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The postpartum *period is crucial in dairy cows and is marked by major physiological and metabolic changes that affect milk production, immune response and fertility. Nutrition remains the most important lever for limiting the negative energy balance and its consequences on general health status in highly selected dairy cows. In order to analyze the effect of a commercial micronutrient on intrinsic parameters, performances and the epigenome of dairy cows, 2 groups of 12 Holstein cows were used: 1 fed a standard diet (mainly composed of corn silage, soybean meal and non-mineral supplement) and the other 1 fed the same diet supplemented with the commercial micronutrient (μ-nutrient supplementation) for 4 weeks before calving and 8 weeks thereafter. Milk production and composition, BW, body condition score (**BCS**), DM intake (**DMI**) and health (calving score, metritis and mastitis) were recorded over the study period. Milk samples were collected on D15 and D60 post-calving for analyses of casein, Na^+ and K^+ contents and metalloprotease activity. Milk leukocytes and milk mammary epithelial cells (**mMECs**) were purified and counted. The viability of mMECs was assessed, together with their activity, through an analysis of gene expression. At the same time points, peripheral blood mononuclear cells (**PBMCs**) were purified and counted. Using genomic DNA extracted from PBMCs, mMECs and milk leukocytes, we assessed global DNA methylation (Me-CCGG) to evaluate the epigenetic imprinting associated with the μ-nutrient-supplemented diet. The μ-nutrient supplementation increased BCS and BW without modifying DMI or milk yield and composition. It also improved calving condition, reducing the time interval between calving and first service. Each easily collectable cell type displayed a specific pattern of Me-CCGG with only subtle changes associated with lactation stages in PBMCs. In conclusion, the response to the μ-nutrient supplementation improved the body condition without alteration of global epigenetic status in dairy cows.*

Keywords: cows, peripartum, micronutrients, methylome, performances

Implications

Nutrition is a key factor for maintaining metabolic homeostasis in dairy cattle during the peripartum period. Individual nutritional optimization is a potentially interesting approach requiring the establishment of reliable biomarkers. This study reports beneficial effect of a diet supplemented with micronutrients on body weight, body condition score and on resumption of ovarian activity with milk production preservation. This study also lays the foundation for testing the use of global DNA methylation status in three different cell populations easily collectable via a minimally invasive procedure (blood cells, leukocytes and epithelial cells of milk), as a new phenotypic parameter.

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Introduction

Dairy cows undergo crucial physiological, hormonal and metabolic changes during the *postpartum* period, with implications for milk production performance, immune responses and subsequent fertility (Drackley and Cardoso, 2014; Sordillo *et al.*, 2016). The discrepancy between energy requirements and energy uptake during this period generates a negative energy balance that is most severe about 2 weeks after calving. Highly negative *postpartum* energy balances increase susceptibility to diseases, including uterine diseases, mastitis and complex disorders (Leblanc, 2012). Nutrition remains a key factor for maintaining metabolic homeostasis in dairy cattle during the peripartum period (Zebeli *et al.*, 2015) and immune responses (Ingvartsen and Moyes, 2013). Targeted micronutrient supplementation might therefore be

beneficial. We investigated the effects of diet enrichment in organic Se from yeast, antioxidants and trace elements from plants and microalgae containing a cocktail of minerals. Trace minerals (e.g. Zn, Mn, Cu and Co) are important for an optimal transition from pregnancy to lactation (Andrieu, 2008). Selenium is a trace element contributing to dairy cow health and performance (Spears and Weiss, 2008; Mehdi and Dufrasne, 2016). Supplementation with certain antioxidants may also improve immunity and lessen disease susceptibility, because peripartum changes in metabolism increase reactive oxygen species (**ROS**) release, exposing cows to greater oxidative stress (Sordillo and Aitken, 2009). We evaluated the effect of micronutrient supplementation on various parameters classically used to assess quality in dairy cows (milk production and composition, DM intake (**DMI**), net energy of lactation and health parameters). A focus was performed at two time points, at day 15 and 60. Milk production is setting into place during the first 15 days *postpartum* and is usually maximal by day 60 (Grossman and Koops, 2003). Environmental changes, such as micronutrient intake, may modify epigenetic imprinting. A Selenium-Epigenome interaction has been reported (Speckmann and Grune, 2015). Selenium enhances homocysteine flux through the transsulfuration pathway. It may also affect one-carbon metabolism, thereby altering DNA methylation potential. Vitamin E, β carotene and Cu also act on the epigenome (Chavatte-Palmer *et al.*, 2018; Huang *et al.*, 2019). We evaluated global DNA methylation as a new phenotypic parameter (Me-CCGG), in readily available cell types, such as peripheral blood mononuclear cells (**PBMCs**), milk leukocytes and milk mammary epithelial cells (**mMECs**), collected at D15 and D60. An epigenetic imprint was observed in PBMCs associated with the lactation period. We demonstrated clear beneficial effects of micronutrient supplementation on dairy cow health.

Material and methods

Animals and experimental design

Holstein cows were housed at the INRA Mejusseaume experimental dairy farm (UMR1348 IEPL, Le Rheu, France). All animal procedures were approved by the Animal Care Committee of the French Ministry of Agriculture, in accordance with French regulations (decree no. 2001–464, May 26, 2001; reference no. *0162503*). The 24 cows (7 primiparous and 17 multiparous) were genotyped by Labogena-DNA (Jouy en Josas, France), with the EuroG10K beadchip (bovine chip designed by Eurogenomics, Amsterdam, the Netherlands, and manufactured by Illumina Inc., San Diego, CA, USA), and their genetic index was calculated by UMT-eBis (Jouy en Josas, France) and expressed as a total merit index (**MI**). The experimental trial began 4 weeks before the expected parturition date. We split the cows into 2 groups of 12 (supplemented (SUPPL) and control (CTRL) groups) balanced for MI, parity, calving date, body condition score (**BCS**) and previous lactation performance for the multiparous cows (Supplementary Table S1). All cows (SUPPL and CTRL) were allowed *ad libitum* access to standard diet provided as a total mixed ration (**TMR**) in accordance with INRA guidelines (INRA 2007), as described in Table 1. The nutritional values of the various dietary components were obtained by chemical analysis (Table 1; LDA, Ploufragan, France). After 15 days of lactation, the proportions of corn silage and non-mineral supplement were modified and straw was removed. Forage consumption is recommended only at early stages of lactation to reduce the risk of subacute ruminal acidosis (Allen, 1996; Coon et al., 2019). Only 1 group of 12 cows (SUPPL cows) received micronutrient supplementation (μ-nutrient supplementation, from PILARDIERE (Saint-Mars-la-Réorthe, France) and XR REPRO (Coubon, France)), as recommended for improving the health and fertility of dairy cows during *postpartum*. The SUPPL cows received 160 g of μ-nutrient per day for 4 weeks before calving and 250 g per day during the first 60 days of lactation. The supplement was mixed with the TMR, as recommended by the supplier. The composition of μ-nutrient is described in Table 2. Feed intake was recorded daily during the lactation period analyzed. Feed was weighed and distributed in two equal-sized portions, at 0830 and

Table 1 *Composition of the basal diet before and after calving for all cows (CTRL and SUPPL groups), expressed as a percentage DM*

Total mixed ration	Before calving	After calving D0–D15	After calving D15–D60
Corn silage	84.7%	51.8%	63.8%
Soybean meal	5%	9.6%	8.60%
Straw	9.1%	1.9%	–
Energy concentrate	1.3%	24.0%	15.5%
Dehydrated alfalfa silage	–	10.8%	10.3%
Urea	–	0.6%	0.6%
Classic mineral supplement	–	1.3%	1.3%
Chemical composition			
DM (g/100 g)	41	53	69
Organic matter (OM) (g/100 g)	944	930	924
Net energy for lactation (NE_L) (MJ/kg)	6.3	6.6	6.6
PDIE (g/kg DM)[1]	78	102	108
PDIN (g/kg DM)[2]	59	95	104
Total nitrogenous matter content (g/kg DM)	100	137	151
Starch (g/kg DM)	296	220	185
NDF (g/kg DM)	416	349	334
ADF (g/kg DM)	234	189	180

The cows were provided *ad libitum* with a total mixed ration (TMR) formulated to meet energy requirements and to exceed the metabolizable protein (MP) requirement (i.e. protein digestible in the intestine (PDI) in the French system) according to INRA guidelines (INRA 2007). The nutritional values of the various components of the diet (organic matter (OM), fiber (NDF, ADF) and starch were obtained by chemical analysis (LDA, Ploufragan, France) The micronutrient supplement accounts for less than 2% of DM and its nutritional value was not taken into account in NE_L and PDI calculations.
[1]Digestible protein in the small intestine supplied by microbial protein from rumen-fermented OM (INRA 2007).
[2]Digestible protein in the small intestine supplied by microbial protein from rumen-degradable protein (INRA 2007).

Table 2 *Ingredients and chemical composition of the μ-nutrient supplement used for supplemented cows (SUPPL group)*

Calcium carbonate ($CaCO_3$), monocalcium phosphate ($Ca(H_2PO_4)$), sodium chloride (NaCl), magnesium oxide (MgO), magnesium carbonate ($MgCO_3$), sugar cane molasses	
Chemical analysis (%)	
P	4
Ca	22
Mg	6
Na	3
Additives	
Vitamins (IU/kg)	
Vitamin A	442 500
Vitamin D_3	98 000
Vitamin E	1100
Trace elements (mg/kg)	
Copper sulfate ($CuSO_4,5H_2O$)	125
Copper chelate of amino acid hydrate	110
Zinc chelate of amino acid hydrate	375
Manganese oxide	250
Anhydrous calcium iodate	75
Cobalt carbonate	33
Sodium selenite	6
Organic Se	4
Antioxidant (mg/kg)	
Ethoxyquin	0.4

1630 h. Before each feed distribution, any uneaten food remaining from the last meal (refusals) was removed and weighed, with the amount recorded daily. Daily feed intake and supply were averaged each week from week 2 to week 8 *postpartum* and over 5 days around D15 and before D60. Energy balance for lactation (NE_L expressed in J/day) and utilizable protein in the diet (protein digestible in the intestine, **PDI**, g/day) were also averaged weekly and over a period of 5 days around D15 and before D60. The NE_L and PDI can be used to determine the difference between net energy intake and net energy needs for lactation, body maintenance, lean tissue growth and activity, according to the INRA method (INRA, 2007).

Dairy cow parameters

For all cows, BW was recorded before and after calving, and weekly from weeks 2 to 8 *postpartum*. Body condition score was recorded every 3 weeks, by the same three experienced assessors, on a scale from 0 (extremely thin) to 5 (extremely fat) with 0.25-point increments. Calving condition, and calf sex, birth weight and growth were recorded. All observations of diseases classically associated with the post-calving period were noted and scored (Leblanc, 2012). An experienced herdsman checked the cows for heat every day. The calving-to-first-service interval was then calculated, with artificial insemination systematically performed after the first detection of heat.

During the experimental period, the cows were milked twice daily, at 0715 and 1715 h. Daily milk yield was averaged weekly from weeks 2 to 8 *postpartum*. Milk samples (50 ml) were collected four times weekly, from the entire batch of milk collected from each cow during the morning and afternoon milkings, for somatic cell count (**SCC**) and milk composition (protein and fat percentages) determinations by a service company (Lillab, Châteaugiron, France). Fat and protein contents and yields were averaged weekly from weeks 2 to 8 *postpartum*. On D15 and D60 post-calving, samples from the morning milking session were also analyzed for total N, non-protein N and non-casein N contents, as previously described (Lollivier *et al.,* 2015). Casein was determined as total N minus non-casein N, and whey proteins were determined as non-casein N minus non-protein N. Milk samples were centrifuged, and skimmed milk was collected and stored at −20°C for determinations of metalloprotease activity by zymography (Yart *et al.,* 2013) and of lactoferrin and α-lactalbumin contents (Boutinaud *et al.,* 2016).

Hematology parameters, cell preparations and flow cytometry analysis

On D15 and D60, unclotted samples collected into K_3EDTA were analyzed with a hematology analyzer by a service company (Institut en Santé Agro-Environnement, Fougères, France). Total leukocyte and neutrophil, lymphocyte, eosinophil and monocyte percentages were determined. For PBMC isolation, additional blood samples (80 ml) were collected from the jugular vein into vacutainer tubes containing K_3EDTA. The PBMC fraction was obtained by centrifugation on Ficoll (Supplementary Material S1). Peripheral blood mononuclear cells were counted and immediately frozen at −80 °C for genomic DNA extraction. At the same time points (D15 and D60), morning milk samples were collected for the purification of mMECs and milk leukocytes (Boutinaud *et al.,* 2015; Supplementary Material S1). Mammary epithelial cells were isolated by positive selection with anti-human cytokeratin antibody-coated magnetic beads. After contact with the magnetic particle concentrator, the supernatant containing the non-selected cells was collected and centrifuged to obtain milk leukocytes. For each mMEC and milk leukocyte preparation, cell counts were obtained, apoptotic and necrotic mMECs were determined and DNA and RNA were obtained (Supplementary Material S1).

Total RNA extraction and gene expression analysis

Total RNA was extracted from mMECs with TRIzol (Thermo Fisher Scientific, Courtaboeuf, France) and purified on RNeasy Mini Kit columns after DNAse treatment (Qiagen, Courtaboeuf, France). Ribonucleic acid was quantified and its integrity assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France). Only samples with an RNA integrity number (**RIN**) >6 were used for reverse transcription-quantitative PCR (**RT-qPCR**) analyses (Boutinaud *et al.,* 2008). This excluded two mMEC samples. Mean RIN was 7.5 ± 0.12, and purity was high on spectrometry (1.96 and 1.35 for A_{260}/A_{280} and A_{260}/A_{230} ratio, respectively). Reverse transcription-quantitative PCR was performed in triplicate with primers for α-lactalbumin (*LALBA*), κ-casein (*CSN3*), *BAX, PRLR* long isoform and three housekeeping genes

(cyclophilin, *R18S* and ribosomal protein large P0; Supplementary Table S2). The housekeeping gene with the most stable expression profile according to NormFinder software (Andersen *et al.*, 2004) was defined as the reference gene: *RPLP0,* with a stability of 0.209, *v.* 0.433 and 0.534 for *R18S* and *cyclophilin*, respectively. The results for each target gene are thus expressed as ratios relative to this selected reference gene by an mRNA quantification method described elsewhere (Boutinaud *et al.*, 2008), after checking for an absence of effect of the various factors (stage and diet) on *RPLP0* gene expression.

Genomic DNA extraction and global DNA methylation analysis

The cell pellets obtained at the end of the purification process (PBMCs, milk leukocytes and mMECs) were used for DNA extraction, as previously described (Supplementary Material S3). Genomic DNA was quantified with a Qubit™ kit (Invitrogen) and 1 μg of genomic DNA was used for global DNA methylation analysis by luminometric methylation assay (**LUMA**). Luminometric methylation assay quantifies DNA methylation at CCGG sites (Me-CCGG), using the restriction enzyme isoschizomers *Msp*I and *Hpa*II (New England Biolabs, Evry, France), as previously described (Attig *et al.,* 2013; Supplementary Material S3). *Eco*RI cleavage was performed simultaneously to determine the amount of genomic DNA initially present. The cleavage sites were quantified by pyrosequencing (PSQ24; Qiagen; Supplementary Material S3). *Msp*I and *Hpa*II cleavages were used to determine the total number of genomic cleavage sites and the number of unmethylated cleavage sites, respectively. The *Eco*RI cleavage values were used to normalize the values obtained for the *Msp*I and *Hpa*II cleavages. The enzymatic cleavages (*Msp*I/*Eco*RI and *Hpa*II/*Eco*RI) were performed in duplicate and two independent pyrosequencing runs were performed for each sample. A bovine genomic DNA sample obtained from adult cow liver was loaded onto each pyrosequencing run (Perrier *et al.,* 2018) for calculation of the inter-assay CV. This coefficient was very low (1.36%), demonstrating the reliability and reproducibility of this method.

Statistical analyses

Data were analyzed by ANOVA, with the MIXED procedure of SAS (SAS Institute, 1999). All data are presented as least-squares means (**LSMs**) and standard errors. The effects of parity (P), lactation week (W; from 2 to 8) or stage (S; D15 *v.* D60), and diet (D; CTRL *v.* SUPPL) were considered as fixed effects, cows were considered as a random effect and interactions (D × S, D × P, P × S and D × P × S) were assessed. A repeated statement was performed, with lactation weeks or stage (D15 and D60) as the 'repeated effect' and cows as the 'subject'. Due to imbalance in the numbers of primiparous and multiparous cows, we did not analyze the effect of parity.

The BW and BCS values obtained before the treatment period were used as covariates (Supplementary Table S1). Calving condition score and time from calving to first artificial insemination were analyzed with an ANOVA MIXED procedure. Only the effect of diet (CTRL *v.* SUPPL) was assessed.

The mRNA data were subjected to log10 transformation for statistical analysis. Spearman's correlation coefficients were calculated with SAS software. Values of $P \leq 0.05$ were considered significant.

Results

Diet effects throughout the trial

We evaluated the effect of μ-nutrient supplementation throughout the trial on various parameters classically used to assess performance in dairy cows. We collected data from calving to D60 of lactation and averaged them from weeks 2 to 8 *postpartum* (Table 3). The μ-nutrient supplementation did not affect milk production or composition (fat and protein contents), DMI or energy balance. However, this supplementation had a significant positive effect on BW ($P = 0.004$),

Table 3 *Effect of* μ*-nutrient supplementation throughout the trial on BW, DM intake (DMI), energy balance for lactation (NEL) and protein digestible in the intestine (PDI) intake, milk yield and milk composition in dairy cows* (n = *24)*

				P-values[1]		
	CTRL	SUPPL	SEM	D	W	W × D
BW (kg)	585	610	6.0	0.004	0.701	0.999
DMI (kg/day)	18.1	18.4	0.29	0.49	<0.0001	0.837
PDI intake (g/day)	1862	1865	29.9	0.94	0.003	0.873
PDI balance (g/day)	−143	−204	54.7	0.41	0.004	0.997
NE_L intake (MJ/day)	120	120	1.9	0.99	<0.0001	0.791
NE_L balance (MJ/day)	−26	−33	4.1	0.20	0.004	0.999
Milk production (kg/day)	33.2	32.8	0.57	0.64	0.219	0.632
Fat content (g/l)	40.4	39.5	0.64	0.31	<0.0001	0.897
Protein content (g/l)	29.5	29.0	0.25	0.13	<0.0001	0.688
Fat yield (kg/day)	1348	1299	31.2	0.26	0.051	0.990
Protein yield (kg/day)	975	945	17.6	0.21	0.020	0.302

Data are presented as the least-squares means (LSM) of values.
[1]*P* value for diet effect (D; control (CTRL) and supplemented (SUPPL) diet groups), week effect (W; 2 to 8 weeks *postpartum*) and week × diet interaction (W × D).

Table 4 *Effect of μ-nutrient supplementation on milk yield and milk composition in dairy cows (n = 24) at two time points in lactation (D15 and D60)*

	CTRL		SUPPL			*P*-values[1]		
	D15	D60	D15	D60	SEM	S	D	D × S
BW (kg)	584	580	614	609	13.93	0.722	0.035	0.955
BCS	1.80	1.51	2.12	1.80	0.13	0.013	0.016	0.934
Milk production (kg/day)	29.6	33.9	31.9	33.5	1.86	0.054	0.597	0.352
Fat content (g/l)	43.6	38.1	41.9	32.9	2.38	0.001	0.131	0.376
Protein content (g/l)	30.9	26.1	31.3	27.8	0.82	<0.001	0.197	0.345
Casein content (g/l)	24.5	21.4	25.6	22.4	0.79	<0.001	0.170	0.991
Whey protein content (g/l)	5.7	5.4	6.0	5.6	0.27	0.093	0.254	0.998
Casein/whey protein ratio	4.3	4.1	4.3	4.0	0.26	0.249	0.994	0.950
Lactose content (g/l)	47.5	48.2	46.5	47.7	1.10	0.255	0.451	0.725
α-Lactalbumin concentration (mg/l)	959	699	1078	804	58.3	<0.001	0.049	0.877
SCC (log(cells/ml))	2.27	2.42	2.32	2.16	0.13	0.927	0.395	0.145
Lactoferrin concentration (mg/l)	88.9	122.3	68.6	90.4	21.47	0.116	0.200	0.733

For milk production, fat and protein contents and somatic cell count (SCC), the data are presented as the least-squares means (LSM) of values obtained for 5 days around D15 or 5 days before D60, time points at which blood and milk were sampled. The BW and body condition score (BCS) were also reported. For all other assays, including determinations of casein, whey protein, lactose, α-lactalbumin and lactoferrin concentrations, the data are presented as the LSM of values obtained at the two time points considered (D15 and D60).

[1] *P* value for diet effect (D; control (CTRL) and μ-nutrient diet (SUPPL) groups, stage effect (S; 15 and 60 days *postpartum*) and diet × stage interaction (D × S).

whereas BW was stable throughout the peripartal period in the control group ($P = 0.701$).

Diet effects at two particular time points in lactation

For the correlation of milk and blood cell parameters with cow performance, we performed more detailed analyses at two times in lactation: days 15 and 60 (Table 4 and Supplementary Table S3). Supplementation with μ-nutrient had a positive effect on BW ($P = 0.035$), associated with a better BCS ($P = 0.016$) as early as D15 of lactation. As expected, milk yield tended to increase with lactation stage ($P = 0.054$), whereas milk fat and protein contents decreased ($P < 0.001$). Milk yield, fat and protein contents did not differ between the CTRL and SUPPL groups.

Both casein and total protein contents decreased from D15 to D60 ($P < 0.001$). However, neither whey protein content nor casein/whey protein ratio varied with lactation stage or diet group. Milk α-lactalbumin concentration decreased with lactation stage ($P < 0.001$) but was higher with than without μ-nutrient supplementation ($P = 0.049$). Lactose content was similar in all groups (lactation stage and diet groups).

No significant differences in milk SCC were noted between groups. The concentration in milk of lactoferrin, which has antibacterial properties, did not differ between groups.

Thus, at D15 and D60 of lactation, μ-nutrient supplementation increased BW and BCS without significantly modifying milk production and quality.

Diet effects on calving condition score, calf birth weight and cow health

Calving condition score was lower in the SUPPL group than in the CTRL group (LSM: 1.16 ± 0.26 and 2.16 ± 0.24, for the SUPPL and CTRL groups, respectively $P = 0.0123$), indicating better calving condition in this group of cows. Mean calf birth weight was 41.1 ± 4.76 kg, with no significant difference between female and male calves (39.8 ± 2.87 and 41.8 ± 5.43 kg, respectively) or diet groups.

Only a few cows presented disease, with no difference between diet groups (3/24 endometriosis, 3/24 mastitis and 1/24 digital dermatitis). The calving-to-first-service interval, attesting to ovarian activity, was calculated, with artificial insemination systematically performed after the cow was observed to be on heat. This interval tended to be shorter in the SUPPL group (75.25 ± 7.6 days) than in the CTRL group (95.66 ± 9.23 days) ($P = 0.116$). However, for multiparous cows, it was significantly shorter in the SUPPL group than in the CTRL group ($P = 0.036$; LSM: 62 ± 10.4 and 113.6 ± 15.6 days, in the SUPPL and CTRL groups, respectively). The difference was not significant for primiparous cows (LSM: 77.6 ± 9.8 and 88.5 ± 13.5 days, in the CTRL and SUPPL groups, respectively).

Thus, μ-nutrient supplementation improved calving condition and tended to decrease the time between calving and first artificial insemination. These findings suggest that μ-nutrient diet supplementation may promote the resumption of ovarian activity.

Diet effects on global DNA methylation patterns and cell counts, viability and activity

Me-CCGG levels were measured by LUMA, with genomic DNA from PBMCs, mMECs and milk leukocytes. Me-CCGG levels were cell type specific (Figure 1): 74.43% with low intra-group variability ($CV_{(PBMC)} = 2.2\%$) for PBMCs, 67.98% with moderate intra-group variability ($CV_{(milk\ leukocytes)} = 5.9\%$) for milk leukocytes and 64.39% with high intra-group variability ($CV_{(mMEC)} = 11.8\ \%$) for mMECs (Table 5).

The Me-CCGG level of PBMCs was higher on D60 than on D15 ($P = 0.020$), with no effect of μ-nutrient supplementation. However, total white blood cell count

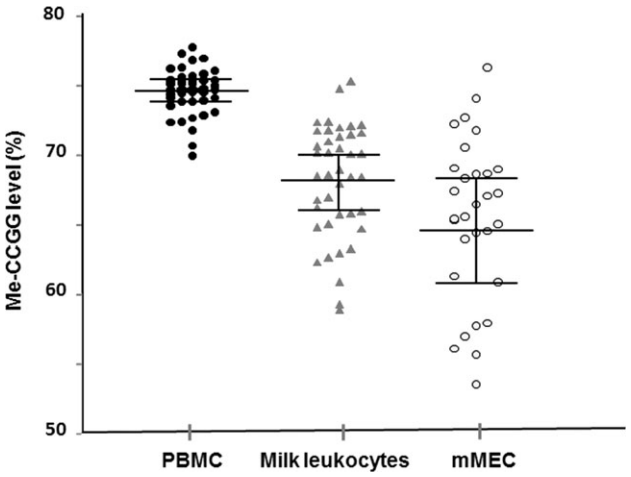

Figure 1 Global DNA methylation (Me-CCGG) in three different purified cell types. Peripheral blood mononuclear cells (PBMC; $n = 44$) were purified from blood samples collected from lactating cows on D15 and D60 *postpartum*. At the same *postpartum* stages, morning milk was collected and used to purify milk mammary epithelial cells (mMEC; $n = 32$) and milk leukocytes ($n = 42$). Genomic DNA was extracted from the three different cell populations and used for the quantification of DNA methylation by LUminometric Methylation Assay (LUMA; expressed in percentage of methylation). Each symbol (black, grey or white) represents one individual sample. The large and small horizontal bars indicate the mean ± SDs, respectively. The difference in Me-CCGG between cell types was highly significant ($P < 2.2 \times 10^{-16}$; approximate Kruskal-Wallis test).

and the proportions of lymphocytes and neutrophils were similar in all groups.

The Me-CCGG level of mMECs did not differ between groups, but the CV between mMEC samples was higher than the CV for PBMCs and milk leukocytes. Milk MEC count was lower at D60 than at D15 ($P = 0.014$) in both diet groups. The percentages of apoptotic and necrotic mMECs were similar at the two time points and in the two diet groups. Bcl-2-associated × protein (*BAX*) gene expression levels were also similar in all groups, consistent with the similar levels of apoptotic activity in all groups analyzed. Similarly, expression of the *CSN3 and LALBA* genes, markers of mammary cell activity, was unaffected by lactation stage or μ-nutrient supplementation. *PRLR* gene expression decreased significantly with lactation stage ($P = 0.049$) and was not affected by μ-nutrient supplementation. Higher mMEC counts were associated with higher levels of milk metalloprotease activity (MMP2; $P = 0.024$) on D15 than on D60, consistent with mammary tissue remodeling and the initiation of lactation. Na^+/K^+ ratio, a marker of epithelium tight junction opening, did not vary. Micronutrient supplementation did not affect any of the mMEC parameters analyzed.

The Me-CCGG level of milk leukocytes remained stable during the study period and was unaffected by μ-nutrient supplementation. However, milk leukocyte count increased with lactation stage ($P = 0.01$).

Me-CCGG levels were clearly cell specific, varying slightly between cell types. Only Me-CCGG in PBMC differed significantly between the two lactation time points analyzed. Global DNA methylation was unaffected by μ-nutrient supplementation, in all cell types.

Discussion

We investigated the health and performance of dairy cows fed a diet supplemented with a commercial micronutrient. This trial was performed with a limited number of genotyped cows managed under highly controlled conditions on an experimental farm. We took into account the total MI calculated from the genotyping results, to limit the genotype × diet interaction. We performed a large phenotypic analysis, including a determination of epigenetic status based on measurements of global DNA methylation, to characterize and describe the effects of μ-nutrient supplementation in detail.

Effects of μ-nutrient supplementation during the peripartum period in dairy cows

Supplementation with a commercial micronutrient currently used by French dairy farmers improved BCS and BW without modifying DMI, NE_L, milk yield or milk components (except for an increase in α-lactalbumin content). The main physical findings were changes in body parameters associated with improvements in calving condition and a shorter interval between calving and first service.

The cocktail of minerals (P, Ca, Mg and Na) included in the μ-nutrient supplementation was equivalent, in terms of its ingredients and their quantities, to the mineral cocktail distributed to the CRTL group in accordance with INRA recommendations (2007). However, the μ-nutrient supplementation also supplied the cows with non-mineral components, such as organic Se from yeast, trace elements (Zn and Cu) and extracts from plants and microalgae containing β-carotene, vitamin A, D_3, E and natural superoxide dismutase (**SOD**). All these components have been reported to be beneficial to cow health. Selenium is an important trace element in cattle nutrition (Ammerman and Miller, 1975; Mehdi and Dufrasne, 2016). It is obtained from yeast as selenomethionine and selenocysteine. These forms are well absorbed by the body and can be used directly for the synthesis of selenoproteins, which act as antioxidants (Speckmann and Grune, 2015). Superoxide dismutase and β-carotene have known beneficial effects on immune function (Spears and Weiss, 2008). Together, natural SOD, β-carotene, Zn and Cu, with Se and vitamin E, help to control the antioxidant system, minimizing free radical production (Politis, 2012). Oxidative stress occurs during the peripartum period, when the production of ROS exceeds the antioxidant defense mechanisms of the body (Abuelo *et al.,* 2015). The presence of antioxidants in the μ-nutrient supplementation studied here may improve body condition, increasing BCS and BW, both of which are considered to be markers of animal health status and performance (Ingvartsen and Moyes, 2013). A general improvement in health status has been shown to be correlated with a shorter interval between calving and first service (Friggens *et al.,* 2010), potentially accounting for our observations. However, one of the limitations of this study was the small number of cows used to analyze reproduction data and the short duration of the trial, making it impossible to draw conclusions about the effects of

Table 5 *Effects of μ-nutrient supplementation in dairy cows on cell proportions and global DNA methylation (Me-CCGG) level for each cell type (blood cells, milk mammary epithelial cells and milk leukocytes) at two time points in lactation*

	CTRL		SUPPL			*P*-values[1]		
	D15	D60	D15	D60	SEM	S	D	D × S
Blood cells								
Whole blood cells (×10^6 cells/ml)	7.50	8.96	9.36	9.46	0.892	0.374	0.184	0.435
Lymphocytes (%)	49.2	44.9	43.3	42.8	5.62	0.590	0.450	0.676
Neutrophils (%)	41.0	44.7	48.7	41.7	4.71	0.608	0.726	0.324
Me-CCGG (%)	74.5	75.3	72.8	75.0	0.75	0.020	0.190	0.221
Milk epithelial cells (MEC)								
mMEC count (×10^6 cells/day)[2]	806	221	1240	463	60.6	0.014	0.194	0.705
Apoptotic mMECs (%)	15.5	20.2	14.9	15.7	2.23	0.216	0.254	0.377
Necrotic mMECs (%)	8.2	9.9	12.3	9.65	2.62	0.865	0.455	0.396
BAX expression (log)[3]	4.22	4.10	4.03	4.07	0.082	0.627	0.182	0.323
CSN3 expression (log)[3]	7.30	7.20	7.15	6.63	0.236	0.194	0.132	0.373
LALBA expression (log)[3]	8.58	8.50	8.48	8.07	0.244	0.307	0.279	0.501
PRLR expression (log)[3]	3.64	3.41	3.56	3.21	0.140	0.049	0.319	0.650
Milk MMP2 activity (log)[4]	6.59	6.38	6.47	6.43	0.065	0.024	0.542	0.121
Milk Na^+/K^+ ratio	0.200	0.203	0.210	0.206	0.010	0.927	0.514	0.686
Me-CCGG (%)	62.7	68.6	67.5	63.9	3.73	0.720	0.972	0.203
Milk leukocytes								
Milk leukocytes (%)	38.4	54.1	35.1	52.3	5.99	0.010	0.673	0.908
Me-CCGG (%)	66.3	66.9	71.1	68	1.63	0.380	0.061	0.189

[1]*P* value for diet effect (D; control (CTRL) *v.* μ-nutrient diet (SUPPL) groups) and stage effect (S; 15 or 60 days *postpartum*).
[2]The rate of MEC exfoliation was defined as the number of MECs exfoliated per day, calculated by multiplying the daily milk yield by the MEC concentration measured during the morning milking session.
[3]The results for each target gene are expressed as a ratio relative to the selected reference gene (*RPLP0).*
[4]Milk matrix metalloproteinase activity measured by zymography.

supplementation on fertility requiring repeated artificial insemination and gestational diagnostics (Lehmann *et al.,* 2017).

Impact of μ-nutrient supplementation on blood and milk cell populations

We tested the use of three different cell populations that could be easily collected with a minimally invasive procedure for the monitoring of epigenetic status and health in dairy cows.

We first determined counts, viability and activity for the various cell populations. These immune cells play an important role in inflammatory responses (Rainard and Riollet, 2006): lymphocytes regulate the induction and suppression of immune responses, whereas neutrophils protect against invading bacteria at the start of acute inflammation. In this study, the proportions of these two cell types were unaffected by stage or diet. However, previous studies reported a significant effect of energy intake (Agrawal *et al.,* 2017) and rumen methionine supplementation (Li *et al.,* 2016) on the transcriptome of polymorphonuclear leukocytes (**PMNLs**) and neutrophils, suggesting that any effect of the μ-nutrient supplementation effect would be more likely to be associated with a regulation of leukocyte activity than leukocyte count.

The relevance of MECs isolated from milk as a valuable noninvasive source of biological samples for mammary gene expression analysis after various treatments has already been discussed (Boutinaud *et al.,* 2015). We found that mMEC count decreased with lactation stage, whereas other parameters (viability, gene expression and apoptotic status) were unaffected by group (diet or stage), except for *RPRL* mRNA levels, which were lower at D60 than at D15. Only metalloprotease activity varied significantly with lactation stage. The high mMEC count on D15, associated with metalloprotease activity, was therefore unexpected (Rabot *et al.*, 2007) and suggested early mammary tissue remodeling linked to the establishment of milk production. These data highlighted the possibility of mMEC release into milk being dependent on two different mechanisms: remodeling by metalloprotease activity at early stages of lactation (D15) and a subsequent renewal of secretory epithelial cells, during intense milk production. The μ-nutrient supplementation had no effect on cell count or gene expression in mMECs, consistent with the absence of change in milk yield or composition.

We report a clear increase in the proportion of milk leukocytes with lactation stage, suggesting changes in the immune capacity of the mammary gland during lactation. Piepers and De Vliegher (2013) also reported changes in the percentages of milk PMNL undergoing apoptosis during the periparturient period as a function of energy balance, reflecting a potential decrease in innate immune capacity. The μ-nutrient supplementation did not affect milk leukocyte counts.

Global DNA methylation in dairy cows

Epigenetic modifications, including DNA methylation, constitute a crucial connection between genome and environment

contributing to sustained changes in gene expression and cellular phenotypes. Global DNA methylation is a potentially useful marker of health status in dairy cows, as already shown in humans, in response to environmental changes (nutrition, lifestyle and stress; Martin and Fry, 2018). We previously showed that LUMA provides a highly reproducible quantification of DNA methylation at CCGG sites (Perrier *et al.,* 2018). In cattle, the genomic distribution of CCGG sites is associated with potential regulatory regions for gene expression: 42.2% of CCGG sites are intragenic, with 7.6% associated with the promoters and transcription start sites of genes and 33.6% of CCGG sites are located in and around CpG islands.

Differences in Me-CCGG levels were found to be cell type specific. In PBMCs, the CV between individual Me-CCGG values was very low. Only lactation stage had a significant effect on Me-CCGG levels in PBMC. The decrease in PBMC Me-CCGG could be interpreted in two ways. Firstly, subtle changes may occur in the proportion of blood cell subpopulations and the granulocyte/monocyte ratio during the transition period (Zecconi *et al.*, 2018). Furthermore, in humans, each blood cell subpopulation has its own global DNA methylation level (Reinius *et al.*, 2012). Secondly, subtle changes in Me-CCGG may be associated with changes in cell activity and gene expression. Further investigations are required to define the methylome of different blood cells subpopulations.

We report here the first determination of Me-CCGG in mMECs. The values obtained were similar to those obtained for mammary gland biopsies (Nguyen *et al.,* 2013). This finding supports the notion that mMECs constitute a noninvasive source of biological material representative of the mammary gland that can also be used for epigenetic analysis. However, the CV was high and unrelated to lactation stage or diet group. This was also the first study to analyze Me-CCGG levels for milk leukocyte DNA. These levels were found to be lower than those in PBMCs, with a moderate CV. The Me-CCGG of milk leukocytes tended to be higher in the μ-nutrient supplementation group than in the control group at the two lactation stages analyzed. Consistent with the genomic distribution of CCGG sites, these differences may be associated with functional changes in gene transcription in milk leukocytes and require further investigation.

It is becoming increasingly evident that micronutrients play a major role in epigenetic imprinting (Ragaller et al., 2009; Chavatte-Palmer *et al.*, 2018; Huang *et al.*, 2019). The Se present in the μ-nutrient supplementation is directly linked to one-carbon metabolism and could act directly on DNA methylation (Mentch and Locasale, 2016). Based on data reported for humans and rodent models, other components may also be involved in epigenetic modifications. In suckling rats, β-carotene supplementation affects the DNA methylation of adipogenesis-related genes (Arreguín et al., 2018). Oxidative stress induces DNA damage, with 8-hydroxy-guanosine as one of the most prevalent oxidation products (Cerda and Weitzman, 1997). This chemical modification of guanine inhibits the methyl group binding to CpG dinucleotides and induces a loss of methylation. Thus, anti-oxidative components could protect genomic DNA from this guanine modification, indirectly helping to maintain normal methylation patterns (Cerda and Weitzman, 1997). We detected positive effects of μ-nutrient supplementation on BW, BCS and reproductive activity. The antioxidant capacity of the components provided by μ-nutrient supplementation may trigger subtle changes in the global level of DNA methylation. We will now perform genome-wide mapping studies to improve our understanding of the subtle changes in cell-specific methylation patterns underlying genome functioning.

Conclusions

We found that, on an experimental farm, the use of a commercial supplement improved BCS and BW in dairy cows. We provide the first description of global methylation analyses on several easily collectable cell types in cows. Our data highlight subtle cell-specific methylome modifications and suggest that whole-genome DNA methylation analysis could be used to determine epigenetic biomarkers of health in dairy cows.

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Declaration of interest

This study was performed primarily for scientific reasons, within a conventional academic laboratory and experimental farm. The μ-nutrient supplementation is a patented commercial premix marketed by PILARDIERE and XR REPRO under the trade name Genial®. J. Zawadzki and E. Mariani currently work for PILARDIERE, an animal nutrition company with commercial interests in feed additives and for XR REPRO, a breeding cooperative, respectively.

Ethics statement

All the animal procedures were approved by the Animal Care Committee of the French Ministry of Agriculture, in accordance

with French regulations (decree no. 2001–464, May 26, 2001; agreement reference no. 0162503).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

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