 0.009$). These results suggest that multivariate analysis of non-invasive milk measures show potential for deriving new resilience phenotypes.
Key Words: resilience, milk metabolites, multivariate modelling, dairy goats

INTRODUCTION

Today, there is growing interest in selecting for resilience, as livestock are expected to face increasingly harsh environmental and climatic conditions. Animal resilience is defined as the ability to overcome short-term environmental disturbances and quickly return to its pre-disturbance state (Colditz and Hine, 2016). In this context, resilience can be seen as an underlying component of longevity since it corresponds to the ability to cope with and recover from challenges to allow the animal to carry on its productive life (Friggens et al., 2017; Scheffer et al., 2018). Longevity corresponds to true longevity (all culling reasons) and functional longevity that includes all culling reasons, except productivity (Sasaki, 2013). Several studies estimated heritability of functional longevity to be around 10% in cattle and goats (Castañeda-Bustos et al., 2017; Nayeri et al., 2017; Palhière et al., 2018). Ithurbide et al. (2022) showed that selection on functional longevity in a commercial population of dairy goats translated into significant differences in longevity and resilience related traits such as better mammary health and lower body fat mobilization during the beginning of the first lactation for goats selected for longer functional longevity. Selection seems to be possible; however, improvements are expected to be slow due to low heritability. This low heritability could be explained by the fact that longevity is a multifactorial trait, i.e., there are other factors than resilience contributing to longevity, and that strong genetic x environmental interactions can be involved (Tsartsianidou et al., 2021). Thus, there is a need to find more direct resilience indicators. Being less

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multifactorial, more direct resilience indicators could have a higher heritability than functional longevity, and allow a more efficient selection and for instance select animals for longevity at an early stage of productive life.

We hypothesized that the metabolic response to short-term feed restriction could provide information about some genetic characteristics of goat resilience. The objective of this study is to explore the existence of underlying resilience components within the time-course of 13 milk metabolites and 1 enzyme activity during an underfeeding challenge imposed to goats of 2 divergent lines of goats for functional longevity. We propose a new statistical approach to model and explore multivariate longitudinal data.

MATERIALS AND METHODS

The experiment was carried out in agreement with French National Regulations for the humane care and use of animals for research purposes. Animals were bred at 2 experimental INRAE Farms: P3R Bourges (UE0332, La Sapinière, Osmoy, France, license to carry out animal experiments: C18–174–01) and Experimental Installation, UMR MoSAR (Route de la Ferme, Thiverval-Grignon, France) close to Paris license to carry out animal experiments: A 78 615 1002). This article followed the STROBE vet guidelines (O'Connor et al., 2016). All procedures performed on animals were approved by the Ethics Committee on Animal Experimentation and the French Ministry of Higher Education, Research and Innovation (APAFIS#8613–2017012013585646 V4 and APAFIS#24314–2019120915403741).

Animals

Following the method developed by (Palhière et al., 2018) and described by Ithurbide et al. (2022), we created 2 functional longevity lines of Alpine goats. Since 2017, we have run the genetic evaluation for functional longevity over 8,787 alpine artificial insemination (**AI**) bucks based on the productive longevity of their daughters (time difference between first kidding and culling) corrected for milk yield. We selected the 16 bucks who had the highest EBV and the 19 bucks who had the lowest EBV among the whole AI bucks population to find the low longevity line (**Low_LGV**) and high longevity line (**High_LGV**), respectively. From 2019 to 2022, 138 goats were bred: 60 Low_LGV goats and 78 High_LGV goats. Among them, 69 were bred in the INRAE P3R Bourges facility and 69 in the INRAE Paris facility (**Table 1**). Within each facility, Low_LGV and High_LGV goats were housed in common pens.

Farm management and animal monitoring in P3R Bourges facility are described in Ithurbide et al. (2022). Briefly, goats were not culled for milk production reasons, which allows a clean assessment of functional longevity. For farm management reasons, low producing goats could not be kept on farm in the Paris facility. Thus, survival data were not available in the latter facility. Weight, chest size and height were measured every month in both facilities during the first year of life. The milk yield (**MY**), milk fat content (**MFC**), milk protein content (**MPC**) and somatic cells score (**SCS**) were measured every month during lactation. Moreover, the EBV of the goats sires for functional longevity, **MY**, **MFC**, **MPC** and **SCS** were estimated and provided from the national genetic evaluation procedure.

Underfeeding challenge

A total of 138 one-year-old primiparous dairy goats were exposed to a 2-d underfeeding challenge during early lactation (35.5 d in milk (DIM) ± 5.6 SD). The design of the underfeeding challenge followed the protocol described in detail in Friggens et al. (2016); briefly, the challenge consisted of a 2-d, straw only feeding. Milk samples were collected for the 4 d pre-challenge, throughout the challenge period, and for 4 d following the challenge. From parturition and for 2 weeks post-challenge animals received a standard lactation diet. At P3R Bourges, the goats received a ration based on lucerne hay offered in collective troughs, complemented

Table 1. Distribution of the 138 goats within the 2 divergent lines selected on high longevity (High_LGV) or low longevity (Low_LGV) bred at INRAE facilities of P3R Bourges and Mosar Paris that underwent the underfeeding challenge during early lactation (36.7 DIM ± 6.2 SD)

	Year of the underfeeding challenge / INRAE facility				
	2020 / P3R Bourges	2021 / P3R Bourges	2021 / Paris	2022 / Paris	*Total*
Low_LGV	15	14	17	14	*60*
High_LGV	18	22	17	21	*78*
Total	*33*	*36*	*34*	*35*	*138*

with concentrate that was dispensed by automatic concentrate feeders and in the milking parlor. At Paris, the lactation diet was offered as a TMR containing on a DM basis: 20% concentrate, 24% hay, 29% Lucerne, 27% beet pulp, and 1% mineral and vitamin supplement (as described by Gindri et al., 2023). Forage and water were offered ad libitum. All goats were milked twice a day.During pre-challenge, challenge and recovery periods respectively 3, 2, and 4 milk samples were collected during morning milking. Fixed standard volume were taken after mixing the total production in the milking jar. The concentrations of 13 milk metabolites and 1 enzyme were measured: glucose-6-phosphate (**Glu6P**), glucose (**Glu**), galactose (**Gal**), β-hydroxy-butyrate (**BOHB**), **isocitrate**, **glutamate**, $\mathbf{NH_2}$ groups, lactate dehydrogenase (**LDH**), **urea**, **choline**, **malate**, **urate**, triacylglycerols (**TAG**), cholesterol (**Chol**). Each goat had 13 milk metabolites and 1 enzyme curves with data points at days −7, −4, −1, 0, 1, 2, 3, 4, 5 and 6 for Bourges and every day from day −4 to 12 in the Paris facility. Day 0 being the last morning milking before the underfeeding challenge that started the same day.

Milk urea was analyzed with a FIAstar 5000 Analyzer (Foss Tecator AB, Höganäs, Sweden) using flow injection analysis (Nielsen et al., 2005). Enzymatic-fluorometric methods were used to analyze TAG and minor milk constituents: LDH activity (Larsen, 2005), BOHB (Larsen and Nielsen, 2005), urate (Larsen and Moyes, 2010), TAG (Larsen et al., 2011), Chol (Larsen, 2012), isocitrate (Larsen, 2014), Glu and Glu6P (Larsen, 2015). Gal in milk was analyzed by an analog procedure to Glu, using b-galactose dehydrogenase (EC 1.1.1.48) to start the fluorometric determination. Moreover, weight, MY, milk composition (MFC, MPC) and udder health indicator (SCS) were measured the same days as the milk samples in both facilities.

Statistical Analysis

Fitting of the individual milk metabolite curves All statistical analysis were done in the R statistical environment (https://www.r-project.org/). To model the individual metabolite concentration curves we used the functional data analysis smoothing method described by Ramsay and Silverman (2005). We used a spline interpolation, i.e., a piece-wise interpolation that joins several low degree polynomial functions at knots (predetermined time points along the time-series of data). We used natural cubic splines: i.e., a piece-wise cubic polynomial that is a continuous when differentiated twice, fixing a minimum degree of the polynomial at 5. The degree of smoothing of the spline was controlled by a roughness penalty.

Three goats exhibited outlier metabolic trajectories with BOHB concentrations in milk above 3 SD. The recordings of these goats were excluded. In addition, 10 implausible data points were excluded from the analysis (10 out of 27594 data points). None of these points belonged to the underfeeding period (d 0 to 2) and each belonged to different goats and metabolite curve. As such, removing these points did not distort the general shape of the curves. **Figure 1** shows the smoothed curves of the 13 milk metabolites and 1 enzyme from one randomly selected goat.

Correction for the year x facility effect with functional regression To minimize the impact of non-genetic factors, such as the global environment, on the metabolic response to the underfeeding challenge, we accounted for the facility x year effect by running a functional regression analysis:

$$Xi(t) = \beta 0(t) + \beta 1(t) \times Year\_i + \xi i(t),$$

where $Xi(t)$ was the milk metabolite curve for the ith goat, $\beta 0(t)$ was the intercept function, $\beta 1(t)$ was a function of time corresponding to the regression coefficient associated with the year-facility effect, $Year\_i$ was a dummy variable corresponding to the year-facility of study of the ith goat and $\xi i(t)$ was the residual term. Using a functional regression coefficient allows a correction of the year-facility effect. The corrected individual curves were then estimated as:

$$Xi\_correct(t) = \beta 0(t) + \xi i(t).$$

Figure 2. sets out a summary of the milk metabolite curves modeling steps and shows smoothed curves of isocitrate milk concentration before and after correction for year-facility effect. Note that, at Paris, animals were reared from weaning until mid-gestation on 2 different diets but these were balanced and equally distributed between years, they were also equally distributed between clusters in the present analyses, and consequently were ignored.

Functional Principal Component Analysis We characterized milk metabolite curves upon challenge using a functional PCA (**FPCA**) for each year-facility-corrected milk metabolite (Yao et al., 2005) using the R package "FDA." Functional PCA is a statistical method for investigating the dominant modes of variation of a functional data set. It allows the time related variation to be captured in a small number of principal components (see **Figure 2**). In other words, FPCA decomposes a set of random function Xj(t) from the j-th metabolite in the following representation:

$$Xj(t) = \sum_{k=1}^{\infty} \gamma_{jk}\, \omega_{jk}(t),$$

where ω_{jk}'s are orthogonal functions across k knots, i.e., the functional principal components (**FPCs)** that are common to all goats, and γjk's are the FPC scores that characterize individual curves.

The first step to this decomposition was to estimate the functional principal components ω_{jk}. Let Cj(s,t) be the covariance function of Xj(t), and it corresponds to a self-adjoint and positive semi-definite operator Cj: L2(τ)→L2(τ) The FPC $\omega_{jk}(t)$'s satisfy the following eigen equation:

$$C_j\omega_{jk} = \rho_{jk}\omega_{jk},$$

where ρjk's are the eigenvalues of Cj and Cj gives the following integral transform:

$$C_j\omega_{jk} = \int_{Ä} C_j(s,t)\,\omega_{jk}(s)\,ds.$$

To obtain the FPCs, we could solve the eigen equations for k = 1,...,K for a fixed K. Equivalently, the solution fits the maximization problem of:

$$maxQ0(\omega_{jk}) = max\langle\, \omega_{jk}, C_j\, \omega_{jk}\rangle,$$

subject to the constraints of $||\omega_{jk}|| = 1$ and $\langle \omega_{jk}, \omega_{jk}'\rangle = 0$ for k′ < k. Given a set of observed trajectories x1,...,xn we have the observed covariance function:

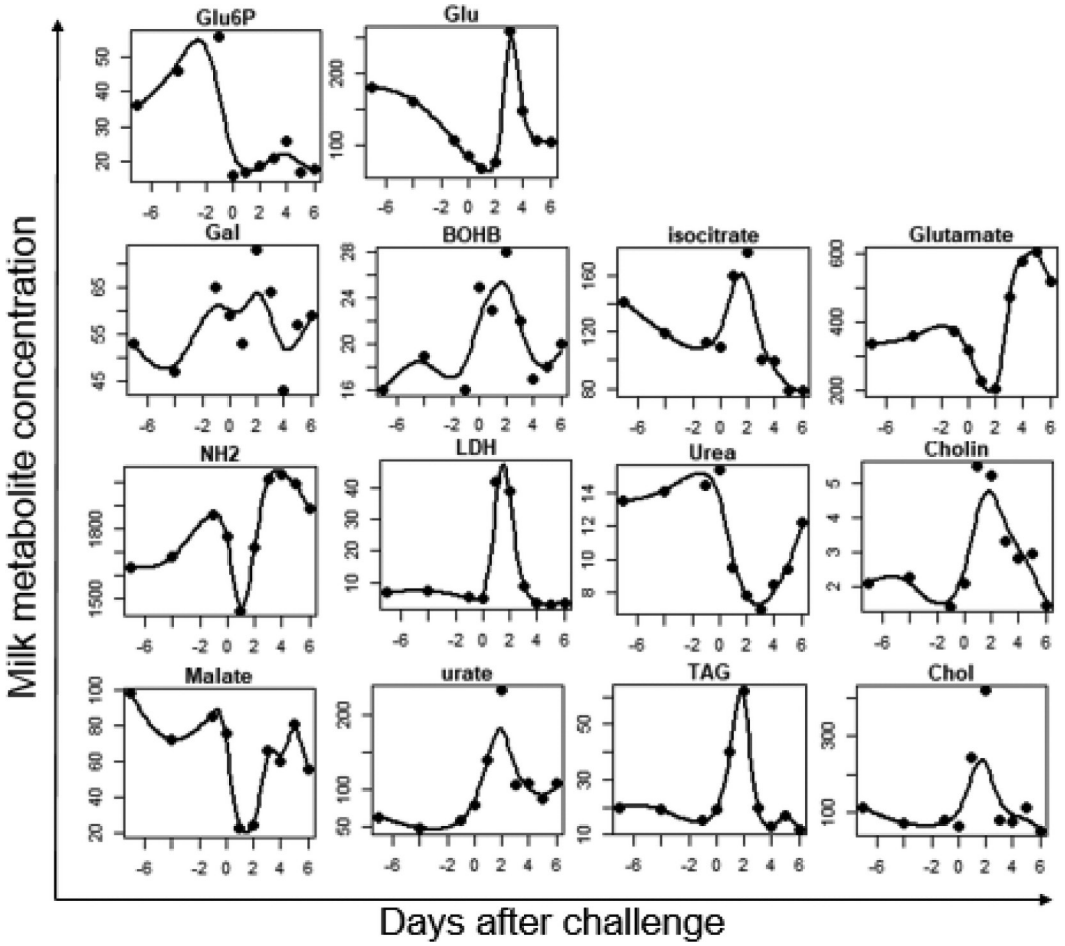

Figure 1. The smoothed curves of 13 milk metabolites and 1 enzyme from one randomly selected goat. Day 0 corresponds to the beginning of the 2 d underfeeding challenge. Points correspond to observed values. The 13 milk metabolites and 1 enzyme are: glucose-6-phosphate (Glu6P, microM), glucose (Glu, microM), galactose (Gal, microM), β-hydroxy-butyrate (BOHB, microM), isocitrate (microM), glutamate (microM), NH_2 (glutamate micro equivalent), lactate dehydrogenase (LDH, UI), urea (mM), choline (mM), malate (microM), urate (microM), triacylglycerols (TAG, mM), cholesterol (Chol, microM). The 138 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges and Mosar Paris.

$$\hat{C}j(s,t) = \frac{1}{n}\sum_{i=1}^{n} \mathrm{xi}(\mathrm{s})\,\mathrm{xi}(\mathrm{t})$$

with its associated operator $\hat{C}j$. For obtaining estimates ω̂jk, we solve the maximization or eigen equation problem by replacing C(s,t) with its empirical version $\hat{C}(\mathrm{st})$.

The second step was the calculation of the individual FPC scores as the projections of Xj(t) onto the FPCs through the inner product:

$$\gamma jk = Xj, \omega jk = \int_{\tau} Xj(t)\,\omega jk(t)\,dt.$$

We chose the minimum number of components that explained at least 90% of the variability. A small number of FPC scores thus characterized each individual milk metabolite curve. The **Figure 2.4.** represents the 3 FPCs for isocitrate and the corresponding scores for one given goat. Note that by construction, the mean values of the FPC equals 0. The 1st FPC plot was positive across the whole period of time, increasing slightly during challenge. The chosen goat has a negative 1st score for isocitrate (1isocitrate), indicating that this goat has a low overall isocitrate concentration. The individual curve plotted in **Figure 2.3** confirms that. Likewise, we can interpret the negative 2isocitrate score value as a high isocitrate concentration compared with pre challenge and recovery concentration, and the nega-

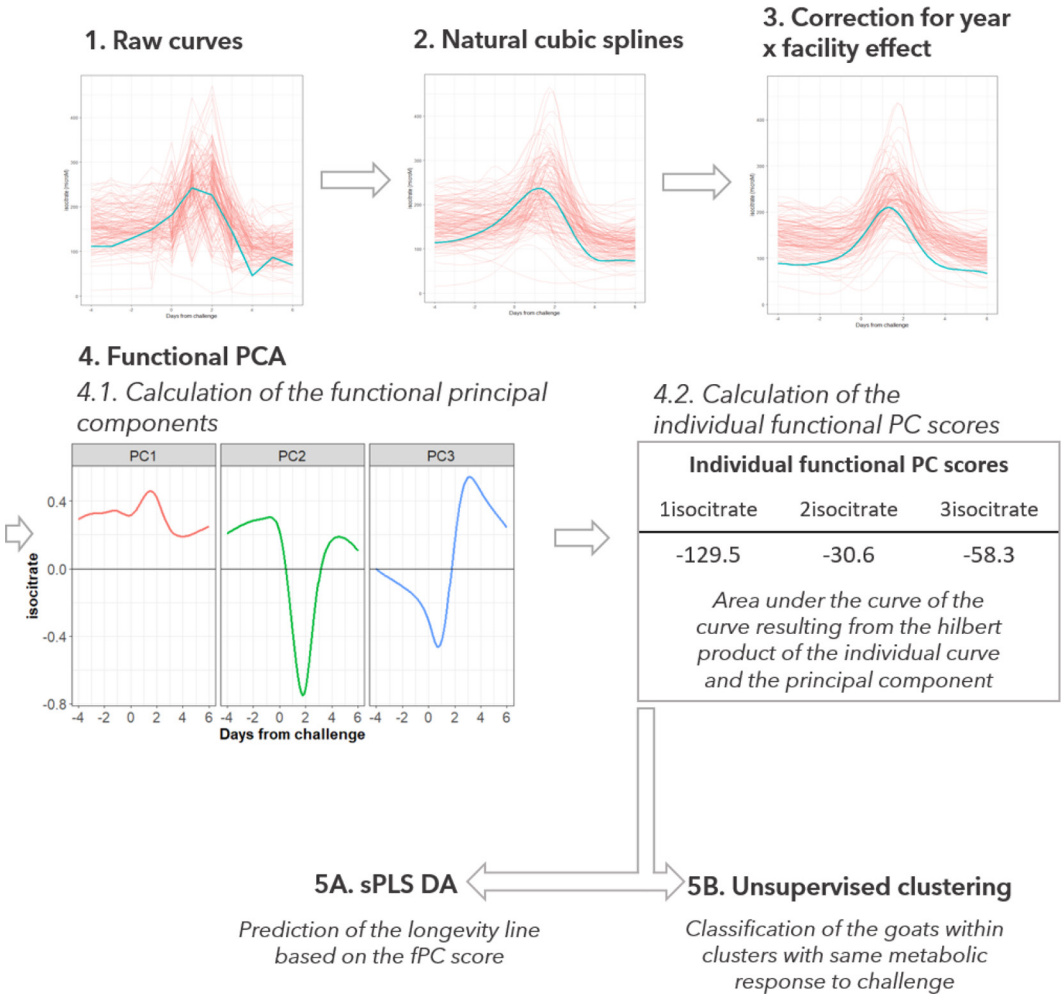

Figure 2. Scheme showing the different stages of analysis of one milk metabolite curves data set (here isocitrate is shown as example). In the raw curves plot (1), smoothed curves plot (2), and curves corrected for year x facility effect (3) each red line correspond to one goat. The bold blue line corresponds to one randomly chosen goat. The functional principal components of the fPCA for isocitrate are plot in 4.1. and the corresponding scores for the randomly chosen goat are shown in 4.2. The fPC scores of the 13 milk metabolites and 1 enzyme are then used to predict the longevity line of the goats with a sPLS DA (5.A) and classify the goats within clusters with the same overall metabolic response to underfeeding challenge (5.B).

tive 3isocitrate as a low post-challenge / pre-challenge concentration ratio compared with other goats.

Prediction of the longevity lines of goats based on the metabolite curves model. For the supervised clustering we used Sparse Partial Least Square Discriminant Analysis (**sPLS-DA**) to evaluate the ability of milk metabolite curves to distinguish the longevity lines of the 138 goats. This is a linear multivariate model which performs classification tasks and is able to predict the class of new samples (R package "MixOmics," Lê Cao et al., 2011). The method integrated a continuous data matrix comprising the individual FPCs of the 13 milk metabolites and 1 enzyme and enzyme and a categorical outcome variable: the line of the goat (High_LGV versus Low_LGV). sPLS-DA seeks the components that best separate the sample groups, and also selects variables that best discriminate between groups using lasso penalization. We chose the number of components using cross validation on a non-sparse model (comprising all variables) and then tuned the number of variables to select on each component using lasso selection. We assessed the final performance of the model using a 5-fold cross validation.

Unsupervised clustering of the milk metabolite curves.

Hierarchical Clustering We ran an unsupervised hierarchical clustering on all the FPCs (Dash et al., 2003, R package "FactoMineR"), to define the different types of metabolic responses to the underfeeding challenge for each goat, and independently of the longevity line. First, a 5-dimensions PCA was run on the FPCs. The PCA hierarchical clustering starts by treating each goat as a separate cluster. Then, it recursively executes the following steps: (1) identify the 2 closest clusters; (2) merge the 2 closest clusters. This process continues until all the clusters are merged together. The final number of clusters was automatically chosen based on the inertia gain, i.e., finding a minimum number of clusters allowing a low intra-cluster variability and a high inter-cluster variability.

To easily understand the differences between clusters we compared the milk metabolite curves between clusters using a permutation test (Ramsay and Silverman, 2005; Sirski, 2012). The test begins by taking the absolute value of a *t*-test-type statistic at each point along the curve:

$$F(t) = \frac{\left[\overline{X1}(t) - \overline{X1}(t)\right]^2}{\sum_i [Xi1(t) - \overline{X1}(t)\Big]^2 + \sum_i [Xi2(t) - \overline{X2}(t)\Big]^2}.$$

Then it uses a permutation test to assess significance, by randomly reordering the curves and recalculating the test statistic with the new groups of curves. We used the default setting of 200 random reorderings. One main advantage of the permutation test is that, unlike parametric tests, it does not assume theoretical probability distributions.

Data used to compare clusters The R package 'survival' was used to compare lifespan between the clusters of goats resulting from the unsupervised clustering. Survival analysis was performed using a Cox model (Cox, 1972). Because of the lack of survival data in Paris data set, the survival analysis was thus run over a sub data set of the 3 clusters, excluding the Paris data.

Analysis of variance tests were used to compare the following data. Weight, chest size and height at the withers were measured at 6 mo old. The estimated breeding values of the sires of the goats for functional longevity and milk performances were also compared between clusters. The weight and milk performance curves (milk yield (**MY**), milk fat content (**MFC**), milk protein content (**MPC**), ratio of fat content to protein content (**F:P ratio**), and somatic cell score (**SCS**)) during the underfeeding challenge were compared between clusters following the exact same methodology as the milk metabolite curves: spline interpolation, correction for year x site effect and permutation test.

RESULTS

Modeling of the individual milk metabolite curves with functional PCA

The smoothed curves of the 13 milk metabolites and 1 enzyme of one randomly selected goat are presented in the **Figure 1,** and **Table 2** shows the distribution of the milk metabolite concentrations during the whole period of sampling among the 138 goats. After smoothing, the general shape of the curve was preserved and the bounce that occurred during the challenge was correctly fitted. Linear regression correctly corrected for the year-facility effect, leading to similar mean curves per year-facility for each metabolite. Between 2 and 4 functional components were necessary to explain 90% of variation for each metabolite. The interpretation of those principal components should be made as follows (**Figure 2**): using the isocitrate components as an example, the first component (PC1) of isocitrate roughly corresponds to a flat line over the whole period, a goat with a higher than average milk isocitrate concentration over the whole period will have a proportionally high score FPCs on this component. The second component (PC2) shows a positive flat line before challenge and a

Table 2. Concentrations of 13 milk metabolites and 1 enzyme collected during 10 morning milkings among 138 goats that underwent an underfeeding challenge during early lactation: 2 samples were taken during underfeeding challenge of 2 d, 4 before and 4 after. The 138 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges and Mosar Paris

	Pre-challenge n = 552 samples		Challenge n = 276 samples		Post-challenge n = 552 samples	
	Mean	(SD)	Mean	(SD)	Mean	(SD)
BOHB (microM)	27.3	(±8.8)	39.8	(±25.1)	25.0	(±7.5)
Chol (microM)	192.0	(±74.4)	480.9	(±192.7)	258.6	(±144.3)
Choline (mM)	1.5	(±0.9)	3.3	(±1.3)	1.5	(±1.0)
Gal (microM)	65.9	(±22.4)	86.5	(±36.2)	66.3	(±29.9)
Glu (microM)	226.8	(±120.6)	117.2	(±57.2)	269.8	(±161.1)
Glu6P (microM)	60.8	(±19.0)	40.9	(±20.9)	55.6	(±20.7)
Glutamate (microM)	250.0	(±95.3)	103.3	(±43.3)	283.2	(±157.8)
Isocitrate (microM)	162.2	(±45.5)	239.3	(±76.5)	118.7	(±42.2)
LDH (UI)	10.2	(±4.7)	45.5	(±27.8)	13.9	(±14.0)
Malate (microM)	98.0	(±51.2)	38.0	(±20.5)	78.6	(±34.1)
NH_2 (glutamate micro eqv)	1687.1	(±377.1)	1356.5	(±300.6)	1893.5	(±414.9)
TAG (mM)	44.7	(±15.5)	83.6	(±31.3)	44.4	(±20.6)
Urate (microM)	58.9	(±31.7)	116.5	(±70.7)	76.2	(±57.7)
Urea (mM)	6.7	(±3.7)	6.5	(±2.1)	4.8	(±3.1)
MY (kg)	3.1	(±0.5)	1.7	(±0.6)	2.5	(±0.6)
MFC (g/kg)	40.5	(±6.2)	65.9	(±15.5)	38.7	(±10.7)
MPC (g/kg)	33.2	(±2.5)	34.8	(±3.9)	32.8	(±2.8)
F:P ratio	1.3	(±0.2)	1.9	(±0.4)	1.2	(±0.3)
SCS	4.9	(±1.8)	5.6	(±3.0)	6.1	(±1.8)

Glucose-6-phosphate (Glu6P), glucose (Glu), galactose (Gal), β-hydroxy-butyrate (BOHB), isocitrate, glutamate, NH_2, urea, choline, malate, urate, triacylglycerols (TAG), cholesterol (Chol) and lactate dehydrogenase enzyme (LDH). Milk performance: daily milk yield (MY), ratio of fat content to protein content (F:P ratio), fat content (MFC), protein content (MPC), somatic cells score (SCS).

shift to negative value during the challenge: a goat with higher than average concentration of isocitrate before challenge and a lower than average concentration post-challenge will get a high score on this component. In total 48 FPCs were attributed to each goat to characterize the variation of the 13 milk metabolites and 1 enzyme through the underfeeding challenge. The FPCs were then used to compare the milk metabolite variations between lines.

Supervised clustering to compare the milk metabolite curves between the 2 longevity lines of goats

The optimal number of components in the sPLS-DA to discriminate the 2 longevity lines of goats was 1. The lasso penalization selected 13 variables for this component (**Figure 3**). Chief among these were Gal (3rd fPCA component), glutamate and urea (respectively 1st and 2nd fPCA component). The balanced error rate (i.e., the percentage of misclassifications) estimated overall for the model was 49.5%. It was 61.3% and 37.7% respectively for Low_LGV and High_LGV lines. Thus, the milk metabolite curves during an underfeeding challenge could not predict the longevity line of the goats coming from the 4 year-sites of experiment. However, if the analysis was run on P3R Bourges and Paris separately, the balanced error (**BER**) rate was respectively 44 and 37%. **Figure 3** shows the contributions of the selected fPCscores to the prediction of the longevity line within Paris and P3R Bourges data sets. When the analysis was made on each of the 4 year-facilities separately, the BER ranged from 30% (Paris 2022) to 39% (P3R Bourges 2021).

Unsupervised clustering on the milk metabolite curves

Description of the clusters. The correlation circle of the PCA applied on the 48 FPCs of the 138 goats is shown in **Figure 4A**. Three clusters were identified: cluster 1, 2 and 3 respectively gathered 36, 53 and 49 goats (**Figure 4B**). Distribution of goats from the 4 year-facility combinations did not differ along clusters nor between longevity lines and the number of kids per kidding ($P > 0.70$). The mean milk metabolite curves per cluster and permutation test result are shown in **Figure 5**. The permutation test over the milk metabolites curves between clusters indicates which milk metabolites were significantly different between clusters. The metabolite curves that were significantly different between clusters are: BOHB, Chol, choline, Glu, Glu6P, glutamate, LDH, malate, NH_2, TAG (permutation test, 5% critical value). Except for TAG and Chol, the dif-

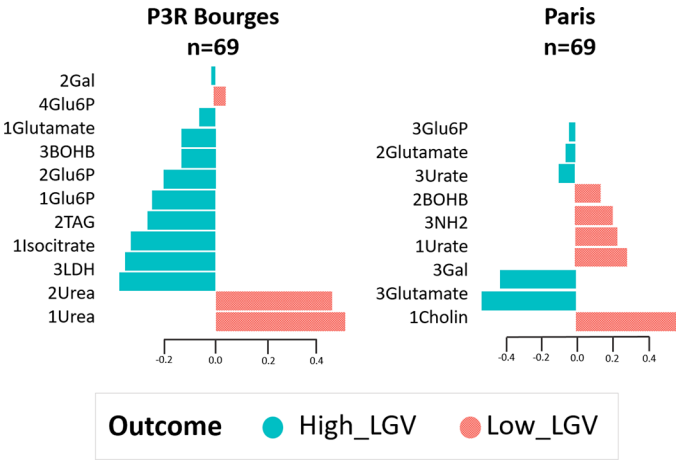

Figure 3. Loading plot from the sPLS-DA to discriminate the 2 lines selected on functional longevity, based on 13 milk metabolites and 1 enzyme curves through a 2-d underfeeding challenge run separately on goats from P3R Bourges facility (left, n = 69) and Paris facility (right, n = 69). Colors indicate the longevity line in which the median is maximum for each fPCscore: red = Low_LGV, blue = High_LGV. Variable names indicate the fPCscore of the metabolite that was selected.

ferences were significant only after the beginning of the feed restriction. For Chol and TAG the values were significantly higher for cluster 2 before day (−2).

Cluster 1 was mainly characterized by lower Glu, malate and glutamate during the recovery period, higher Gal and Chol during the recovery period, higher BOHB, TAG and choline during challenge and lower NH_2 and Glu6P during challenge and early recovery. The cluster 2 was mainly characterized by lower TAG, Choline, BOHB, LDH and Chol during challenge. The cluster 3 was mainly intermediate between clusters 1 and 2 with the exception of a higher LDH during challenge.

Comparison of resilience related features between clusters. The Kaplan–Meier survival curves of the 3 clusters are displayed in **Figure 6**. The Cox analysis shows poorer survival of goats belonging to the cluster 1 relative to both clusters 2 and 3 ($P = 0.04$, hazard ratio = 2.63 and $P = 0.02$, hazard ratio = 3.70 respectively, **Table 3**). Note that a Cox analysis comparing cluster 1 relative to the rest of the goats (i.e., cluster 2 and 3 merged) shows a more significant effect (hazard ratio = 2.97, $P = 0.009$). **Table 4** presents ANOVA results between clusters. No significant difference could be seen in sire's EBV (longevity, milk yield, milk components and SCS), morphology at 6 mo old and days in milk at the beginning of the challenge ($P > 0.05$). **Figure 7** shows the mean curves of the milk performance and weight within the 3 clusters through a 2-d underfeeding challenge. Cluster 1 showed higher F:P ratio and MFC, during and after challenge, as well as higher MPC during challenge and higher SCS after challenge (permutation test, 5% critical value).

DISCUSSION

Context

This study presents an innovative design using longevity lines exposed to a short-term challenge with repeated measures of multiple milk metabolites. We hypothesized that the metabolic responses to a short-term feeding restriction would characterize a resilience mechanism that has an impact on goat survival within herd. Repeated measurements over time were of great value in understanding the temporal aspect of resilience (Döring et al., 2015). Moreover, animal resilience is a complex trait as it involves many interconnected physiological regulations and metabolic pathways. Novel data analysis methods of 13 milk metabolites and 1 enzyme concentrations over time allowed us to both grasp the time varying aspect of the process and some of its complexity. Several studies report the modeling of a physiological response to short-term perturbation (Sadoul et al., 2015; Friggens et al., 2016). Those models made strong assumptions concerning the shape of the curves to decipher the different components of the reaction (pre-challenge baseline, response, recovery). To deal with the complexity of the metabolic pathways that we explored, and reduce the number of assumptions made a priori, we used spline interpolations as they were flexible and do not make a priori assumptions regarding curve shapes. Both the sparsity of the time points and the heterogeneity of variance between days (a sharp difference occurred during the 2 d of underfeeding challenge) made it difficult to settle on a proper roughness penalty that would be strong enough to prevent boundary effects and flexible enough to capture the bounce during challenge. That is why we used natural cubic splines, fixing a minimum degree of the polynomial at 5. The natural cubic spline is considerably 'stiffer' than a polynomial in the sense that it has less tendency to oscillate between data points. Imposing a minimum complexity via the natural cubic spline allowed both a small boundary effect and a good fitting of the sharp increases and decreases during the underfeeding challenge. Moreover, Friggens et al. (2016) showed an interesting variability in the reaction to the challenge but also strong correlation between the pre-supposed components of the reaction suggesting redundancy among them. This is why we decided to use FPCA which allowed an efficient dimension reduction since each principal component is orthogonal to the others, avoiding any redundancy. In a sense, one can see FPCA as an alternative piecewise

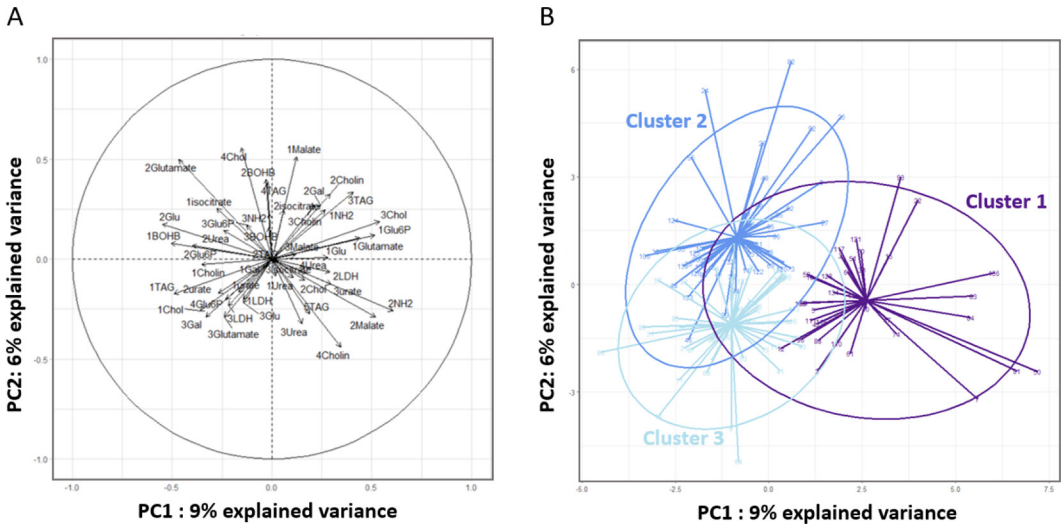

Figure 4. Hierarchical clustering on PCA of the 138 goats described with 48 functional Principal Component scores (FPCs) of 13 milk metabolites and 1 enzyme curves through a 2-d underfeeding challenge. The 138 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges and Mosar Paris. A- Correlation circle of the PCA applied on the 48 FPCs of the 138 goats. B- Individual plot of the PCA. The colours correspond to the 3 clusters determined by the hierarchical clustering.

modeling since the individual curves can be estimated as the linear combination of the functional principal components weighted by the FPC scores (**Figure 2**), but with automatically optimized components rather than pre-supposed components.

Findings of supervised clustering

The prediction of the longevity line of goats by the sPLS-DA was associated with a 49.5% error rate, showing that no discrimination of the genetic line was possible by this approach. There may be several explanations for this high error rate. First, as previously stated, the selection for functional longevity might lead to a large intra-line variability i.e., many factors affecting the longevity could be selected. Ithurbide et al. (2022) showed that the high longevity line of goats had higher body weight and lower fat to protein ratio in milk at the beginning of the first lactation, suggesting that the better survival of the High_LGV line was linked with lower body fat mobilisation. However, the nature of 2 d underfeeding challenge we imposed in the present study does not exactly mimic the challenges that can be naturally undergone during the beginning of the first lactation. The differences in the metabolic reaction to the early lactation-related energy deficit and a negative energy balance induced by feed restriction have been investigated in dairy cows (Gross and Bruckmaier, 2015).

Moreover, functional longevity is a complex trait, and selection for better longevity can result in animals with different kind of resilience or robustness mechanism (resilience or resistance to diseases for example). The present study only explored one aspect of the resilience: the energy metabolism. This diversity of the possible underlying components of longevity reduces the statistical power of the analysis (some goats could be considered High_LGV because they have good genetic value for disease resilience despite low energy metabolism resilience).

Despite the finding that the 2-d underfeeding challenge we used was shown to induce acute metabolic and production deviations (Friggens et al., 2016), resilience and longevity may reflect a broader range of (short- and long-term) coping mechanisms to a diversity of challenges such as heat waves, behavioral stress, infectious diseases. We found a large batch effect between the 4 year x facility combinations of the study. That finding is corroborated by several studies that showed large farm to farm variability in either the proportion of variance explained or in the panel of dynamic features which best predicted resilience (Adriaens et al., 2020; Krogh et al., 2020; Poppe et al., 2020). We chose to apply a linear functional regression to deal with this batch effect. This functional linear regression relied on the hypothesis that the difference we observe between years was not due to resilience related differences. This correction was necessary to run an unsupervised clustering, but not for the sPLS DA. We decided to present the result of the sPLS DA based on FPCA over the milk metabolite curves corrected for year-facility effect to compare the conclusions of the 2 approaches based on the same FPC scores. However, the sPLS DA based on the non-corrected curves did not result in better

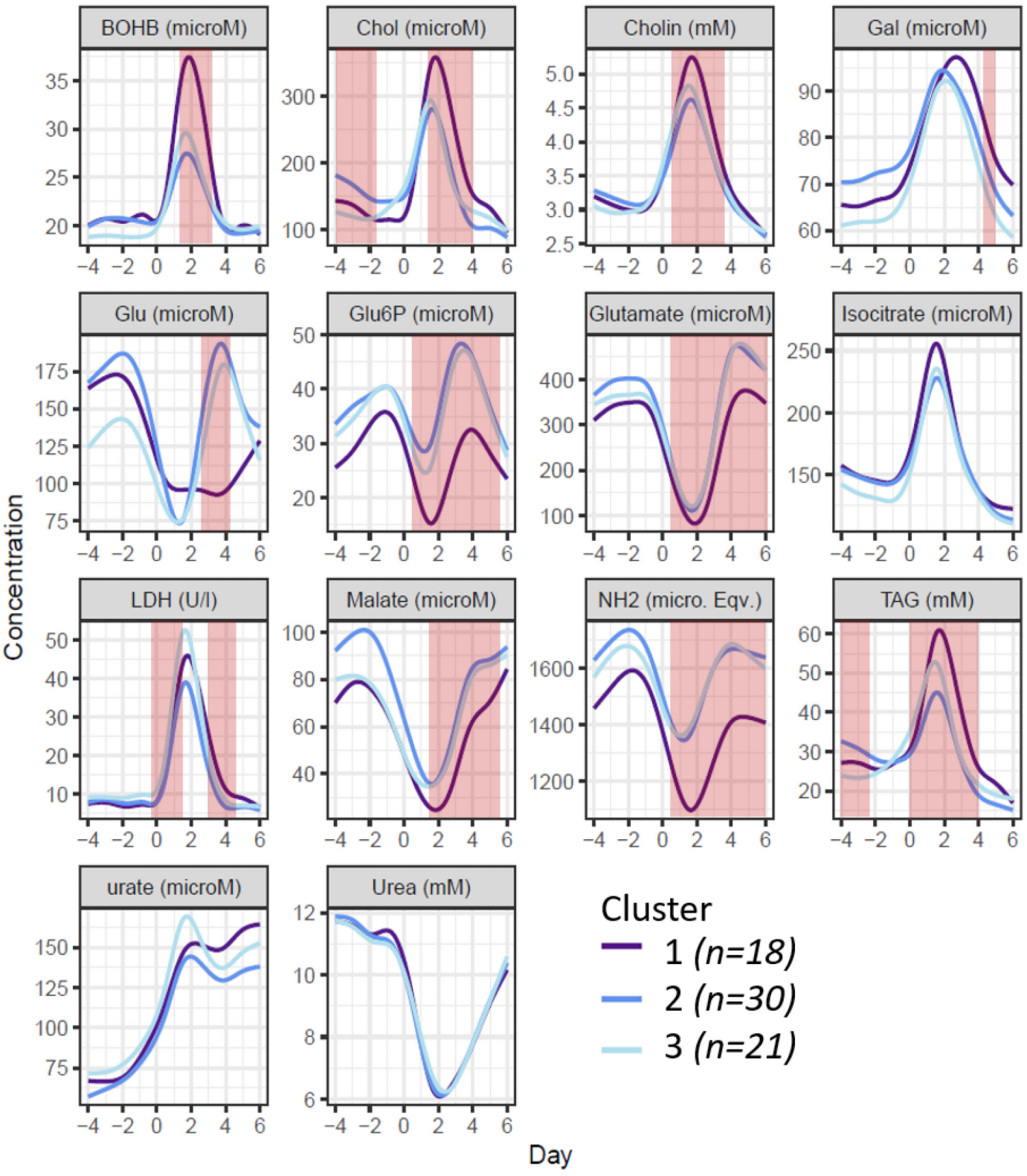

Figure 5. Mean curves of the milk 13 milk metabolites and 1 enzyme within the 3 clusters identified by unsupervised clustering in 138 goat through a 2-d underfeeding challenge. These curves are corrected for the year x facility effect with a functional linear regression. The red area indicates the time period during which the variables are significantly different between clusters (permutation test, 5% critical value).

Table 3. Hazard ratios (HR) with 95% lower and upper CI from Cox hazard model for culling data in 69 goats of the 3 clusters identified by unsupervised clustering. The unsupervised clustering defined 3 overall metabolic responses to a 2-d underfeeding challenge based on milk metabolite curves. The 69 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges

Risk factor	HR	CI		*P*-value
Cluster 1 vs 2	2.63	1.07	6.25	0.04
Cluster 1 vs 3	3.70	1.25	11.11	0.02
Cluster 2 vs 3	0.70	0.24	2.06	—

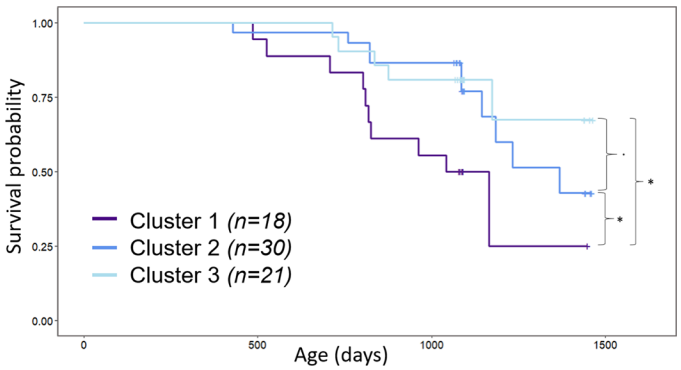

Figure 6. Plot of Kaplan–Meier curve showing survival against time in 69 goats of the 3 clusters identified by unsupervised clustering. The unsupervised clustering defined 3 overall metabolic responses to a 2-d underfeeding challenge based on milk metabolite curves. The 69 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges. The cox model analysis showed significantly poorer survival of the cluster 1 over cluster 2 and 3 (* $P < 0.05$).

prediction (results not shown). The prediction run separately on the 2 years of experiments in Paris and the 2 years in Bourges showed better results (respectively 37 and 44% BER). This suggests a possible interaction between the longevity line and the environment, as well as the importance to further study those effects. The housing and the staff differed between the 2 facilities. As explained in the materials and methods section, the diets were different between facilities. Feed quality can also vary between years due to the prevailing weather, and other factors. The 2 farms had performance levels similar to commercial farms, as described in Ithurbide et al. (2022).

The previous points highlighted that, even if the selection for functional longevity implied differences for several resilience traits (Ithurbide et al., 2022), it did not result in 2 strictly different metabolic responses to the underfeeding challenge but rather to a large variability of response that overlapped between the 2 longevity lines of goats. This led us to explore the diversity of responses to the challenge without any preliminary hypothesis on the level of resilience of the goats, i.e., without taking into account line, through the unsupervised clustering of the metabolic responses to the underfeeding challenge.

Findings of unsupervised clustering

The unsupervised clustering based on the fPCscores of all the 13 metabolites and the activity of 1 enzyme was a powerful method to explore the diversity of metabolism responses to underfeeding. This analysis defined 3 clusters of metabolic response to the underfeeding challenge. The survival of goats of cluster 1 was lower than cluster 2 and 3, with an estimated hazard ration equal to 2.97 ($P = 0.009$) i.e., at any age of life, a goat from the cluster 1 had 2.97 times more risk of being culled than other goats (cox model analysis). It should be noted that survival records were only available for goats in the Bourges facility.

Interestingly, the cluster that was associated with the lowest survival (cluster 1) had the highest milk TAG, Choline, Chol and BOHB concentrations during chal-

Table 4. The ANOVA testing (LSMEANS) for the difference between the 3 clusters identified by unsupervised clustering in 138 goats through a 2-d underfeeding challenge. The ANOVA included the year x site effect. The 138 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges and Mosar Paris

	Number of records	cluster 1	cluster 2	cluster 3	p_value
EBV_{sire} for functional longevity (days)	124	−1.82	23.59	6.30	—
EBV_{sire} _MPC (g/L)	124	0.50	0.02	0.39	—
EBV_{sire} _MFC (g/L)	124	−0.04	0.00	0.13	—
EBV_{sire} _SCS	124	100.7	100.6	98.4	—
EBV_{sire} _MY (kg)	124	15.06	14.43	4.75	—
Weight_6mo (kg)	137	34.0	33.0	33.8	—
Chest_6mo (cm)	136	71.4	71.2	71.0	—
Height_6mo (cm)	137	66.2	66.7	66.7	—
DIM (days)	138	34.7	35.8	35.9	—

Estimated breeding values of the goats' fathers for functional longevity (EBV_{sire} for functional longevity), milk protein content (EBV_{sire} _MPC), milk fat content (EBV_{sire} _MFC), somatic cells score (EBV_{sire} _SCS) and milk yield (EBV_{sire} _MY). Morphology at 6 mo of age: weight (Weight_6mo), chest size (Chest_6mo), height at the withers (height_6mo). Days in milk at the beginning of the challenge (DIM).

lenge and recovery periods (**Figure 6**). Milk Chol and Choline are shown to be associated with milk TAG (Billa et al., 2020). Increased milk fat content and BOHB during the underfeeding challenge suggests higher body fat mobilization in cluster 1 (Bjerre-Harpøth et al., 2012; Pires et al., 2022). A possible interpretation is that a high body fat mobilization during short-term feed restriction is linked with lower resilience mechanism. This was confirmed by the higher F:P ratio and higher MFC of the cluster 1 during challenge (**Figure 7**). Interestingly Ithurbide et al. (2022) showed that Low_LGV goats had higher F:P ratio during early lactation, indicating a link between resilience and body fat mobilization.

Cluster 1 was also defined by lower Glu and Glu6P during the recovery period and from the beginning of challenge respectively. Milk G6P is synthetized in the mammary gland from Glu and is a precursor for NADPH via the pentose phosphate pathway that provides reduction equivalents for preventing oxidative stress and also for reductive biosyntheses (Garnsworthy et al., 2006). Several studies report an increased G6P milk concentration during feed restriction (Chaiyabutr et al., 1981; Faulkner and Peaker, 1982; Larsen et al., 2016; Billa et al., 2020). Zachut et al. (2016) suggested that the increase in milk Glu6P concentrations observed at the onset of lactation may be due to activation of the pentose phosphate pathway in mammary epithelial cells. The Glu6P increase would meet the NADPH requirements for the attenuation of cellular oxidative stress during periods of increased fatty acids oxidation. The lower Glu6P among cluster 1 goats might indicate a lower ability to mitigate oxidative stress. Surprisingly, cluster 1 also presented higher Gal concentration dur-

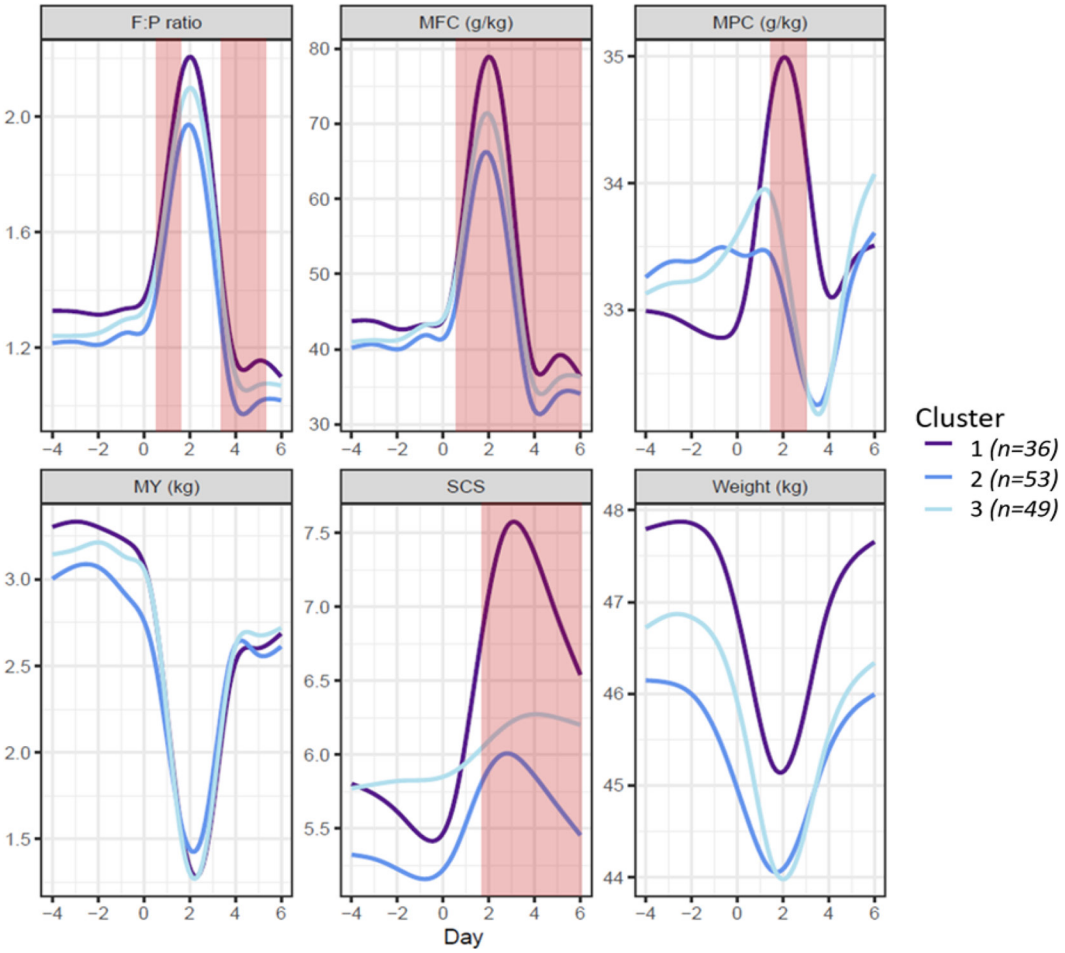

Figure 7. Mean curves of the milk performance and weight within the 3 clusters identified by unsupervised clustering in 138 goat through a 2-d underfeeding challenge: daily milk yield (MY), ratio of fat content to protein content (F:P), fat content (MFC), protein content (MPC), somatic cells score (SCS) and weight (Weight). These curves are corrected for the year x facility effect with a functional linear regression. The red area indicates the time period during which the variables are significantly different between clusters (permutation test, 5% critical value). The 138 goats belonged to 2 divergent lines selected on high longevity or low longevity bred at INRAE facilities of P3R Bourges and Mosar Paris.

ing the recovery period. Similarly to Glu6P, Gal is synthetized in the mammary gland from Glu. That might indicate that Glu is preferably used for Gal synthesis rather than Glu6P among cluster 1 animals, which might increase oxidative stress. Interestingly, Ben Abdelkrim et al. (2023) found that Glu and BOHB milk concentrations were part of the most informative milk components for determining membership of clusters of milk metabolite curves through a 2 d underfeeding challenge in late lactating dairy goats.

The glutamate, malate and NH_2 decrease during challenge tended to be greater in cluster 1 with a slower increase during recovery. Overall, cluster 1 corresponds to goats that have stronger modifications of milk metabolite concentrations during challenge. The idea that a better resilience is associated with smaller metabolic variations is explored in several articles. For example lower variation and autocorrelation of the daily milk yield (Poppe et al., 2020) or the relative height of the milk yield maximum compared with the milk yield in late lactation (Arnal et al., 2019). The comparison of the milk composition and SCS between clusters showed that cluster 1 had significantly higher SCS during the recovery period. Milk SCS in goats is an indicator of inflammation and bacterial mastitis (Paape et al., 2001; Luengo et al., 2004; Moroni et al., 2005). Interestingly cluster 1 also showed higher LDH concentration around d 4 after the beginning of the challenge. Endogenous LDH in milk originates mainly from somatic cells, leucocytes and invading microorganisms (Larsen, 2005) and is an indicator of inflammation (Krogh et al., 2020). Increased LDH during feed restriction could be partly explained by cell damage of mammary tissue during the challenge period and was also reported in Ben Abdelkrim et al. (2023). Inflammation imposes a metabolic burden, because it requires glucose and other limiting nutrients in ruminants, and may explain decreased concentrations of glucogenic milk metabolites concomitant with increased SCS in cluster 1 (Bouvier-Muller et al.,. 2016; Kvidera et al., 2017). Our study suggests that the goats of the cluster 1 were characterized by lower resilience mechanisms, related both to energy metabolism and the inflammatory system.

CONCLUSION

This study presented the curves of 13 milk metabolites and 1 enzyme through an underfeeding challenge among 138 early lactating primiparous goats selected for extreme functional longevity. A novel functional PCA approach was used to model the milk metabolites curves, allowing to address the dynamic and multifactorial patterns of the responses. The approach did not discriminate the 2 longevity lines, highlighting a large variability within lines. Unsupervised clustering of such profiling however showed distinct metabolite curves associated with length of productive life in the flock. Moreover, we found that Cholesterol, Glu6P, Glu, TAG and BOHB were the most discriminating metabolites for the cluster. These results confirm that multivariate analysis of non-invasive milk measures shows potential for deriving new resilience phenotypes.

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