BIOMARQ'LAIT -Identification of biomarkers in milk to monitor the nutritional status of dairy cows

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Abstract

The aim of the BIOMARQ'LAIT project was to identify molecules that could be measured in milk, in order to use a non-invasive approach to determine the capacity of dairy cows to cope with an energy deficit. In this project, numerous milk components have been studied in whole milk, fat globules, extracellular vesicles and mammary epithelial cells. A panel of candidate biomarkers consisting of metabolites, proteins and microRNAs was built up. The project then led to the development of equations to predict the concentrations of three metabolites of interest based on the mid-infrared spectra of milk. The new references acquired during the project were then disseminated to stakeholders in the dairy sector through institutional, technical and scientific communications. An educational kit was also produced to provide farmers and students with a better understanding of the energy deficit in dairy cows.

Keywords: dairy cattle, feed restriction, microRNA, protein, metabolite, mid-infrared spectrometry

1. Introduction

In the current socio-economic context, improving feed efficiency is a key determinant of the competitiveness of dairy farms. Precise management of short periods when homeostasis is disrupted by physiological transitions (start of lactation) or environmental changes (change in feed intake following a climatic hazard, for example) that can cause an energy deficit in dairy cows, presents a real opportunity to improve feed efficiency (Bradford *et al.,* 2016). During these periods, the ability of animals to endure a change in their environment without damaging their production potential (maintaining a metabolic status that limits the onset of metabolic diseases, maintaining a capacity for milk synthesis and a number of cells in the udder to maintain a good production potential) differs from one individual to another (Jorgensen *et al.,* 2016).

The aim of the BIOMARQ'LAIT project was to exploit this individual variability by **identifying molecules that can be measured in milk, using a non-invasive approach to determine the capacity of dairy cows to adapt to an energy deficit** caused either by the start of lactation (change in requirements linked to the emergence of milk production needs), or by a hazard (change in intake following a climatic hazard, for example). Milk is a particularly rich source of information, containing a wide range of biomolecules

such as metabolites, proteins (Miranda *et al.,* 2013), mRNAs (Boutinaud *et al.,* 2015) and microRNAs (Laubier *et al,* 2015) which are present in fat globules (Canovas *et al.,* 2014), somatic cells including exfoliated mammary epithelial cells (Hervé *et al.,* 2016) and extracellular vesicles (Valadi *et al.,* 2007). These microRNAs are small non-coding RNAs (~ 22 nucleotides) that control many biological processes such as cell proliferation, growth and death, immune response and metabolism, are involved in breast function (Le Guillou *et al.,* 2012) and are affected by dietary deficiencies (Mobuchon *et al.,* 2015).

As these molecules are time-consuming and costly to assay, they cannot currently be used routinely. However, high throughput measurement of this type of trait is a major challenge. On the one hand, it would enable breeders to monitor their animals effectively during periods of risk, and on the other, it would provide a new phenotypic measure to enhance genetic selection for animal robustness. As a result, the second objective of the project is to **develop a method for using these biomarkers in the field,** the technology chosen being mid-infrared spectrometry (MIR). This tool for monitoring the nutritional status of dairy cows should provide farmers with the leverage they need to adapt their animals' feed on a case-by-case basis in order to avoid the metabolic disorders typically observed at the start of lactation and to ensure that it continues under the best possible conditions. This objective helps to reduce feed wastage, as there is no need to preventively increase the energy intake of the whole batch or herd when only a few cows need additional supplementation at any given time.

Lastly, **the new references acquired in this project were intended to be passed on to the stakeholders** via livestock consultants and agricultural and agronomic education.

This project was carried out in partnership between **the French Livestock Institute (IDELE)**, **INRAE (UMR H, GABI and PEGASE)**, and **L'Institut Agro Rennes-Angers**.

2. Inventory of practices for identifying nutritional stress in livestock farming

Surveys were carried out in January 2019 to **understand the perception of energy deficit in dairy cows, identify the indicators used and take stock of the practices put in place to manage it**. These surveys were carried out by 20 agronomist students from the "Sciences and Engineering in Animal Production" (SIPA) specialisation at L'Institut Agro Rennes-Angers by telephone or face-to-face. Each target audience (dairy farmers and consultants) was surveyed using specific questionnaires, consisting of closed and open-ended questions. A total of 67 dairy farmers and 14 consultats (technical advisors, chambers of agriculture, veterinary clinics, farmers' associations) responded to the questionnaire.60 of the 81 people surveyed were located in the west part of France.

This survey showed that dairy farmers and their technical support staff are still not very familiar with the concept of energy deficit and how to manage it on their farms (Gelé *et al.,* 2020). A paradox between the inability of some farmers to define energy deficit and their very good practical knowledge of the phenomenon, the sensitive periods and the means available to recognise it was highlighted. **More than half of the farmers who responded had already noticed energy deficient cows in their herd.** Body condition score is the indicator they use the most, while technical advisors prefer production data and milk composition. The actions taken to limit the energy deficit mainly involve improving the ration, either overall or by specifically increasing energy intake. Around 20% of farmers would like to have easily accessible indicators or equipment that would enable them to better anticipate the risks of an energy deficit.

3. Identification in milk of biomarkers of the adaptive response of dairy cows to an energy deficit

Two feeding restriction protocols were used to **characterise the adaptive response of dairy cows to a reduction in energy intake across different phenotypes** and to assess the impact of the intensity and duration of this challenge. Molecules present in milk were quantified to assess their potential as biomarkers of this response.

3.1 Feed restriction protocols

3.1.1 Short and intense restriction (SI) protocol

This protocol was implemented in 2016 at the Marcenat experimental farm (INRAE, Herbipôle, https://doi.org/10.15454/1.5572318050509348E12) on 20 cows (10 Holstein and 10 Monbéliarde) in mid-lactation. During pre- and post-restriction, the cows were fed an *ad libitum* ration consisting of maize silage (66.3%), barley straw (8.0%), maize grain (7.6%), soya meal (17.4%) and a mixture of minerals and vitamins (0.7%). **During the experimental period, these cows were fed at a low level for 6 days (intake limited to 50% of estimated energy requirements before the start of the restriction).** Milk and blood sampling kinetics were used to study responses to restriction (from D-2 to D6) and refeeding (D+2 to D+8). The short duration of the restriction period meant that each cow could be considered as its own control.

3.1.2 Long and moderate restriction (LM) protocol

Carried out in 2016 at the IE PL (Installation Expérimentale Production Laitière) experimental farm (INRAE UMR PEGASE, https://doi.org/10.15454/yk9q-pf68), this protocol involved 19 Holstein cows (9 control and 10 restricted) in mid-lactation. In the pre- and post-restriction periods, the cows were fed an *ad libitum* ration consisting of maize silage (60%), energy concentrate (15%), soya meal (15%) and dehydrated alfalfa (10%). **During the experimental period, the cows were divided into 2 groups and fed either: 1) at a high level (same ration as in the pre- and post-experimental periods), or 2) at a low level (80% of their individual feed intake capacity) for 4 weeks (restriction period).** Milk and blood samples were taken at D-5, D5, D9, D30, D+5, D+9 and D+30.

3.2 Phenotyping in milk and blood

3.2.1 Phenotyping in blood

Plasma metabolite concentrations and blood hormone profiles were phenotyped in both protocols in the pre-experimental period to serve as a control, in the experimental period to assess the impact of restriction, and in the post-experimental period to assess the dynamics of the return to basal state. **Plasma metabolites** were measured in complete kinetics for the SI trial and on 4 occasions for the LM trial. Non-esterified fatty acids (NEFA), glucose, β-hydroxybutyrate (BHB) and urea were assayed using an automated assay system (ARENA 20XT, Thermo Fisher Scientific, Kone Instrument Corp., Espoo, Finland) and specific kits, while glutamine, NH_2 and glutamate were assayed by Aarhus University in Denmark. **Blood hormones** (insulin and IGF-1) were assayed on 4 occasions for the LM trial by the University of Bern in Switzerland, but were not assayed for the SI protocol because the kit was no longer on the market.

3.2.2 Phenotyping in milk

In addition to milk yield (MY) and the fat (FC) and protein (PC) contents, milk fine composition in terms of fatty acids (FA) and lactoproteins was also phenotyped in both protocols on several occasions before, during and after restriction. **Milk fatty acids** were quantified by gas chromatography (GC) at INRAE UMRH for the SI protocol in complete kinetics, but it was not possible to carry out the measurements for the LM protocol. **Lactoprotein** assays were carried out on defatted milk using liquid chromatography-mass spectrometry (LC-MS) at INRAE UMR GABI on the 19 cows in the LM trial on 5 occasions, and on 8 Holstein cows in the SI trial on 4 occasions.

The target molecules were phenotyped in whole milk. The potential biomarkers identified were metabolites, microRNAs and proteins, as well as the rate of exfoliation of mammary epithelial cells

(MECs). MicroRNAs were also measured in different milk compartments: fat globules (FG), extracellular vesicles (EV) and MECs.

The levels of milk metabolites (BHB, uric acid, isocitrate, glucose, glucose-6-phosphate, glutamate and NH_2 **) were assayed** using fluorometric methods by the University of Aarhus in Denmark in complete kinetics for the SI trial, and 4 times for the cows in the LM trial.

Milk MECs were counted 4 times for the LM protocol. The MECs were first purified using an immunomagnetic separation method (Boutinaud *et al.,* 2008) and then their concentration was measured at INRAE UMR PEGASE. The MECs exfoliation rate was then obtained by multiplying the MECs concentration in the milk by the volume of milk produced per day.

The miRNomes were extracted from a subgroup of 8 individuals for each experimental protocol, with one sample before restriction and one after restriction for each compartment (whole milk with the RNA Now kit by INRAE UMR GABI, FG with the Trizol LS kit by UMR H, MECs with the RNA Now kit by UMR PEGASE, and EV with the miRVana kit by the Excilone laboratory). Sequencing was carried out by the GenomEast platform of the IGBMC. A wide range of concentrations was observed depending on the compartment, and in the end the lack of biological material meant that sequences could not be analysed using EVs for the LM protocol.

Proteome analysis was carried out by the PAPPSO platform in Jouy-en-Josas on defatted milk from 32 samples chosen for miRNome analysis, i.e. 8 cows x 2 test-days (before and during restriction) per protocol.

3.3 Characterisation of the adaptive response of cows to feed restriction

The adaptive response of the animals was evaluated independently in each of the protocols, by analysing the phenotypes (basal level before restriction, deviation during restriction, ability to return to basal level after the end of restriction) collected in both SI and LM trials: zootechnical data, milk fine composition in FA and proteins and the hormones and metabolites in the plasma. **These analyses revealed the response kinetics over the duration of the restriction, as well as a return to the basal state during *ad libitum* re-feeding, and then characterised these responses as a function of the duration and intensity of the restriction** (Billa *et al.,* 2020; Leduc *et al.,* 2021a).

3.3.1 A plasma response proportional to the intensity of restriction

In the SI protocol, **restriction resulted in an increase of more than 600% in NEFAs** and BHB, indicating increased mobilisation of energy reserves. Plasma glutamate concentrations also increased during restriction, while those of glucose, glutamine, urea, NH_2 and insulin decreased. In the LM protocol, the plasma response showed a 355% increase in NEFAs in the restricted cows. Conversely, IGF-1 and insulin decreased in these same cows, with no effect on blood glucose levels. In both protocols, values returned to basal levels in the post-experimental period.

3.3.2 A response in milk linked to the feed intake and lipomobilisation

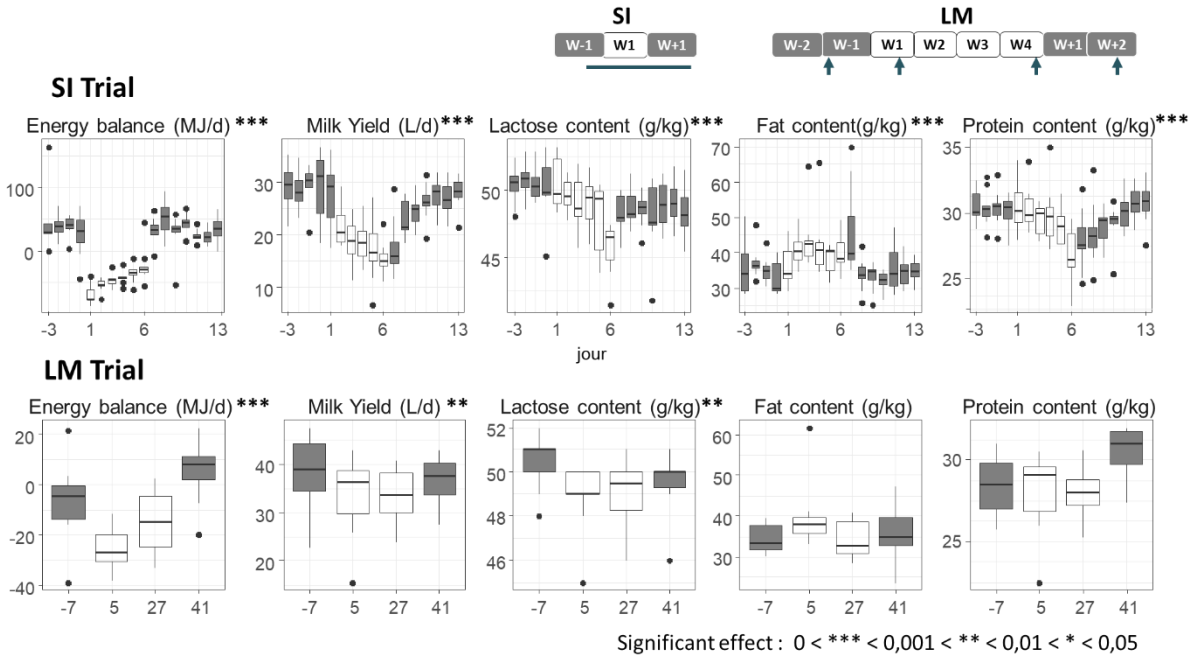

Figure 1: Cow Response to restriction in the short and intense (SI) and long and moderate (LM) restriction protocols.

In the SI trial, restriction led to a 64% drop in feed intake and a 34% drop in MY. Rates fell significantly: -25% for FC, -36% for PC and -34% for lactose content (Figure 1). The effects observed were more important in the Montbéliarde breed than in the Holstein breed. A decrease in *de novo* FA synthesis and an increase in long-chain FA content (particularly C18:0, C18:1c9 and C16:0) were observed during the restriction period, suggesting lipomobilisation. Restriction also affected the lactoprotein profile: concentrations of α_{S1} , α_{S2} and β caseins decreased significantly during dietary restriction.

The LM restriction led to similar trends to the CI protocol, although to a lesser extent: a 20% drop in feed intake and a 7.6% drop in MY in cows in the Restricted batch compared with the Control batch during the experimental period (Figure 1). This difference was no longer significant in the *ad libitum* re-feeding phase. Similarly, an increase in FC (+5%), and a decrease in PC (-13%) and lactose content (-13%) were observed during the restriction phase. During this trial, only the concentration of α-casein$_{S2}$ decreased.

3.4 Identification of a panel of biomarkers of response to feed restriction

3.4.1 Effect of feed restriction on target molecules

Comparative analysis of the concentrations of these molecules before and during the feed restriction phase confirmed their value as biomarkers. This analysis was carried out independently for each protocol, in order to assess the impact of the severity of the restriction.

More exfoliated MECs in restricted cows

A significant increase in MECs exfoliation in milk and an increase in the viability of exfoliated MECs were observed during the restriction phase of the LM protocol (Hervé *et al.,* 2019). The effect of feed restriction does not appear to be persistent on MECs exfoliation. Na^+ content was higher in control animals,

suggesting a disruption of tight junctions during restriction. However, this observation is not sufficient to validate the hypothesis that the increased rate of MECs exfoliation is linked to a loss of integrity of the mammary epithelium.

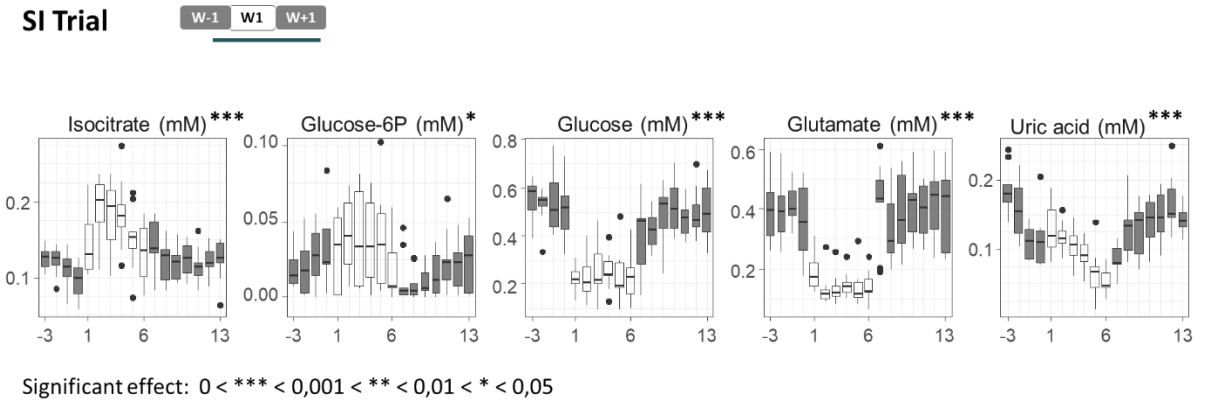

Figure 2: Changes in milk metabolite levels in response to restriction (short and intense SI restriction trial)

Variations in metabolite concentrations depends on the intensity of restriction

Blood metabolites are already used as markers of energy deficit, but milk metabolites, which are easier to access, have only recently been studied. A review of the literature identified BHB, glucose, glucose-6-phosphate, uric acid, citrate and isocitrate as potential candidates. However, no studies comparing the effect of different levels of feed restriction on milk metabolites have been identified.

In the **SI protocol**, there was a sharp drop in glucose and glutamate concentrations, and a smaller drop in BHB, uric acid and NH_2 , as well as an increase in isocitrate and glucose-6-phosphate during feed restriction (Figure 2). The kinetics are more or less rapid depending on the metabolites. Concentrations of glutamate, isocitrate and NH_2 returned to pre-challenge values the week following the restriction period, while those of BHB, glucose and uric acid did not return to their pre-challenge values until the second week post-restriction (Billa *et al.*, 2020). A correlation matrix showed strong correlations between energy balance and milk glucose (0.62) and glutamate (0.59), which correlate well with plasma metabolites and milk FAs. The other milk metabolites studied were also significantly correlated with energy balance (0.46, -0.25, -0.41 for BHB, glucose-6-phosphate, and isocitrate, respectively).

In the **LM protocol**, there was an increase in glutamate, galactose and isocitrate, a decrease in creatinine (suggesting muscle protein mobilisation) and glucose-6-phosphate, with a return to normal after restriction. Variations in BHB, glucose, glutamate and NAGase were not significant. Uric acid decreased throughout the restriction period before returning to basal levels on refeeding. The energy deficit observed in this protocol was less important than that observed in the SI protocol. The results obtained were consistent for isocitrate, galactose and uric acid, which responded in the same way as in the SI protocol. On the other hand, the contradictory results observed for glutamate suggest an effect of the intensity of restriction on the adaptive response, and in particular a possible blockage of the pentose phosphate pathway during intense restriction (Leduc *et al.,* 2021b).

Six proteins affected by restriction of any intensity

The study of the impact of restriction on milk proteomes is the first of its kind (Leduc *et al.,* 2022). Among the 232 proteins found in the milk proteome of **the SI trial**, the abundance of 160 proteins varied with feed restriction: 43 were found in the milk only during feed restriction, one only before the start of feed

restriction, and the abundance of 77 and 39 proteins respectively increased or decreased during feed restriction. Of the 194 proteins found in the milk proteome of **the LM trial**, the abundance of 8 proteins varied with feed restriction: 2 proteins were found only in milk during feed restriction, the abundance of 5 proteins increased and that of one decreased during feed restriction. Six proteins were similarly affected by LM and SI restriction, five of which are normally present in plasma. The effects observed were greater in the SI trial, demonstrating a strong impact of the intensity of the restriction. An *in silico* analysis was used to identify the metabolic pathways in which proteins differentially abundant during restriction are involved. Sixty-six proteins are involved in protein metabolism, 14 in lipid metabolism, 13 in carbohydrate metabolism and 49 in the immune response. These variations in the proteome reflect the adaptation of mammary metabolism to energy stress, as well as a loss of integrity of the mammary epithelial barrier and altered immune function.

Five microRNAs identified in whole milk as potential biomarkers

This study is the first to characterise the impact of feed restriction on milk microRNAs. The analysis revealed an average of 21 million readings per sample, with considerable heterogeneity between samples but homogeneity between the different milk compartments studied. 2,896 microRNAs were detected, 1,493 of which were already known in the bovine species. Of the 1,096 microRNAs abundant enough to be informative, 10% were exclusive to one milk compartment and the abundance of 1,027 of them varied between compartments, revealing a specific miRNome for each milk fraction. Dietary restriction affected the abundance of 159 miRNAs, with the EV and whole milk compartments most affected, while FG and exfoliated MECs were little or unaffected. No diet-dependent differentially present microRNAs were detected in common between the same compartment in both protocols, demonstrating **a strong effect of restriction intensity**. For the known microRNAs that varied during restriction, an *in silico* analysis was used to predict their targets and study the metabolic pathways in which they are involved. The 17 known variable microRNAs in sufficient quantity were used to predict 1378 target genes, including 14 coding for proteins affected in the SI trial. The 41 metabolic pathways involved reflect the modification of certain key pathways in lactation linked to lipid and protein metabolism, the cell cycle and the response to stress. **Five microRNAs were finally identified as potential biomarkers, mainly in whole milk because they are easier to use** (Leduc *et al.,* 2023).

3.4.2 Identification of biomarkers of the adaptive response using integrative analysis

Secondly, all the data common to the LM and SI trials was analysed in an integrative manner in order to identify the biomarkers of the adaptive response of dairy cows to an energy deficit. Two consecutive approaches were used, resulting in a **panel of biomarkers** of energy deficit in dairy cows.

Passage from an ad libitum state to a restricted state followed by a return to the initial state.

A **kinetic test** using redundancy analysis of the day effect was used to identify the kinetic changes during the transition from an *ad libitum* state to a restricted state followed by a return to the initial state. This analysis also made it possible to identify the markers associated with these state transitions, including protein (PC), butyrate (FC) and lactose levels, and milk metabolite concentrations. We noted the importance of variations in isocitrate in the transition to the restricted state in both protocols, and variations in glucose and glutamate in the return to an *ad libitum* state in the SI protocol. However, this approach did not allow us to take into account all the potential biomarkers, in particular proteomes and miRNomes.

Identification of a panel of biomarkers of energy deficit

The second approach, using **multi-omics analysis** (DIABLO method), aimed to identify a panel of potential biomarkers of energy deficit, by integrating data on macro components, metabolites, major proteins, miRNomes and milk proteomes from both feed restriction trials. This approach makes it possible to predict the energy balance of cows with 10% of the variables, separated into blocks: a block of fat globule miRNomes, a block of milk miRNomes, a block of proteomes and a block consisting of MY, milk

components and milk metabolites. The analysis was first carried out separately on each of the SI and LM trials, revealing 16 markers common to both trials, including 13 microRNAs, lactose level, serotransferrin already identified in the proteome analysis and milk yield, to which glutamate and uric acid can be added if the threshold is lowered to 20% of the variables for the 'other' block.

After integrating all the results, these approaches revealed that decreases in **milk yield**, **lactose level** and **uric acid** concentration, increases in **isocitrate** and **serotransferrin** concentrations and variations in **microRNA** abundance in whole milk and fat globules were systematic during the LM and SI feed restriction trials. These candidate biomarkers are in addition to **α_{S2}-casein** , the best candidate among the lactoproteins measured by HP-LC, **proteins common to the 2 trials** measured by LC-MC/MS, and milk **glutamate. These results pave the way for the development of a panel of non-invasive biomarkers of energy deficiency.**

4. Validation of biomarkers identified in energy-deficient cows of different origins

A third experimental protocol was set up to allow **initial validation of the biomarkers on a larger number of animals and during energy deficits of different origins**: one physiological, at the start of lactation, and the other environmental, induced by a dilution of the energy value of the ration. As no omics data was generated during this trial, biomarker validation in this trial was therefore limited to production data (MY, macro components), FA, major proteins and milk metabolites.

4.1 DEFFILAIT protocol

The measurements were carried out as part of a trial set up during the ANR DEFFILAIT programme at INRAE experimental farm of the INRAE UMR PEGASE (IEPL), on around thirty Holstein cows chosen to be representative of the herd's variability. These cows calved in autumn 2017 and were fed *ad libitum* on a constant diet (a total mixed ration based on maize silage, dehydrated alfalfa, soya meal and production concentrate) for the first few months of lactation. **In mid-lactation, all the cows underwent a change of diet in March 2018, moving from a control diet (2 weeks) to a restricted diet (5 weeks) after a week of dietary transition.** The restricted ration was diluted in energy and protein with straw and aimed to reduce milk production by 20% while maintaining *ad libitum* intake. The quantities of feed offered and refused were measured to determine the quantities of dry matter intake.

4.2 Phenotyping in milk and blood

Among the cows in the DEFFILAIT trial, **milk and blood samples were taken at 22 ± 1 days of lactation (D21) from 34 cows that calved in autumn 2017, then at D-7 and D+7 in relation to the dietary transition.** The phenotypes used to assess the cows' adaptive response were measured: milk yield, fat, protein, lactose levels, milk and blood metabolites and minerals, plasma hormones, major lactoproteins and MECs. Analyses were carried out jointly by the same laboratories as for the SI and LM protocols.

4.3 Effects of the energy deficit at the start of lactation and due to feed restriction

The effects of early lactation energy deficit and feed restriction were assessed by comparison with values measured prior to restriction in order to evaluate the concordance between the adaptive response of cows when the energy deficit is of physiological origin and when it is due to feed restriction and to validate the biomarkers identified on a new feed restriction trial. Differences between each sampling day were obtained using a pairwise comparison of marginal means estimated on the same linear mixed model that included the sampling day as a fixed effect and the cow as a random effect.

4.3.1 A different response to energy deficit in early lactation

At the start of lactation, the cows had a negative energy balance, as expected (Figure 3).
Blood NEFA and BHB levels were higher than in the control and restricted periods, suggesting mobilisation of body reserves. Blood glucose and MY levels were not different from the pre-restriction period.

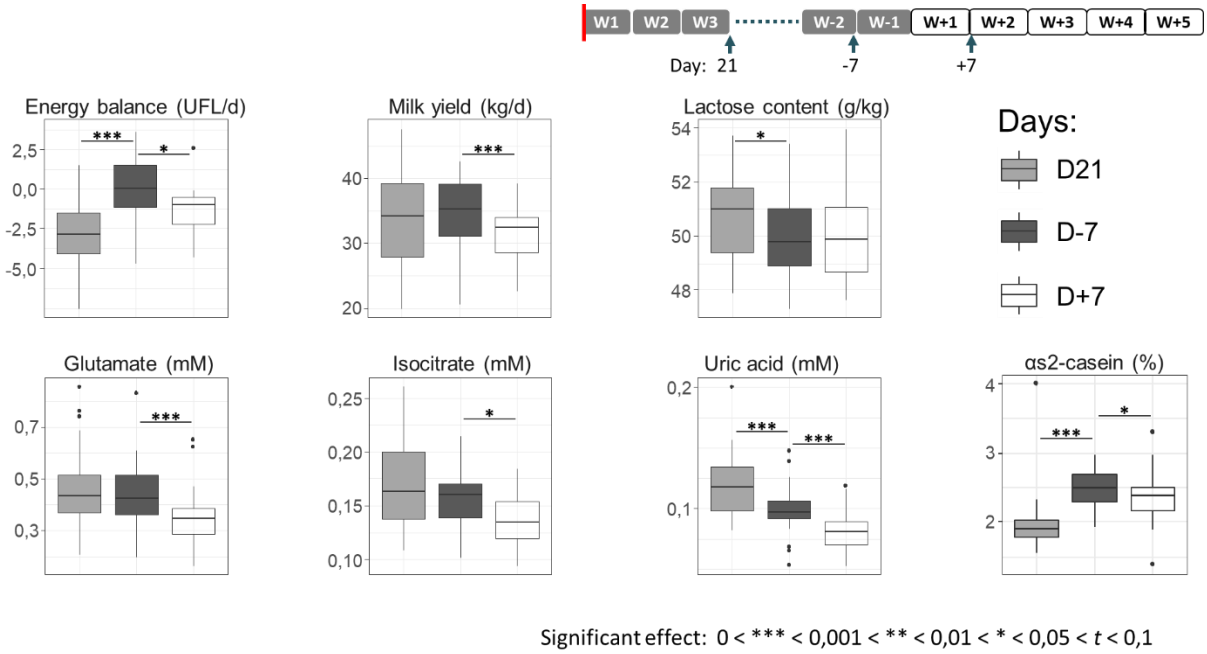

Figure 3: Variations in energy balance, milk production and milk components in the DEFFILAIT trial.

Among the candidate metabolites, glucose-6-phosphate, uric acid and lactose levels were higher at D21 than in the control and restricted periods, while glucose and galactose levels were lower. Glutamate and isocitrate levels were similar at D21 and in the control period, but higher than those measured in the restricted period. Milk concentrations of α_{S2}-casein, α-LA and β-LG were lower than those measured mid-lactation, at D-7. **These results suggest a different response depending on whether the energy deficit is physiological (early lactation) or due to a feed restriction**. In fact, only the glucose-6P concentration increased, whether in the case of an energy deficit at the start of lactation or with an energy deficit induced by severe feed restriction. However, the results obtained at the start of lactation are difficult to interpret given the significant time lag (5 months) with the other measurement periods. In fact, the energy deficit was compounded by the effect of advanced lactation. However, in the first months of lactation, milk composition is known to change significantly, and this trial did not allow the effect of energy deficit on milk composition to be differentiated from that of advanced lactation.

4.3.2 Dairy cows adapt in response to feed restriction

The feed restriction resulting from the change of diet was of low intensity. Milk production fell by 9% (compared with a 20% drop in intake) between before and after the dietary transition and was accompanied by a significant 48% increase in the rate of exfoliation of MECs in milk. **The results obtained are consistent with those obtained with the LM protocol and suggest an adaptation of the mammary tissue of dairy cows in response to restriction**. Fat, protein and lactose contents decreased very slightly. Blood BHB, glucose and urea levels decreased. No variation in body condition score or blood NEFA content was observed in the cows after the change in diet, suggesting that they did

not need to mobilise their body reserves. Variations in milk metabolite levels were observed but in different ways to those observed at the start of lactation. In this trial, a decrease in all milk metabolite concentrations (galactose, glucose, glucose-6P, glutamate, isocitrate) was observed with the change in diet, whereas the results of the SI protocol showed an increase in isocitrate and glucose-6P concentrations and a decrease in glutamate concentration. During mid-lactation feed restriction, at D+7, α_{S2}-casein and α-LA concentrations decreased while κ-casein concentrations increased. These results suggest a different response depending on the intensity of the restriction (mobilisation of reserves or not).

5. Monitoring energy deficit using mid-infrared spectra of milk

A tool for monitoring nutritional status using mid-infrared spectrometry was developed so that the results obtained could be used by farmers to adapt the ration of cows requiring it. Among the biomarkers identified, equations were developed to estimate the concentrations of glutamate, uric acid and isocitrate, for which no equations existed.

5.1 728 MIR spectra associated to metabolite concentration used

Data from the SI and DEFFILAIT protocols were used to carry out this work, representing a total of 728 pairs of MIR spectra - milk metabolite concentration, including 644 data from the SI trial and 84 data from the DEFFILAIT trial.
A Principal Component Analysis (PCA) carried out on the 728 MIR spectra confirmed that it seemed possible to discriminate between cows according to their diet using MIR spectra. Two datasets were then created:

- A **calibration set** comprising 70% of the data, on which the equations were developed using sparse partial least square regression (SPLS). For glutamate, two equations were developed: the first without transformation of the data, and the second after Log transformation of the data.
- A **validation set** comprising 30% of the data on which the equations developed have been applied to calculate their performance.

5.2 Promising prediction of glutamate concentrations

Initial results show that milk glutamate concentrations are fairly well predicted by the MIR spectrum, both with and without log transformation, with a calibration R^2 of around 0.80 (Figure 4). Isocitrate is a little less well estimated, with a calibration R^2 greater than 0.60, while the prediction of uric acid is not sufficient ($R^2 < 0.50$). The equations developed could be used in conjunction with existing equations (lactose, C18:1c9, BHB, citrate, etc.) to create a panel of biomarkers in a simple and affordable way for routine use.

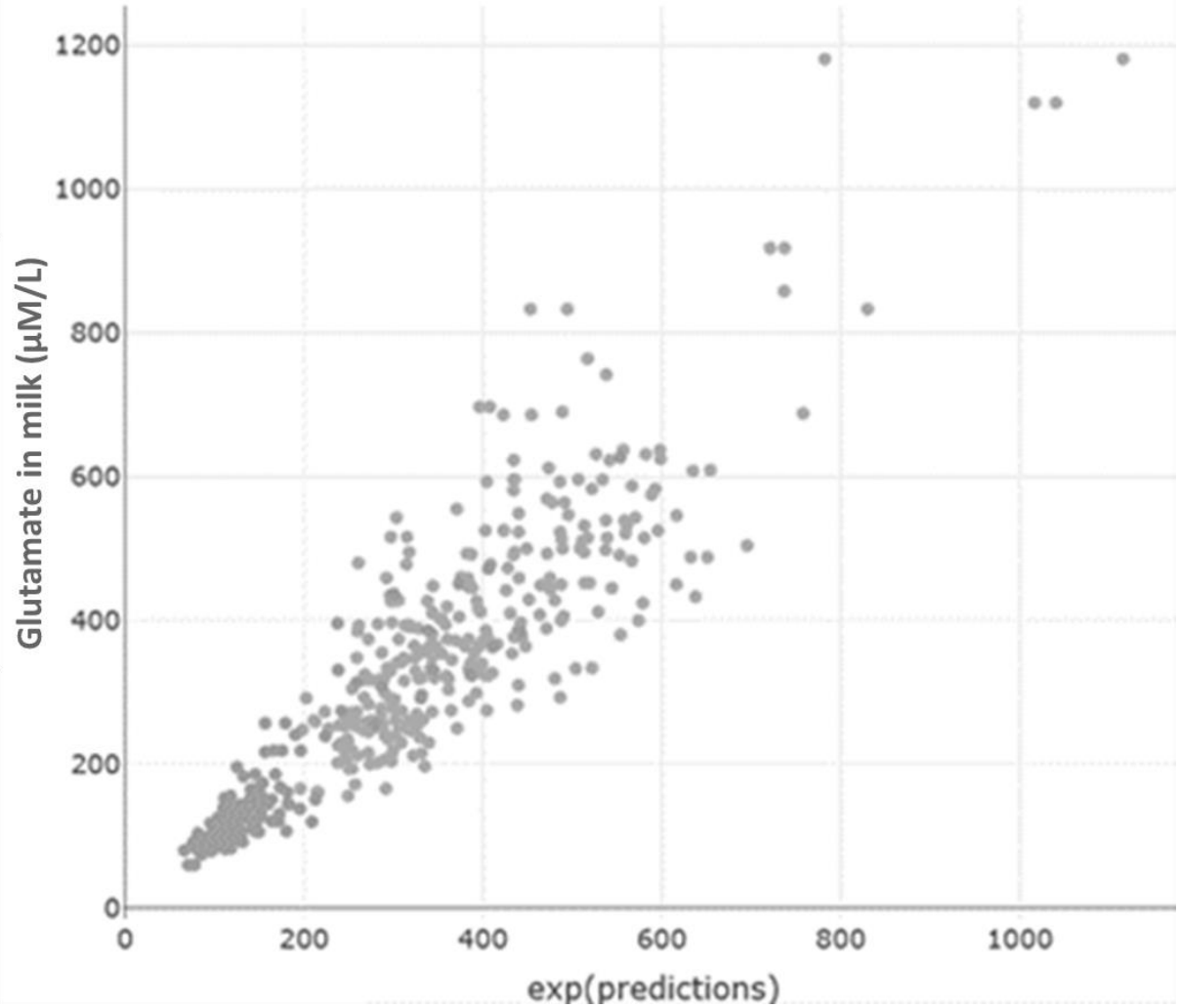

Figure 4: Predicted glutamate values (x-axis) compared with measured values (y-axis). Results on calibration set after log transformation.

6. Production of teaching aids for livestock farmers, technical advisors and agricultural and agronomic teachers

Teaching aids aimed at farmers and technical advisors, as well as agricultural and agronomy teaching staff, have been produced in order to transfer to current and future farmers and advisors the knowledge needed to prevent, identify and correct energy deficiencies on dairy farms. Surveys carried out among 21 zootechnics teachers in the Bac Technologique STAV, BP REA and BTS PA courses, as well as an exchange with the FCEL Nutrition expert group, finally showed that expectations regarding teaching aids were fairly shared between technicians and teachers. The surveys also revealed that targeting farmers directly was not necessarily appropriate, and that it would be preferable to offer support to technicians, who could then raise awareness among the farmers they support.

It was therefore decided to produce a **single educational kit** to meet all the expectations of the target groups. The kit includes both basic and more complex elements of knowledge, enabling the desired level to be adapted, from awareness-raising (in groups of livestock farmers, for example) to more or less advanced learning (BTS or agricultural engineering classes). These three media can be used independently and in any order.

The kit includes the following components

- 1 **short video** explaining what energy deficiency is, what its consequences are and how to prevent and detect it,
- 3 **technical sheets** covering the same topics but in greater detail, enabling technical audiences to learn more,
- 1 fun **assessment quiz** using the Plickers application, suitable for teachers to assess students' level of knowledge and for technicians to raise awareness among farmers.

This educational kit is available free of charge from www.idele.fr and can be passed on to technical advisers by the FCEL network, to agricultural colleges by the DGER and to engineering schools by IDELE.

7. Conclusion and future prospects

The BIOMARQ'LAIT project has led to significant scientific advances in characterising the effects of different types of dietary restriction on the fine composition of milk. The results obtained on the miRNomes of the various milk compartments and on the proteomes are original achievements. Taken together, these results provide a better understanding of the metabolic pathways affected by dietary restriction in the mammary gland. These include protein, lipid and carbohydrate metabolism linked to milk production, remodelling of the mammary epithelium *via* apoptosis and exfoliation, and the immune response.

A panel of molecules identified as biomarkers of the nutritional status of cows was identified and included macro-components and metabolites (lactose, isocitrate, glutamate and uric acid), proteins (α_{S2}-casein, apolipoprotein A-IV, α-1B-glycoprotein, angiotensinogen, and serotransferrin), and 5 whole milk microRNAs and 8 fat globule microRNAs.

The results have been disseminated to various target audiences by means of communications to the technical public (livestock farmers and advisors) *via* **the specialist press and trade shows,** numerous scientific communications including **six articles published in peer-reviewed journals** and a seventh currently being written, and an **educational kit** for transferring knowledge to professionals and agricultural and agronomic education.

The use of these results in the field requires methods for measuring the biomarkers identified that are simple, rapid and affordable to implement. For milk metabolites, equations based on MIR spectrometry are currently being developed and could be used in conjunction with existing equations. For proteins, and serotransferrin in particular, the development of specific ELISA-type immunochemical assays is under consideration. Finally, a microfluidic chip for the direct detection of microRNAs in fluids is currently being developed for mouse milk (Horny *et al.,* 2021) and is being adapted for bovine milk.

Ethics
The authors declare that the experiments were carried out in compliance with the applicable national regulations.

Declaration on the availability of data and models
The data supporting the results presented in this article are available on request from the author of the article.

Declaration on Generative Artificial Intelligence and Artificial Intelligence Assisted Technologies in the Drafting Process.
The authors used artificial intelligence in the translation process from French to English.

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Marine Gelé wrote the article, and all the co-authors read and approved it.

Declaration of interest
The authors declare that they do not work for, advise, own shares in, or receive funds from any organisation that could benefit from this article, and declare no affiliation other than those listed at the beginning of the article.

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