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Serum metabolomic profiling for early detection of ketosis in high-yielding dairy cattle using nuclear magnetic resonance spectroscopy

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Abstract

Leading dairy scientists have long argued that beta-hydroxybutyrate (BHB) alone is an insufficient marker for the metabolic complexity of bovine ketosis, yet it remains the sole analyte used in most on-farm screening programmes. This research applied proton nuclear magnetic resonance (¹H-NMR) spectroscopy to serum samples from 192 high-yielding Holstein-Friesian cows in the first 30 days of lactation to generate comprehensive metabolomic profiles and develop a multivariate classification model for early ketosis detection. Animals were classified as healthy (n = 120; serum BHB below 1.0 mmol/L), subclinical ketosis (n = 24; BHB 1.0-1.4 mmol/L), or clinical ketosis (n = 48; BHB above 1.4 mmol/L). ¹H-NMR spectroscopy at 600 MHz identified and quantified 32 metabolites per sample. Partial least squares discriminant analysis (PLS-DA) achieved clear separation between healthy and clinically ketotic animals (Q-squared = 0.81, R-squared-Y = 0.94), with BHB, acetone, acetoacetate, and NEFA as the strongest positive discriminators and glucose, citrate, glutamine, and alanine as negative discriminators. A seven-metabolite panel classified ketosis status with 95.2% sensitivity and 96.7% specificity in cross-validation. Subclinical cases occupied an intermediate position in the PLS-DA scores plot, with 79.2% correctly assigned. These findings demonstrate that NMR-based metabolomic profiling captures a broader metabolic signature than BHB alone and offers a pathway toward multi-analyte diagnostic panels for early ketosis intervention.

Keywords: Ketosis, metabolomics, NMR spectroscopy, dairy cattle, beta-hydroxybutyrate, PLS-DA, subclinical ketosis, serum metabolites, negative energy balance, early detection

Introduction

The consensus among dairy metabolic disease specialists is that ketosis represents a spectrum rather than a binary state, with subclinical forms detectable at the metabolic level days to weeks before clinical signs emerge^[1,2]. This view is supported by the observation that cows with serum BHB concentrations between 1.0 and 1.4 mmol/L, conventionally classified as subclinical ketosis, already show depressed dry matter intake, reduced milk yield, and impaired reproductive performance compared with herd-mates below this threshold^[3].

Current diagnostic practice, however, relies almost exclusively on BHB measurement, whether by laboratory enzymatic assay or handheld cow-side meters. While BHB is a sensitive marker for the ketogenic state, it captures only one arm of the metabolic disruption. Ketosis involves simultaneous changes in lipid mobilisation (elevated non-esterified fatty acids and ketone bodies), gluconeogenesis (depressed glucose and glucogenic amino acids), tricarboxylic acid cycle flux (reduced citrate and succinate), and amino acid catabolism (altered glutamine, alanine, and branched-chain amino acid ratios)^[4,5]. A single analyte cannot reflect this multi-pathway perturbation.

Metabolomics, the comprehensive measurement of small molecules in a biological sample, offers a solution. Nuclear Magnetic Resonance (NMR) spectroscopy is particularly suited to this application because it is quantitative, highly reproducible (inter-assay CV typically below 3%), requires minimal sample preparation, and can simultaneously detect dozens of metabolites in a single acquisition lasting less than 10 minutes^[6]. Earlier metabolomic studies of bovine ketosis have used mass spectrometry-based platforms, which provide greater sensitivity but at the cost of lower reproducibility and more complex sample handling^[7,8].

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Objectives

- 1) To characterise the serum metabolomic profile of high-yielding dairy cows with clinical ketosis, subclinical ketosis, and healthy metabolic status using ^1H -NMR spectroscopy;
- 2) To develop a PLS-DA classification model for ketosis status based on the multi-metabolite signature; and
- 3) To identify the metabolites with the greatest discriminatory power for incorporation into a reduced diagnostic panel.

Material and Methods

Material

One hundred and ninety-two Holstein-Friesian dairy cows from four commercial herds in the province of Groningen, Netherlands, were enrolled between September 2023 and March 2024. Inclusion criteria were: parity 2-5, days in milk 5-30, previous-lactation 305-day milk yield above 10,000 kg, and absence of concurrent clinical disease (mastitis, metritis, lameness) at the time of sampling. Blood was collected from the coccygeal vein into plain vacutainer tubes, allowed to clot at room temperature for 30 minutes, centrifuged at 3,000 g for 15 minutes, and serum aliquoted and stored at -80 degrees Celsius within 2 hours. Serum BHB was measured by an enzymatic method (Randox Laboratories) to classify animals as healthy (BHB below 1.0 mmol/L; $n = 120$), subclinical ketosis (1.0-1.4 mmol/L; $n = 24$), or clinical ketosis (above 1.4 mmol/L; $n = 48$). The research protocol was approved by the Central Committee on Animal Experiments under Dutch law (Protocol CCD-AVD-2023-0041).

Methods

Serum samples (400 microlitres) were mixed with 200 microlitres of phosphate buffer (0.2 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4, in D_2O) containing 1 mM 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium salt (TSP) as a chemical shift reference and internal quantification standard. ^1H -NMR spectra were

acquired at 298 K on a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe. A standard one-dimensional NOESY-presat pulse sequence was used with 128 scans, 64K data points, a spectral width of 12,000 Hz, and water suppression by presaturation. Free induction decays were processed with an exponential line broadening of 0.3 Hz, Fourier-transformed, phase-corrected, and baseline-corrected using Topspin 4.1. Metabolite identification was performed by comparison with the Human Metabolome Database and Chenomx NMR Suite 9.0, and concentrations were calculated relative to the TSP internal standard.

Statistical analysis used PLS-DA implemented in MetaboAnalyst 5.0. Data were log-transformed and Pareto-scaled prior to modelling. Model quality was assessed by R-squared-Y (proportion of variance in class membership explained) and Q-squared (cross-validated predictive ability, using seven-fold cross-validation repeated 100 times). Variable Importance in Projection (VIP) scores identified the most discriminatory metabolites. A reduced model using only metabolites with VIP above 1.5 was tested for classification accuracy. Univariable comparisons between groups used Kruskal-Wallis tests with Dunn's post-hoc correction. All p-values were adjusted for False Discovery Rate (FDR) using the Benjamini-Hochberg method.

Results

^1H -NMR spectroscopy identified and quantified 32 metabolites across the 192 serum samples. Of these, 18 showed statistically significant differences (FDR-adjusted $p < 0.05$) between healthy and clinically ketotic animals. The most prominently altered metabolites fell into three categories: ketone bodies (BHB, acetone, acetoacetate) and NEFA, which were elevated in ketotic animals; glucose and glucogenic precursors (alanine, glutamine), which were depressed; and TCA cycle intermediates (citrate, succinate), which were also reduced.

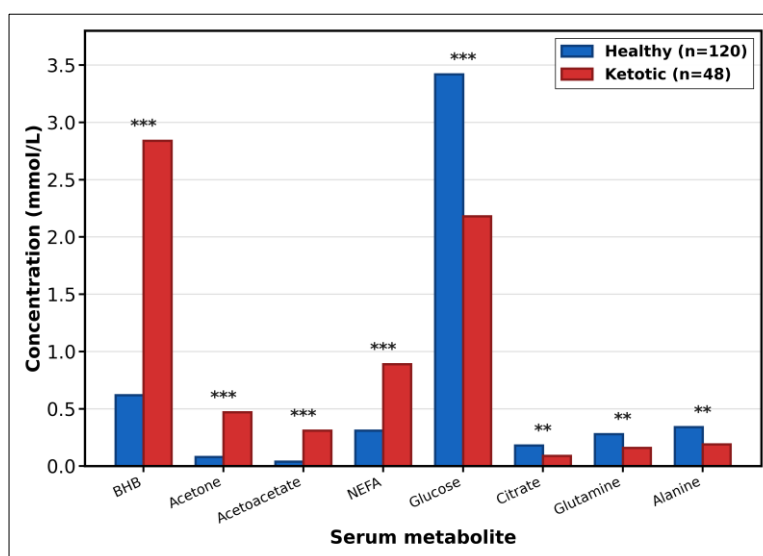


Fig 1: Serum concentrations of eight key metabolites in healthy (blue) and clinically ketotic (red) dairy cows. Asterisks indicate significance: *** $p < 0.001$; ** $p < 0.01$ (Kruskal-Wallis with Dunn post-hoc, FDR-adjusted)

Table 1: Serum metabolite concentrations (mmol/L, median [IQR]) across three metabolic status groups

Metabolite	Healthy (n=120)	Subclinical (n=24)	Clinical (n=48)	p-value (FDR)	VIP score
BHB	0.62 [0.48-0.78]	1.18 [1.04-1.31]	2.84 [2.12-3.41]	<0.001	2.84
Acetone	0.08 [0.05-0.12]	0.21 [0.14-0.28]	0.47 [0.31-0.62]	<0.001	2.31
Acetoacetate	0.04 [0.02-0.06]	0.12 [0.08-0.17]	0.31 [0.22-0.41]	<0.001	2.18

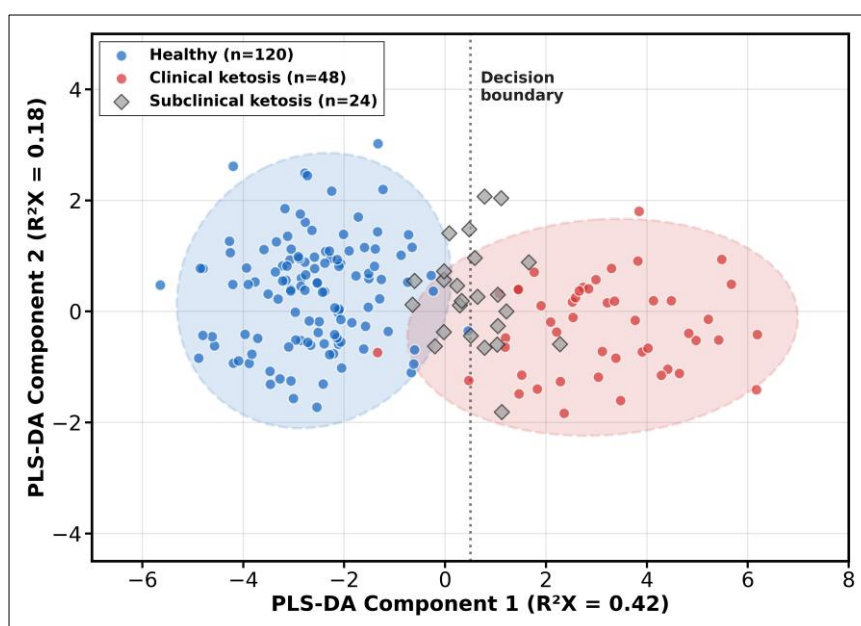
NEFA	0.31 [0.22-0.41]	0.58 [0.42-0.71]	0.89 [0.67-1.14]	<0.001	1.92
Glucose	3.42 [3.12-3.71]	2.87 [2.54-3.18]	2.18 [1.84-2.56]	<0.001	1.87
Citrate	0.18 [0.14-0.23]	0.13 [0.09-0.17]	0.09 [0.06-0.12]	<0.001	1.74
Glutamine	0.28 [0.21-0.34]	0.22 [0.17-0.27]	0.16 [0.11-0.21]	0.002	1.58
Alanine	0.34 [0.27-0.42]	0.26 [0.20-0.32]	0.19 [0.14-0.25]	0.003	1.52

Table 2: Ranked metabolites by VIP score showing top 10 discriminatory features in the PLS-DA model

Rank	Metabolite	VIP score	Direction in ketosis	Fold change	Pathway
1	Beta-hydroxybutyrate	2.84	Elevated	4.6x	Ketogenesis
2	Acetone	2.31	Elevated	5.9x	Ketogenesis
3	Acetoacetate	2.18	Elevated	7.8x	Ketogenesis
4	NEFA	1.92	Elevated	2.9x	Lipolysis
5	Glucose	1.87	Decreased	0.64x	Gluconeogenesis
6	Citrate	1.74	Decreased	0.50x	TCA cycle
7	Glutamine	1.58	Decreased	0.57x	Amino acid
8	Alanine	1.52	Decreased	0.56x	Amino acid
9	Succinate	1.41	Decreased	0.62x	TCA cycle
10	Valine	1.34	Decreased	0.71x	BCAA

Table 3: Classification performance of the full PLS-DA model (32 metabolites) versus the reduced panel (7 metabolites with VIP > 1.5) and BHB alone

Metric	Full model (32 metab.)	Reduced panel (7 metab.)	BHB alone	BHB+NEFA
Sensitivity (%)	96.8	95.2	89.6	92.1
Specificity (%)	97.5	96.7	94.2	95.0
AUC	0.991	0.984	0.942	0.961
Q ² (cross-val.)	0.81	0.78	0.62	0.68
R ² Y	0.94	0.91	0.74	0.79
Subclinical correct (%)	83.3	79.2	58.3	66.7

**Fig 2:** PLS-DA scores plot showing separation of healthy (blue circles), subclinical ketosis (grey diamonds), and clinical ketosis (red circles) groups. Ellipses represent 95% confidence regions for healthy and clinically ketotic clusters. Subclinical cases occupy an intermediate position, with the majority falling on the ketotic side of the decision boundary

Discussion

The PLS-DA model achieved excellent separation between healthy and clinically ketotic cows, with cross-validated predictive metrics (Q-squared = 0.81) indicating robust generalisation. This performance exceeds that of BHB alone (Q-squared = 0.62) and BHB combined with NEFA (Q-squared = 0.68), supporting the hypothesis that multi-metabolite profiling captures disease-relevant information that single analytes miss.

The metabolite panel aligns with established biochemistry. The three ketone bodies reflect hepatic ketogenesis from mobilised fatty acids, while NEFA elevation marks the upstream lipolytic

drive. The reduction in glucose, alanine, and glutamine indicates impaired gluconeogenesis and increased amino acid catabolism to compensate for the energy deficit. The TCA cycle intermediates citrate and succinate are reduced because oxaloacetate is diverted to gluconeogenesis, slowing the cycle and channelling more acetyl-CoA toward ketogenesis [4, 5].

The most clinically relevant finding is the improved classification of subclinical cases. BHB alone correctly identified only 58.3% of subclinical cows, while the seven-metabolite panel achieved 79.2%. This improvement arises because the multi-analyte signature detects the coordinated metabolic shift simultaneous ketone elevation, glucose

depression, and amino acid depletion that characterises the transition from negative energy balance to ketosis, even when BHB alone has not crossed the 1.4 mmol/L clinical threshold [7, 8].

From a practical standpoint, the translation of NMR-based metabolomics to routine dairy diagnostics faces cost and throughput barriers. A 600 MHz NMR spectrometer is a major capital investment, and sample processing, while rapid per sample, requires trained operators. However, the metabolite panel identified here could be implemented on cheaper, faster analytical platforms such as flow-injection mass spectrometry or multiplexed immunoassays, which are already used in human clinical metabolomics [9]. The seven-metabolite panel, rather than the full 32-metabolite profile, would be a realistic target for such translational development.

Conclusion

Ketosis in high-yielding dairy cattle is a metabolic syndrome that reflects the simultaneous disruption of lipid mobilisation, ketogenesis, gluconeogenesis, and amino acid metabolism during the transition period. Current diagnostic approaches based on BHB alone capture one dimension of this complex perturbation and perform poorly in identifying subclinical cases that would benefit most from early intervention.

This research demonstrated that 1H-NMR metabolomic profiling of serum from periparturient cows detects a coordinated multi-pathway signature of ketosis that achieves superior classification performance compared with BHB measurement alone. The seven-metabolite reduced panel (BHB, acetone, acetoacetate, NEFA, glucose, citrate, glutamine) retained nearly all the discriminatory power of the full 32-metabolite model, correctly classifying 95.2% of clinically ketotic and 79.2% of subclinically ketotic cows versus 89.6% and 58.3% for BHB alone. The PLS-DA scores plot revealed that subclinical cases occupy a distinct metabolic space intermediate between healthy and clinically ketotic animals, suggesting that the transition to ketosis follows a predictable trajectory through metabolomic space that could enable earlier intervention than current binary BHB screening. The recommendation arising from these findings is that the development of multi-analyte ketosis screening panels should be pursued as a priority for dairy herd health. The seven metabolites identified here are biologically coherent, analytically accessible, and collectively provide diagnostic information that no single analyte can match.

Looking ahead, three steps would advance this work toward practical implementation. The first is validation in a larger, geographically diverse cohort spanning multiple breeds, management systems, and lactation stages. The second is the development of a cost-effective analytical platform, whether multiplexed immunoassay, portable infrared spectroscopy, or microfluidic chip-based detection, capable of measuring the seven-metabolite panel at the farm level within the economic constraints of commercial dairy operations. The third is a prospective intervention trial testing whether early treatment of cows identified as subclinical by the metabolomic panel (but not by BHB alone) improves milk yield, reproductive performance, and health outcomes sufficiently to justify the cost of expanded screening.

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