Monitoring the Transition Period in Dairy Cows through $^1$H NMR-Based Untargeted Metabolomics

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Abstract: The metabolic alterations associated with the increase in milk production make the transition period critical to the health of dairy cows, usually leading to a higher incidence of disease in parturient animals. In this manuscript, we describe the use of NMR-based untargeted metabolomics to follow how these changes impact the serum metabolome in a group of 28 transition dairy cows with no initial clinical diseases. Principal component analysis (PCA) of serum $^1$H NMR data from four weeks before calving to 8 weeks after parturition allowed us to clearly identify four stages during the transition period. Pairwise comparisons using orthogonal partial least square discriminant analysis (OPLS-DA) and univariate data analysis led to the identification of 18 metabolites that varied significantly through these stages. Species such as acetate, betaine, and creatine are observed early after calving, while other markers of metabolic stress, including acetone, $\beta$-hydroxybutyrate (BHB), and choline, accumulate significantly at the height of milk production. Furthermore, marked variations in the levels of lactate, allantoin, alanine, and other amino acids reveal the activation of different gluconeogenic pathways following parturition. Concomitant with a return to homeostasis, a gradual normalization of the serum metabolome occurs 8 weeks after calving. Correlations of metabolite levels with dietary and metabolic adaptations based on animal parity could also be identified. Overall, these results show that NMR-based chemometric methods are ideally suited to monitor manifestations of metabolic diseases throughout the transition period and to assess the impact of nutritional management schemes on the metabolism of dairy cows.

Keywords: dairy cattle; metabolic stress; NMR-based metabolomics; serum metabolomics; transition period

1. Introduction

During the transition period from late pregnancy to early lactation, dairy cows suffer dramatic changes in their metabolism due to the decrease in dry matter intake (DMI) occurring even before calving and the increased energetic needs associated with milk production [1]. These changes lead to a state of negative energy balance (NEB), which results in the activation of lipid and protein catabolic pathways [2,3]. If the NEB is severe or prolonged, it affects milk production and, more importantly, has effects on the immune and endocrine systems that are detrimental to animal health and reproductive performance [4–7]. Indeed, the transition period is characterized by an increase in the incidence of production diseases such as mastitis, metritis, ketosis, and hepatic lipidosis [3,8]. Furthermore, the process of continuous genetic selection that has been employed over recent decades to improve milk production yields has also led to an exacerbation of problems associated with metabolic stress [9,10].
Since the animal health issues observed during the transition period translate into important economic losses, considerable efforts have been dedicated to better understand the metabolic changes of dairy cows in peripartum. Several studies have focused on monitoring metabolites associated with NEB, particularly increases in the concentration of non-esterified fatty acids (NEFA) in blood [11,12], which are usually followed by increases in β-hydroxybutyrate (BHB), acetone, and acetoacetate levels [13]. The variation in the concentration of glucose, insulin and other hormones, and blood proteins also correlates with the metabolic challenges experienced by the animals around calving [6,11,14]. However, production diseases are etiologically interrelated and cannot be considered in isolation [8], and thus integrative analytical approaches in which groups of metabolites are monitored simultaneously are desirable. Metabolomic methods offer this possibility [15], and consequently they have been successfully employed to investigate dairy cattle health. For example, targeted metabolomics employing mass spectrometry (MS) have been used to investigate mastitis, metritis, ketosis, and hepatic lipidosis in periparturient cows [16–20], and untargeted approaches based on nuclear magnetic resonance (NMR) spectroscopy were also applied to the study of several of these conditions [20–24].

Targeted and untargeted MS-based metabolomics have also been applied to follow metabolite levels in transition cows in an attempt to identify biomarkers that could evidence early stages of disease [25–29]. Most of these studies have been conducted under confined dairy production systems. However, maximizing the proportion of pastures in the diet is a pivotal factor for minimizing production costs [30]. It is well known that grazing dairy cows do not have sufficient DMI to sustain the high milk production that could be achieved with their genetic potential [31], and supplements and concentrates are therefore given to achieve the desired production levels and minimize the magnitude and duration of the NEB. In contrast with total mixed ration (TMR) diet schemes, the prediction of nutrient availability in mixed systems where herbage is the primary diet component is complex because it includes uncertainties associated with grazing behavior. In this scenario, the availability of propionate and other glucogenic metabolites for liver demands may be limited so as to maintain adequate glucose supply for the mammary gland of the high-producing dairy cows. Thus, the characterization of alternative precursors and pathways involved in de novo glucose synthesis and the identification of liver and peripheral tissue adaptations to lactation could contribute to the optimization of herd nutritional management strategies. Moreover, this knowledge could be instrumental in the design of preventive medicine programs aimed at decreasing costly production diseases. With this in mind, we investigated the suitability of NMR-based untargeted metabolomics as a tool to follow changes in the serum metabolome of a group of dairy cows with no initial clinical conditions and access to pastures following calving.

2. Materials and Methods

2.1. Animals and Sample Collection

The experimental protocol was approved by the Comisión Honoraria de Experimentación Animal, Universidad de la República (111130-002261-13). The study was performed on a group of 26 Holstein, Jersey, and Holstein × Jersey crossbred cows managed together at the dairy farm of the Facultad de Veterinaria, Universidad de la República (San José, Uruguay). It included 17 heifers and 9 multiparous cows that had between three and seven calvings. Their diet consisted of a concentrate and silage based TMR before calving, and grazing was offered as an option after that (Table S1). Monitoring was carried out by trained veterinarians from ~30 days prepartum to ~60 days postpartum. Animals with clinical manifestations of hypocalcemia, retained placenta, metritis, or moderate or severe mastitis (i.e., grades 2 or 3) were excluded from the study [32–35], while those developing mild clinical mastitis (i.e., grade 1) or subclinical ketosis that did not show systemic affection were not [35,36]. Bleeding was carried out every 15 days before calving, and once a week immediately after milking and before grazing after parturition. Blood
samples were drawn from the coccygeal vein into tubes without anticoagulant, centrifuged at 3000 g for 10 min, and the resulting serum stored at −20 °C until analyzed.

2.2. Clinical Biochemistry

Cholesterol, total protein, albumin, urea, and calcium concentrations in serum were determined with commercial colorimetric kits (Weiner Laboratorios S.A.I.C., Rosario, Argentina), using a Vitalab Selectra 2 automated biochemistry analyzer. The globulin concentration was determined following the ACS ACOD method (NEFA-C kit, Wako Chemicals USA Inc., Richmond, VA, USA), and enzymatic methods were employed to obtain concentrations of BHB in all samples (Ranbut kit, Randox Laboratories Ltd., Crumlin, UK). Intra and inter-assay coefficients of variation for all commercial serum determinations were less or equal than 10%.

2.3. NMR Spectroscopy

Blood serum samples were allowed to thaw at room temperature, and 400 µL aliquots were mixed with 200 µL of a 0.9% NaCl solution in D$_2$O and transferred to 5 mm NMR tubes (NE HL5 7, New Era Enterprises Inc., Vineland, NJ, USA). All NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 NMR spectrometer operating at $^1$H and $^{13}$C frequencies of 500.13 MHz and 125.76 MHz, respectively, and equipped with a z-gradient TXI probe. To attenuate broad signals from proteins and lipids, 1D $^1$H spectra were obtained using a water suppressed Carr Purcell Meiboom-Gill sequence with a $\tau$ of 0.4 ms and an $n$ of 80 [37,38]. A spectral width of 10 KHz, a data size of 32 K, and a total of 128 scans were employed to record each spectrum, using a relaxation delay of 4 s between scans. When required, gradient enhanced HSQC and 1D TOCSY spectra were acquired using standard pulse sequences provided with the spectrometer.

2.4. Data Processing

All free induction decays were zero-filled to 64 K points and apodized with a 0.3 Hz exponential window function prior to Fourier transformation. $^1$H NMR spectra were manually phased and baseline corrected using MNova (version 10.0, MestreLab Research, S.L., Santiago de Compostela, Spain), and referenced to the lactate methyl resonance at 1.25 ppm present in all serum samples. Manually selected regions of the spectra were then aligned, and the data was normalized to the total spectral area after excluding the residual water resonance signal (4.60–5.00 ppm). Spectra were then segmented into bins of 0.001 ppm between 0.5 and 9.0 ppm [39]. The resulting data matrix was then exported as a text file for use in multivariate analyses.

2.5. Metabolite Concentrations

The molar ratios of unambiguously identified serum metabolites were obtained by integration of their $^1$H signals in the NMR spectrum of each sample. Together with the concentration of BHB obtained through enzymatic methods for the same sample, these estimations were employed to compute absolute metabolite concentrations.

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was performed using GraphPad Prism (version 7.0, GraphPad Software Inc., San Diego, CA, USA). Multivariate statistical analyses, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA), were carried out with the PLS_Toolbox package (version 8.5, Eigenvector Research Inc., Manson, WA, USA) implemented for MATLAB (revision 2014a, The MathWorks Inc., Natick, MA, USA). For all models, the data was mean-centered and scaled using a Pareto factor [40]. Analysis of the data was first performed with PCA, which reduces the dimensionality and facilitates the identification of data clusters or trends [41–43]. The PCA scores plot was
also employed to identify strong outliers outside the 95% significance region of Hotelling’s T2 ellipse. OPLS-DA models were derived from the complete data matrix, as well as from subsets in the 6.0 to 9.0 ppm range. Cross-validation of all OPLS-DA models was achieved using the random subset method, which involved 20 iterations over data split into 10 equally sized parts. Receiver operating characteristic (ROC) curves were plotted, and area under the curve (AUC) values were calculated to ensure the goodness of fit of the resulting models [44,45]. A permutation test with 200 iterations was also performed to determine the degree of over-fitting and further validate the discriminant analyses [46]. The results from these validations are provided as Supplementary Materials.

3. Results and Discussion

3.1. 1H NMR Spectra of Serum Samples

A representative 1H NMR spectrum of a serum sample used in the study is shown in Figure 1. In addition to signals corresponding to lipoproteins, sugars, and amino acids, resonances from metabolites such as acetate (1.84 ppm), acetone (2.16 ppm), allantoin (5.30 ppm), betaine (3.19 and 3.83 ppm), BHB (1.12, 2.22, 2.33, and 4.06 ppm), choline (3.08, 3.36, and 3.90 ppm), citrate (2.45 and 2.61 ppm), citrulline (1.43, 1.73, 3.00, and 3.63 ppm), creatine (2.96 and 3.86 ppm), creatinine (2.97 and 3.98 ppm), formate (8.38 ppm), glycerolphosphocholine (3.15, 3.54, 3.60, 3.83, and 4.23 ppm), isobutyrate (1.04 and 2.55 ppm), lactate (1.25 and 4.03 ppm), phosphocholine (3.17, 3.58, and 4.10 ppm), trimethylamine (TMA) (2.84 ppm), trimethylamine N-oxide (TMAO) (3.28 ppm), and tyramine (6.85 and 7.05 ppm) can be assigned by comparison to literature data [22,37,47]. These assignments were, in some cases, further corroborated with the aid of HSQC and 1D TOCSY spectra.

As discussed earlier, several of these species are related to NEB-related metabolic stress occurring in the transition period of the lactating cow.

![Figure 1. Representative 1H NMR spectrum of a dairy cow serum sample. The signals corresponding to allantoine (1), α-glucose (2), β-glucose (3), BHB (4), lactate (5), myo-inositol (6), TMAO (7), betaine (8), phosphocholine (9), glycerolphosphocholine (10), choline (11), creatinine (12), creatine (13), TMA (14), citrate (15), acetone (16), O-acetylglycoprotein (17), N-acetylglycoprotein (18), acetate (19), citrulline (20), lysine (21), alanine (22), valine (23), isobutyrate (24), isoleucine (25), leucine (26), and L1 lipoprotein (27) are annotated.]

3.2. NMR-Based Serum Metabolomics

The initial step in the study involved the identification of groups among the animals through peripartum. For that purpose, we carried out a PCA considering the data derived from the 1H NMR spectra for all serum samples. The resulting score plot showed no differentiation according to parity or breed (Figure 2a,b, respectively). Similar results were
obtained when principal components capturing less variance were considered in the score plots (Figures S1 and S2). However, when a continuous coloring scheme based on days around calving was employed to color the scores, four clear clusters were identified. These groups involved samples from animals four weeks before calving (C − 4), and one, four, and eight weeks after calving (C + 1, C + 4, and C + 8, respectively), and are colored blue, light-blue, yellow, and red in Figure 2c, respectively. Blood biochemistry data and other information on animals involved in each of these stages are summarized in Table 1.

![Figure 2](image URL)

**Figure 2.** PCA score plot obtained from $^1$H NMR spectral data for all serum samples. The plot was colored based on breed (a), parity (b), or using a continuous coloring scheme which varies as a function of the transition period day relative to calving (c).

| Animal Data | Stage       |
|-------------|-------------|
|             | C − 4      | C + 1 | C + 4 | C + 8 |
| Days in stage | −36 to −22 | 3 to 11 | 23 to 34 | 39 to 68 |
| N ($H, J \times J$) | 23 (19, 2, 2) | 20 (18, 0, 2) | 24 (20, 2, 2) | 20 (17, 1, 2) |
| Parity ($PP, MP$) | 15, 8 | 12, 8 | 15, 9 | 13, 7 |
| Disease ($SCK, MCM$) | 0, 9 | 2, 6 | 6, 10 | 2, 11 |
| Milk production (L/day) | - | 23 ± 7 | 28 ± 6 | 25 ± 7 |
| **Biochemistry** | | | | |
| Albumin (g/L) | 31.00 ± 2.19 | 29.64 ± 2.91 | 29.44 ± 5.67 | 31.42 ± 2.78 |
| Urea (mM) | 2.40 ± 1.31 | 2.82 ± 1.10 | 2.65 ± 1.11 | 3.43 ± 1.46 |
| Cholesterol (mM) | 2.71 ± 0.43 | 2.29 ± 0.50 | 3.81 ± 0.75 | 5.09 ± 0.93 |
| Total protein (g/L) | 79.61 ± 7.00 | 74.23 ± 7.19 | 78.13 ± 7.72 | 80.52 ± 6.64 |
| Globuline (g/L) | 48.90 ± 6.50 | 44.59 ± 5.17 | 48.69 ± 5.12 | 49.10 ± 4.60 |
| BHB (mM) | 0.38 ± 0.21 | 0.56 ± 0.32 | 0.78 ± 0.63 | 0.65 ± 0.35 |
| NEFA (mM) | 0.29 ± 0.18 | 0.63 ± 0.22 | 0.68 ± 0.36 | 0.47 ± 0.20 |

*a* H, J, and $H \times J$ represent Holstein, Jersey, and Holstein × Jersey crosses, respectively. *b* PP and MP indicate primiparous and multiparous, respectively. *c* SCK and MCM indicate subclinical ketosis and mild clinical mastitis (i.e., grade 1), respectively.

Table 1. Animal and blood biochemistry data for cows within each stage identified through multivariate analysis of $^1$H NMR data.

Having identified four peripartum stages, we investigated variations in the serum metabolome, taking prepartum metabolite levels as reference. This was achieved through a pairwise comparison of the C − 4 against the C + 1, C + 4, and C + 8 groups, respectively, using OPLS-DA and univariate data analysis (Figure 3, Figure S3 and Table 2). In all cases, the large positive peak at 1.25 ppm observed in the OPLS-DA loading plots corresponds to a sharp drop in lactic acid concentration after calving. While there are several sources of this metabolite, including dehydrogenation of pyruvate through the glyoxalase system in the metabolism of fats, carbohydrates, and proteins [48], anaerobic bacterial fermentation in rumen and colon likely leads to the high levels of lactate observed before calving in
this case [49,50]. As concluded in a recent large-scale NMR-based serum metabolomics study of clinically healthy dairy cattle [29], serum lactate can indeed be associated with an increased intake of fermentable carbohydrates such as those present in the TMR prepartum diet [51,52]. At the same time, the marked decrease in lactate levels following calving suggests that this metabolite is used to support glucose demands through gluconeogenesis by activation of the Cori cycle [53,54]. This, together with an increase in lipid metabolism through β-oxidation and the activation of the alanine cycle at the height of milk production (vide infra), is also consistent with the observed two- to three-fold rise in blood glucose levels after calving (Table 2), and could help explain why none of the animals had clinical manifestations of ketosis during the study.

One week after calving, negative values in the OPLS-DA loading plot for acetate, betaine, and creatine were observed (Figure 3a), indicating an increase in the levels of these metabolic stress markers [22,28]. The rise in the BHB and NEFA concentrations determined biochemically is also in agreement with this finding (Table 1). Overall, these data agree with the increase in lipid mobilization that reportedly takes place in early lactation [14,55]. As collected in Table 2, increases in asparagine, citrate, citrulline, glucose, and lysine are observed as well. This indicates alterations in the citric acid, urea, glycolytic, and gluconeogenic pathways immediately following parturition [14,28], and confirms that the animals enter a NEB condition. The rise in the levels of circulating allantoin (Table 2), a metabolite linked to purine catabolism, is consistent with higher ruminal microbial yields associated with the increase in DMI as well as the inclusion of forage in the diet after calving [56].

In addition to acetate and BHB, the loading plots for the OPLS-DA contrasting the C − 4 and C + 4 groups show that the levels of other metabolic stress markers, such as acetone, choline, and formate rise significantly four weeks postpartum (Figure S3b). Similarly, univariate data analysis indicates two- to four-fold increases for the species identified in the C + 1 stage, including acetate, BHB, betaine, and glucose, as well as for creatine and creatinine (Table 2). Greater accumulation of allantoin is also observed, which at this stage is likely to be associated not only with higher bacterial protein yields caused by the increase in DMI, but with an increase in tissue damage [57]. Indeed, the accumulation of alanine points to an activation of the alanine cycle, where muscle protein is catalyzed to free liver gluconeogenesis [2,58]. The higher levels of acetate and citrate in blood also reveal a further increase in lipomobilization through lipolysis and β-oxidation. This, in turn, triggers the ketogenic pathway, leading to the marked rise observed in the concentrations of BHB, lysine, and, ultimately, acetone [28,57]. Not surprisingly, these pronounced changes occur when animals reach the peak of milk production (Table 1) and corroborate with the large shifts in metabolism that are required to meet energy demands during this period. Indeed, based exclusively on BHB levels, nearly one out of every four animals developed subclinical ketosis at this stage [36].

Table 2. Average metabolite concentrations during peripartum determined by 1H NMR (mean ± std. dev.) and fold changes with respect to prepartum levels.

| Metabolite | Concentration (mM) | Fold Change |
|------------|-------------------|-------------|
|            | C − 4             | C + 4       | C + 8       | C + 8/C − 4 | C + 1/C − 4 | p     | C + 1/C − 4 | p     |
| Acetate    | 0.83 ± 0.80       | 1.36 ± 1.22 | 1.99 ± 2.17 | 1.86 ± 1.13 | 1.63 ± 0.94 | 2.40 | 0.0091 | 2.25 <0.0001 |
| Acetone    | 0.11 ± 0.15       | 0.12 ± 0.08 | 0.17 ± 0.16 | 0.12 ± 0.09 | 1.14 ± 0.35 | 1.62 | 0.0724 | 1.10 0.3734 |
| Alanine    | 0.18 ± 0.17       | 0.26 ± 0.22 | 0.43 ± 0.46 | 0.51 ± 0.39 | 1.50 ± 0.78 | 2.48 | 0.0071 | 2.88 0.0002 |
| Allantoin  | 0.07 ± 0.07       | 0.16 ± 0.16 | 0.24 ± 0.27 | 0.20 ± 0.12 | 2.16 ± 0.16 | 3.41 | 0.0021 | 2.81 <0.0001 |
| Asparagine | 0.61 ± 0.42       | 1.71 ± 2.20 | 2.36 ± 2.90 | 1.92 ± 1.27 | 2.82 ± 0.11 | 3.88 | 0.0074 | 3.16 <0.0001 |
| Betaine    | 0.18 ± 0.15       | 0.34 ± 0.38 | 0.56 ± 0.63 | 0.43 ± 0.26 | 1.92 ± 0.32 | 3.15 | 0.0035 | 2.44 0.0001 |
| BHB        | 0.38 ± 0.20       | 0.56 ± 0.32 | 0.78 ± 0.62 | 0.65 ± 0.35 | 1.46 ± 0.35 | 2.04 | 0.0028 | 1.71 0.0009 |
| Citrate    | 0.10 ± 0.09       | 0.26 ± 0.25 | 0.39 ± 0.50 | 0.22 ± 0.18 | 2.18 ± 0.99 | 4.01 | 0.0085 | 2.29 0.0035 |
| Citrulline | 0.13 ± 0.12       | 0.26 ± 0.31 | 0.48 ± 0.65 | 0.28 ± 0.23 | 2.01 ± 0.44 | 3.75 | 0.0069 | 2.18 0.0039 |
| Creatine   | 0.04 ± 0.04       | 0.10 ± 0.14 | 0.15 ± 0.22 | 0.10 ± 0.09 | 2.51 ± 0.20 | 3.70 | 0.0105 | 2.33 0.0029 |
| TMA        | 0.36 ± 0.04       | 0.23 ± 0.07 | 0.13 ± 0.19 | 0.10 ± 0.19 | 1.54 ± 0.32 | 2.13 | 0.0484 | 1.68 0.0298 |
| α-Glucose  | 1.00 ± 0.88       | 2.27 ± 2.54 | 3.50 ± 4.15 | 2.91 ± 2.04 | 2.28 ± 0.14 | 3.51 | 0.0033 | 2.29 <0.0001 |
| β-Glucose  | 0.92 ± 0.81       | 2.19 ± 2.61 | 3.49 ± 4.74 | 2.81 ± 2.08 | 2.38 ± 0.41 | 3.77 | 0.0868 | 3.04 <0.0001 |
| Lactate    | 3.60 ± 3.10       | 1.16 ± 0.63 | 1.67 ± 1.13 | 1.60 ± 0.77 | 2.23 ± 0.58 | 2.16 | 0.0015 | 2.26 0.0006 |
| Leucine    | 0.12 ± 0.10       | 0.06 ± 0.03 | 0.06 ± 0.03 | 0.03 ± 0.03 | −1.83 ± 0.16 | −1.93 | 0.0036 | −3.80 <0.0001 |
| Lysine     | 0.08 ± 0.07       | 0.23 ± 0.31 | 0.37 ± 0.48 | 0.26 ± 0.20 | 2.63 ± 0.16 | 4.16 | 0.0027 | 2.97 0.0001 |
| Valine     | 0.25 ± 0.17       | 0.22 ± 0.07 | 0.24 ± 0.08 | 0.26 ± 0.09 | −1.02 ± 0.58 | 1.04 | 0.3938 | 1.14 0.1901 |
Metabolome data at C + 8 is of particular interest. Although cows are still at their peak of milk production, DMI also increases at this stage. As a result, animals tend to normalize their energy balance, which is evidenced by a decrease in the levels of NEFA (Table 1). This is also reflected in the OPLS-DA loading plot presented in Figure 3c, which reveals that the prepartum and C + 8 group are differentiated by metabolites such as acetate and glucose, but not by markers of severe metabolic stress present in the C + 4 group. In a similar way, univariate analysis shows that the metabolites with large fold changes for the C + 4 stage now present smaller variations when compared to their levels before calving. This indicates a trend towards the normalization of the metabolic pathways identified as altered by our analyses of the C + 1 and C + 4 groups and is consistent with the animals exiting the NEB condition and regaining homeostasis.

Figure 3. Score and loading factor plots obtained from the OPLS-DA between prepartum (C − 4) and first (C + 1) (a), second (C + 4) (b), and third (C + 8) (c) postpartum groups. The metabolites that differentiate the prepartum from each postpartum group are annotated in the loading factor plots. The R²Y and Q²Y coefficients for each model were 0.80 and 0.72, 0.89, and 0.37, and 0.94 and 0.87, respectively, and their ROC curves had AUC values of 1.00, 0.85, and 0.99 (see Figures S4–S9).

As stated above, no differences based on animal parity were observed when all the data were analyzed together. However, we decided to investigate if variations related to
this condition could be evidenced within each of the four stages. For that purpose, a PCA for each of the peripartum group datasets was carried out. While no significant effects were observed at any stage after calving, a clear difference between primiparous and multiparous cows was evident in the C − 4 group (Figure 4a). Indeed, an OPLS-DA of these data readily reveals that the concentrations of lactate, acetate, and BHB are higher in multiparous cows before calving, while accumulation of creatine, TMAO, choline, glucose, and lipoproteins is observed in heifers (Figure 4b). These data likely reflect the feeding and herd behaviors of multiparous cows, which are known to be better adapted to prepartum TMR diets and to dominate primiparous animals, and thus have a greater DMI [59,60]. The higher intake of fermentable nutrients in these animals leads, in turn, to higher levels of volatile fatty acids in their bloodstream. In addition, heifers continue growing through pregnancy, and thus the concentration of anabolic hormones, such as IGF-1, is usually greater. This impacts the availability of nutrients in the mammary gland and could contribute to lowering the levels of circulating lactate and ketone bodies in these animals [61]. Few studies have analyzed the effect of parity on the entire serum metabolome of the lactating cow [29], and this is to our knowledge one of the first reports linking the observed variations with a combination of dietary and metabolic adaptations.

![Figure 4](image_url)

**Figure 4.** PCA score plot obtained from $^1$H NMR spectral data for prepartum (C − 4) serum samples colored according to animal parity (a), and score and loading factor plots obtained from the OPLS-DA between primiparous and multiparous dairy cows within this group (b,c). The metabolites that differentiate the two groups are annotated in the loading factor plots. The $R^2_Y$ and $Q^2_Y$ coefficients for the model were 0.82 and 0.56, and its ROC curve had an AUC value of 0.93 (see Figures S16 and S17).

### 4. Conclusions

As detailed in the previous sections, NMR-based metabolomics allowed us to follow the changes of 18 serum metabolites during the transition period of dairy cows. The approach identified four clear stages through peripartum based on the metabolome composition and revealed alterations in important metabolic pathways. In particular, the data shows the sequential activation of several gluconeogenic routes after calving, including the Cori and alanine cycles. Analysis of metabolomic profiles also showed that the contribution of the former gluconeogenic pathway is affected by animal parity before calving. Although recent studies have yielded comparable results at two well-defined moments in the transition period [28], our findings indicate that markers of subjacent metabolic diseases, such as ketosis and liver lipidosis, are present through most of the lactation period but peak four weeks after calving. Furthermore, the results presented here are consistent with data obtained using targeted MS-based metabolomics [27].

Overall, our findings indicate that NMR-based methods are ideally suited to readily evaluate and follow the impact of nutritional management on the metabolic health of dairy herds throughout the transition period. We are currently using this approach to assess the effects of different postpartum dietary schemes on animal condition. In addition, the combined use of metabolomic data from serum, urine, and other biofluids to achieve higher specificity will be evaluated [47,62]. Work in this and related areas is underway, and our findings will be reported in due course.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/dairy2030028/s1, Table S1 and Figures S1–S17: Postpartum diet composition, additional PCA and OPLS-DA plots, and permutation test plots and ROC analysis curves for all OPLS-DA models.

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