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Metabolic clusters of early-lactating dairy cows based on blood β-hydroxybutyrate trajectories and predicted from milk compounds

Muluken Girma,[1]*† S. Heirbaut,[1] K. Hertogs,[2] X. P. Jing,[3] M. Q. Zhang,[1] P. Lutakome,[1,4] K. Geerinckx,[5] S. Els,[5] B. Aernouts,[5] L. Vandaele,[6] and V. Fievez[1]*

[1]Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Sciences and Aquatic Ecology, Ghent University, 9000 Ghent, Belgium
[2]Inagro, 8800 Rumbeke-Beitem, Belgium
[3]State Key Laboratory of Grassland and Agro-Ecosystems, International Centre for Tibetan Plateau Ecosystem Management, School of Life Sciences, Lanzhou University, Lanzhou 730000, China
[4]Department of Agricultural Production, College of Agricultural and Environmental Science, Makerere University, PO Box 7062, Kampala, Uganda
[5]Hooibeekhoeve, 2440 Geel, Belgium
[6]ILVO, 9090 Melle, Belgium

ABSTRACT

High-yielding dairy cows encounter metabolic challenges in early lactation. Typically, BHB, measured at a specific time point, is employed to diagnose the metabolic status of cows based on a predetermined threshold. However, in early lactation, BHB is highly dynamic, with high interindividual variability in its time profile. This could limit the effectiveness of the single measurement and threshold-based diagnosis and could contribute to the disparities in reports linking metabolic status with productive and reproductive outcomes. This research examines the trajectories of BHB to unveil intercow variations and identify latent metabolic groups. We compiled a dataset from 2 observational studies involving a total of 195 lactations from multiparous Holstein Friesian cows. The dataset encompasses measurements of BHB, nonesterified fatty acids (NEFA), and insulin from blood samples collected at 3, 6, 9, and 21 DIM, along with weekly determinations of milk composition and fatty acids (FA) proportions in milk fat. In both experiments, milk yield (MY) and feed intake were recorded daily during the first month of lactation. We explored interindividual and intraindividual variations in metabolic responses using the trajectories of blood BHB and evaluated the presence of distinct metabolic groups based on such variations. For this purpose, we employed the growth mixture model, a trajectory clustering technique. Our findings unveil novel insights into the diverse metabolic responses among cows, encompassing both trajectory patterns and the magnitude of blood BHB concentrations. Specifically, we identified 3 latent metabolic groups: the quickly increasing BHB (QuiBHB) cluster (≈10%) exhibited a higher initial BHB concentration than other clusters, peaked on d 9 (average maximum BHB of 2.4 m*M*) and then declined by d 21; the slowly increasing BHB (SloBHB) cluster (≈23%) started with a lower BHB concentration, gradually increased until d 9, and reached the highest BHB concentration at d 21 (1.6 m*M* serum BHB at the end of the experimental period); and the low BHB (LoBHB) cluster (≈67%) began with the lowest serum BHB concentration (serum BHB <0.75 m*M*) and remained relatively stable throughout the sampling period. Notably, the 3 metabolic groups exhibited significant physiological disparities, which were evident in blood NEFA and insulin concentrations. The QuiBHB and SloBHB cows exhibited higher NEFA and lower insulin concentrations as compared with the LoBHB cows. Interestingly, these metabolic differences extended to MY and DMI during the first month of lactation. The elevated BHB concentrations observed in QuiBHB cows were linked with lower DMI and MY as compared with SloBHB and LoBHB cows. Accordingly, these animals were considered metabolically impaired. Conversely, SloBHB cows displayed higher MY along with increased DMI, and thus the elevated BHB might be indicative of an adaptive response for these cows. The QuiBHB cows also displayed higher proportions of UFA, MUFA, and total C18:1 FA in milk during the first week of lactation. Prediction of the QuiBHB cows using these FA and test day variables resulted in moderate predictive accuracy (area under the receiver operating characteristic curve >0.7). Given the limited sample size for the development of prediction models and the variation in DIM among samples in the same week, the result is indicative of the predictive potential of the model and room for model

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*Corresponding authors: markosethio@yahoo.com and veerle.fievez@ugent.be
†Current address: Department of Animal Sciences, Wollo University, PO Box 1145, Dessie, Ethiopia.

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

optimization. In summary, distinct metabolic groups of cows could be identified based on the trajectories of blood BHB in early lactation.

Key words: metabolic status, β-hydroxybutyrate, trajectory, fatty acid

INTRODUCTION

In the transition from late pregnancy to early lactation, dairy cows undergo significant metabolic and physiological changes. Early lactation milk production to a large extent relies on the mobilization of triglycerides (**TAG**) from adipose tissues into nonesterified fatty acids (**NEFA**; Bauman and Bruce Currie, 1980; Bauman, 2000). Blood NEFA could be used by the mammary gland as an energy source for milk synthesis or directly incorporated into milk fat (Drackley et al., 2001; Ingvartsen, 2006). In the liver, NEFA either undergo β-oxidation followed by complete oxidation in the tricarboxylic acid cycle (**TCA**) or incomplete oxidation into ketone bodies such as BHB, acetoacetate, and acetate, or NEFA are re-esterified into TAG (White, 2015). Finally, NEFA are transported to peripheral tissues, which often experience insulin insensitivity (Sano et al., 1993; Sasaki, 2002; De Koster and Opsomer, 2013) and which rely on NEFA and ketone bodies as an energy source (Lucy, 2001, 2008; White, 2015). The latter is of particular importance to some tissues (e.g., the brain) that cannot easily transport the hydrophobic NEFA across cells (Pownall, 2001) and therefore cannot use them as an energy source. Accordingly, when glucose availability is limited due to copious and prioritized milk production, these tissues rely on hydrophilic and easily transportable ketone bodies as alternative energy sources (Laffel, 1999). These modifications in energy metabolism and partitioning are evolutionary adaptation mechanisms of cows to cope with the negative energy balance during early lactation. Therefore, elevated levels of ketone bodies in the blood, commonly evaluated by blood BHB concentration, are considered a normal phenomenon in early lactation (Baumgard et al., 2017; Horst et al., 2021). However, excess ketone bodies in the blood, known as hyperketonemia (**HYK**) or ketosis, could indicate NEFA overload beyond the liver's adaptive capacity (White, 2015). Moreover, a positive association has been reported between HYK (blood BHB ≥1.2mmol/L) and the risk of infectious and metabolic disorders, and up to 60% of cows might be exposed to HYK in early lactation (McArt et al., 2011).

However, a high interanimal variation is observed in cows' metabolic adaptation to metabolic challenges in early lactation (Herdt, 2000; Kessel et al., 2008; Pryce et al., 2016). This has been observed among cows kept under similar management conditions, revealing the biological variation among cows (Kessel et al., 2008; Gross and Bruckmaier, 2015, 2019). Such observations signal the potential for phenotyping cows into metabolic performance groups for management as well as breeding purposes. In addition to variation in the blood BHB concentration, there is also high intracow variation in the metabolic profile of cows in early lactation as revealed by the dynamics of blood metabolites level (McArt et al., 2012). For instance, it has been shown that cows differed in the time of the onset and duration of ketosis, marked by the variation in the trajectories of blood NEFA and BHB (McArt et al., 2012). However, to our knowledge, no studies have so far considered the time profiles of metabolic biomarkers in the blood to classify cows into different metabolic groups. The objective of this study was to identify metabolic groups among early-lactating cows using the trajectories of BHB in blood and characterize them in terms of other blood metabolites, fatty acids (**FA**) in milk fat, and production parameters. Furthermore, the broader use of blood parameters for identifying metabolic status is limited given the invasiveness and practical challenges associated with blood collection. Building on previous findings that highlighted the predictive ability of FA in milk fat for assessing the metabolic status of cows in early lactation (Jorjong et al., 2015; Girma et al., 2023; Heirbaut et al., 2023), this study also aimed to explore the potential of milk FA in conjunction with test day variables for predicting trajectory clusters.

MATERIALS AND METHODS

In this paper, 2 datasets from 2 different experiments are compiled. Experiment 1 was undertaken at the research farm of ILVO (Flanders Research Institute for Agriculture, Fisheries, and Food, Melle, Belgium) from October 2018 until October 2020. This research monitored multiparous Holstein Friesian cows (n = 120 lactations) starting from 1 wk before calving until the third week of lactation. Experiment 2 was undertaken by Ghent University at Hooibeekhoeve (Geel, Belgium), a Belgian dairy research farm from January 2021 to January 2023. In this experiment, 86 lactations of 73 unique multiparous Holstein Friesian cows were monitored starting from 4 wk before calving through the first 35 d after calving. The combined dataset consists of blood parameters: BHB, NEFA, insulin, and milk parameters including milk yield, milk fat, protein and lactose, milk BHB as well FA in milk fat. Moreover, additional farm data including parity, disease events, and feed intake were included. The experiment conducted at the research farm of ILVO (Flanders Research Institute for Agriculture, Fisheries, and Food, Melle, Belgium) was carried out following approval (2018/329) granted by the Ethical Committee of the institute. Likewise, the research conducted at Hooibeekhoeve was undertaken with approval

from the institutional animal care and use committees of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering, Ghent University (2020-078). The experimental protocols, management, and housing of both experiments are described in the following sections.

Housing and Ration

The detailed protocol of experiment 1 has been reported previously (Heirbaut et al., 2023), and a summary is presented here. Dry and lactating cows were housed separately in a naturally ventilated freestall barn with a slatted floor. From imminent calving (e.g., pelvic ligament relaxation, teat filling) until d 3 after calving, cows were housed in maternity pens with straw bedding within the same building. In case of disease, cows stayed for a longer period in these pens. From 3 wk before calving, cows received the partial mixed ration (**PMR**) of the lactating cows supplemented with a dry cow mineral premix and 1 kg of balanced concentrate per cow per day. The Belgian-Dutch net energy system (Van Es, 1975) and intestinally digestible protein-rumen undegradable protein balance-system (**DVE-OEB**) were used to formulate the ration (Van Duinkerken et al., 2011). Individual feed intake was monitored throughout the trial using roughage intake control (**RIC**) feeding bins (Insentec, Hokofarm Group, Marknesse, the Netherlands), except during the period around calving. During lactation, concentrate intake was monitored at the automatic concentrate providers (Greenfeed, C-Lock Inc., Rapid City, SD; DeLaval, Tumba, Sweden) and in the herringbone milking parlor (DeLaval, Tumba, Sweden). The cows had access to water ad libitum.

In experiment 2, 73 multiparous cows were monitored for around 9 wk. Cows entered the study at 4 wk before calving and were group-housed in a barn with a slatted floor. Subsequently, the cows were transferred to individual straw pens within the same barn. After the initial days postpartum, the cows were relocated to a slatted floor pen with a capacity for 8 cows, maintaining a stocking density of less than 1 cow per cubicle. After calving, experimental cows went to the trial group with RIC feeding bins (Insentec, Hokofarm Group, Marknesse, the Netherlands) until 35 d of lactation. The diet consisted of a PMR supplemented with individually distributed balanced concentrate which was given according to milk production through an automatic concentrate provider (DeLaval, Tumba, Sweden). The composition of the ration could vary slightly depending on the nutritional characteristics of the available roughage, but the nutritional content of the PMR remained constant. The PMR was formulated at the herd level following the Belgian-Dutch net energy system (Van Es, 1975), and DVE-OEB (Van Duinkerken et al., 2011) was used to formulate the ration. Moreover, a constant roughage-to-concentrate ratio was maintained during the trial and met the dietary structural needs at the herd level (De Brabander et al., 2002). The feed was supplied fresh once a day in the RIC bins, which were completely emptied every day, and the leftovers were quantified. Cows had ad libitum access to water. The chemical composition of the diets used in both experiments is summarized in Supplemental Table S1 (see Notes; Girma et al., 2024a)

Sampling and Measurements

Blood Sampling and Analysis. In both experiments, blood samplings were undertaken on d 3, 6, 9, and 21 after calving. Samples were taken from the coccygeal vessels (days, 3, 6, and 9) or the jugular vein (at 21 DIM) in the morning at 1000 h using an 18G needle and Venoject System (Terumo). Blood was collected in serum blood tubes (10 mL; SST II Advance Tubes, BD Diagnostics, Plymouth, United Kingdom) and kept at room temperature for 30 min before centrifugation. Samples were then centrifuged at 1,500 × *g* for 15 min at room temperature, after which serum samples were divided into aliquots and stored at −20°C.

Serum BHB and NEFA were analyzed using Randox RANBUT kits (reference no. RB1008) and Randox NEFA kits (reference no. FA115), respectively, employing enzymatic assays. For the analysis of insulin from the serum samples, the Bovine Insulin ELISA kit from Mercodia (Bio-connect Diagnostics) was used. All analyses of blood parameters in experiment 1 were carried out in the laboratory of Dierengezondheidszorg Vlaanderen, Torhout, Belgium). All blood parameters of experiment 2 were analyzed by the Laboratory for Animal Nutrition and Animal Product Quality (Ghent University, Ghent, Belgium).

Milk Sampling and Analysis. In experiment 1, dairy cows were milked twice daily at 0530 h and 1630 h in a 2 × 7 herringbone milking parlor, and their milk yield (kg/d) was recorded electronically. To determine milk performance, milk samples (27 mL) were collected from the cows in a representative way during the morning milking daily from d 3 to 23 after calving. Samples were stored at 4°C and contained preservatives, specifically sodium azide (maximum concentration 0.02% m/m) and bronopol (maximum concentration 0.005% m/m).

In experiment 2, cows were milked using an automatic milking system (**AMS**; DeLaval, Tumba, Sweden), and milk yield was recorded per milking. The 24-h daily milk yield was calculated from an average of all milkings recorded in the last 96 h using an approach suggested

for AMS (Lazenby et al., 2003). Milk subsamples (15 mL) were collected once weekly every Thursday from the morning milking and stored in the refrigerator at 4°C until transport and analysis. The samples contained preservatives, specifically sodium azide (maximum concentration of 0.02% m/m) and bronopol (maximum concentration of 0.005% m/m).

Milk samples from both experiments were analyzed for milk fat, protein, lactose, urea, BHB, SCC, SFA, UFA, MUFA, and total C18:1 by Qlip laboratory (Zutphen, the Netherlands), which performed routine DHI analysis using Fourier-transform infrared spectrometry (**FTIR**; Milkoscan FT6000, Foss Electric). Milk fat and protein were determined according to ISO 9622:2013 (International Organization for Standardization, 2013). Milk FA were estimated based on the mid-infrared spectra from in-house established equations.

Data Analysis

Data Compilation and Cleaning. The current report employed the data collected after calving in both experiments. Experiment 1 originally enrolled 102 unique cows, among which 18 cows were monitored for 2 consecutive lactations. Data from 3 cows were excluded because of the cows' death, and the final data involved 99 unique cows. Moreover, BHB and NEFA data were missing for 1 cow on the d 9 sampling. In the case of experiment 2, out of 86 lactations, 2 cows' complete data were missing, as the cows had been removed from the experiment due to death or abortion. Moreover, 4 cows had 1 missing data point for either of the blood parameters from the 4 repeated samplings, whereas 6 cows had more than 2 missing blood parameters. For the current study, we selected cows that had at least 2 records of blood parameters after calving. With this cleaning, from experiment 2, we kept 78 lactations from 65 unique cows for further analysis. The combined data of the 2 experiments had 195 lactations from 164 unique cows. The daily milk yield data of these cows were accessed from the electronic database for both experiments. The 2 experiments were different in the protocol for milk sample collection to determine the milk parameters profile. To compile milk performance data, the Thursday data were filtered from the daily milk performance data of experiment 1 to align with the milk sample collection protocol of experiment 2.

Clustering Cows Using Trajectories of Blood BHB. In this paper, we used the longitudinal data of blood BHB to cluster cows based on the trajectories of this metabolite. Before the clustering procedure, data imputation for missing blood parameters and outlier management was undertaken using the "tsrobpre" package (Narajewski et al., 2021) in R version 4.3.0 (R Foundation for Statistical Computing), which was developed specifically for time series data. Following this, we conducted a mixture model analysis to identify metabolic groups with distinct trajectories of BHB during the experimental period. Outcome variables of repeated measures are usually analyzed using a growth curve model or mixed model analysis with the assumption of a homogeneous population that can be represented by a single population mean trajectory (Muthén, 2004). However, in some cases, a group of individuals might have distinct trajectories (intraindividual variation) that cannot be represented by a single population curve. In such instances, mixture modeling techniques prove valuable because they can uncover latent groups characterized by distinct trajectories. These techniques model multiple growth curves as functions of time, revealing classes of individuals that share similar developmental patterns of the outcome variable that may not be readily apparent in the raw data (Muthén, 2008; van der Nest et al., 2020). From the available longitudinal clustering methods, we employed the growth mixture model (**GMM**), which was reported to have better performance compared with other methods (Martin and von Oertzen, 2015; N. Den Teuling [Eindhoven University of Technology, Eindhoven, North Brabant, the Netherlands], S. Pauws [Tilburg University, Tilburg, Noord-Brabang, the Netherlands], and E. van den Heuvel [Eindhoven University of Technology], unpublished data). The GMM can identify latent classes in a population that have distinct trajectories for the selected parameter(s) in a specified time window (Nguena Nguefack et al., 2020). In doing so, GMM considers the interindividual and intraindividual variation in the parameter of interest (Muthén, 2008; van der Nest et al., 2020). The overall exploration of clustering followed the steps recommended for model-based trajectory clustering (van der Nest et al., 2020). In each step, models with various model specifications were built and compared using the Bayesian information criterion, which is the most commonly used evaluation metric for model-based clustering methods. Cluster distribution, the average posterior probability of assignment, and graphical analysis were employed to further refine optimal cluster determination. The steps followed in this process are summarized in Supplemental Table S2 (see Notes; Girma et al., 2024a). In brief: (1) The GMM was first checked with a single trajectory whether or not to include cow as a random factor. In this step, including cow as a random factor was determined advantageous as the model performance was improved. (2) The model was built with 1 to 6 clusters to check whether the BHB data could be represented by a single trajectory or if latent metabolic groups were present

with different trajectories. In this step, the presence of more than 1 latent class was observed and the optimal cluster number was determined to be 3. (3) A model with 3 clusters was checked for different model specifications, which included the determination of constant or varying residual variance among clusters and the determination of similar or varying random factor variance among clusters. (4) Step 3 was repeated and DIM was introduced as random factor in the model. (5) In the last step, a polynomial DIM was introduced in the model as a fixed covariate. The polynomial term did not improve model metrics and also did not affect the distribution of cows in the 3 clusters. The "lcmm," "flexmix," and "latrend" packages in R were used to develop and evaluate the GMM with different specifications (Leisch, 2004; Proust-Lima et al., 2017; Den Teuling, 2023). The final GMM had 3 trajectory clusters with different intercepts, fixed variances of slopes, and varying variances of residuals for each cluster.

Statistical Analysis

Descriptive analyses were conducted to summarize data. To describe the distribution of metabolic clusters across parity groups and experiment (farm) chi-squared was used. Clusters were compared for their milk parameters, and feed intake during the experimental period. As repeated measures from the same cow could be correlated (e.g., Gröhn et al., 1999), the assumption of independence would be violated if the analysis did not account for such correlations. Thus, the between-cow variation was checked for its significance, and a mixed model approach was followed to compare all milk yield and composition parameters, feed intake, and blood metabolites among clusters. Where repeated measures were done weekly, cow was taken as a random factor and parity was included as an additional covariate. In the case of daily measured parameters, cow was taken as a random factor, and parity and DIM were included as additional fixed covariates. The inclusion of DIM as a random factor was also explored for mixed model analyses of DMI, MY, BHB, NEFA, and insulin, whereas the inclusion of experiment (farm) as a random factor was explored for all mixed model analyses. The DIM as a random factor was significant ($P < 0.001$) and included in the DMI, MY, and BHB models, whereas the experiment was included in the DMI, C16:0, UFA, MUFA, total C18:1, and insulin models. Furthermore, to account for the nonlinear association between DIM and both daily MY and DMI within the first 35 d of lactation, a quadratic term for DIM was incorporated into the mixed models of both parameters.

Predicting Metabolic Clusters

The potential for the prediction of metabolic clusters using milk FA and test day variables has been explored. The FA, quantified using FTIR, encompassed C4:0, C6:0, C16:0, C18:0, total C18:1, UFA, and MUFA. The test day variables incorporated factors such as parity, DIM, and FTIR-predicted milk composition parameters including fat, protein, and BHB. Parity was categorized into 3 groups: parity 2, parity 3, and parity ≥4. A one-versus-all approach was followed to predict each cluster, and multiclass classification was done to predict the 3 clusters. For this, models were created using a 5-fold cross-validation technique, repeated 100 times. Stratified sampling was applied within each fold to ensure that cows from all clusters were sampled in proportion to the original dataset. The "groupdata2" package in R was employed for this purpose (Olsen, 2023). Given the imbalanced distribution of the quickly increasing BHB (**QuiBHB**) and slowly increasing BHB (**SloBHB**) cows, an ensemble imbalanced data management technique called underbagging was used to predict these clusters. The technique employed the random forest algorithm as a weak learner and was executed using the "ebmc" package in R (Chen, 2022). In the case of the low BHB (**LoBHB**) cows' prediction, a random forest algorithm was employed without an imbalanced data management technique, as these cows were not the minority. The multiclass classification model was built using random forest algorithm, whereas the "SmoteClassif" function of the "UBL" package (Paula et al., 2023) in R was used to balance the data. Considering that the milk parameters data were collected weekly during the first 3 wk of lactation, distinct models were developed for each week's data separately.

RESULTS

Metabolic Clusters

Figure 1 illustrates the BHB trajectories of the 3 clusters. We refer to the 3 metabolic clusters as the QuiBHB, SloBHB, and LoBHB clusters. The QuiBHB cluster exhibits a higher initial BHB concentration compared with the other clusters, peaking on d 9 and then declining by d 21. The SloBHB cluster begins with a lower BHB concentration, gradually increasing until d 9, and maintaining a higher BHB concentration at d 21. The LoBHB cluster starts with the lowest serum BHB concentration, remaining relatively stable throughout the sampling period. As such, both the trajectory and the concentration of serum BHB throughout the first 3 wk in lactation differ between the clusters.

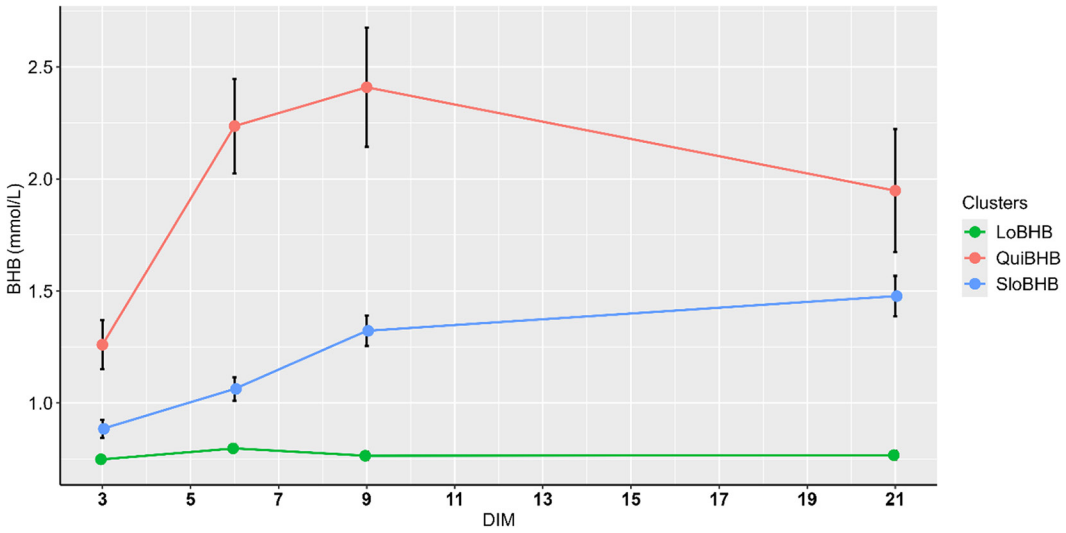

Figure 1. Mean and SE of the time profile of blood BHB concentration for metabolic clusters identified by growth mixture models using the trajectories of BHB. Mixed model analysis was done to describe the association between DIM, clusters, parity, and their interaction with milk yield per day. QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

The distribution of cows across the 3 metabolic clusters and parity groups is presented in Table 1. Two-thirds of the cows are classified into the LoBHB group, and among these, second parity cows were around half of the cows classified in this group. The lowest number of cows were classified into the QuiBHB groups and two-thirds of the cows in this group were parity 3 or higher.

Trajectories of Blood Metabolites

Figure 2 displays the NEFA time profiles of the 3 metabolic clusters identified using blood BHB trajectories. The QuiBHB cows had consistently higher mean NEFA concentrations until at least 9 DIM, whereas SloBHB cows showed intermediate levels until 9 DIM, but reached comparable concentrations with QuiBHB cows around or after 21 DIM. The LoBHB cows exhibited lower blood NEFA concentrations throughout the sampling period. Notably, NEFA concentrations started declining from 6 or 9 DIM for LoBHB and QuiBHB cows, but for SloBHB cows, the average concentration after 21 DIM was similar to the concentration within the first week after calving.

In Figure 3, the serum insulin time profiles for cows in the 3 metabolic clusters are shown. LoBHB cows had higher insulin concentrations compared with the other clusters throughout the sampling period. Until 9 DIM, SloBHB cows had higher insulin concentrations than QuiBHB cows, but the difference narrowed, and both clusters showed comparable insulin concentrations by 21 DIM.

Table 1. Distribution of cows in the 3 metabolic clusters by parity groups[1]

	Parity			Farm (experiment)	
Cluster	2	3	≥4	HBH	ILVO
QuiBHB	5	18	64	5	14
SloBHB	8	15	35	21	24
LoBHB	6	12	32	52	79
Statistics	$\chi^2 = 4.10$, P-value = 0.392			$\chi^2 = 2.32$, P-value = 0.298	

[1]Metabolic clusters are identified using the trajectories of blood BHB concentration: QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

Milk Yield, Composition, and Fatty Acids Profile

Table 2 and Table 3 present summaries of milk yield, composition, and milk fatty acid comparisons among the metabolic clusters during the 3 weekly observations following calving. Table 2 presents milk parameters for which the interaction between clusters and week was not significant ($P < 0.05$) whereas Table 2 presents parameters for which the interaction was significant ($P < 0.05$). No difference was observed among clusters in milk fat content and the concentration of C4:0 and C16:0 FA in milk fat, whereas the LoBHB cows had lower milk protein compared with the other clusters' cows. The QuiBHB cows had a higher ratio of milk fat to protein as compared with the LoBHB cows, but no difference was observed between the SloBHB and other clusters' cows.

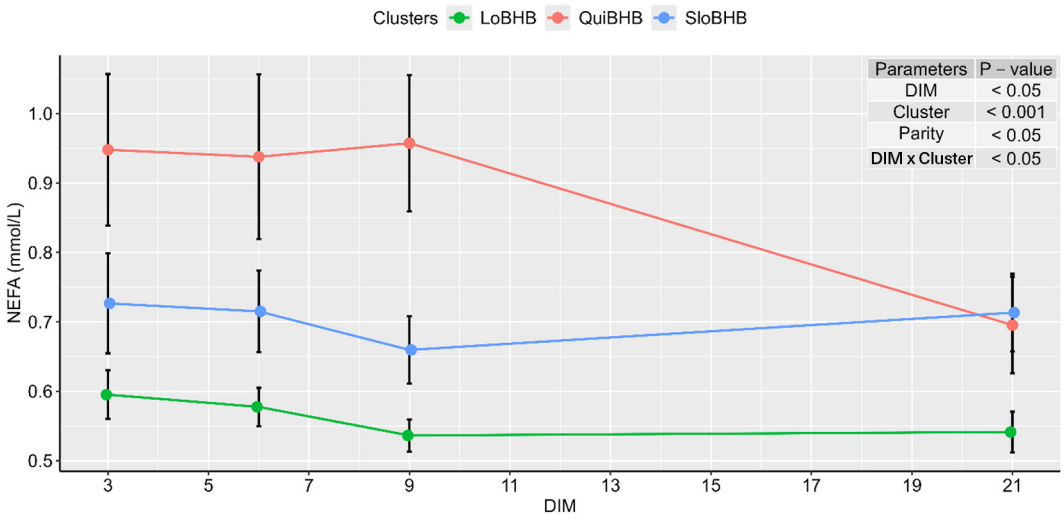

Figure 2. Mean and SE of the time profile of blood NEFA concentration for metabolic clusters identified by growth mixture models using the trajectories of BHB. A mixed model analysis was done to describe the association between DIM, clusters, parity, and their interaction with milk yield per day. QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

During week 1 (3 to 9 DIM), we observed no difference in daily milk yield among the clusters. However, in both week 2 (10 to 16 DIM) and week 3 (17 to 24 DIM), the SloBHB cows demonstrated a higher daily milk yield in comparison to the QuiBHB group. Figure 4 provides additional result of the milk yield per day comparison among clusters during the first 35 DIM.

Although the LoBHB cows exhibited similar daily milk yield as the SloBHB cows, we observed a tendency toward greater yield ($P = 0.096$, week 2; $P = 0.056$, week 3) when compared with the QuiBHB group. In week 1, the QuiBHB cows yielded a greater fat- and protein-corrected milk (**FPCM**) yield than the LoBHB cows, but by week 3, their FPCM yield was the lowest. In week 2, no significant disparity was observed in FPCM yield among the clusters.

In week 1, the QuiBHB cows displayed a higher proportion of both UFA and MUFA in milk fat compared

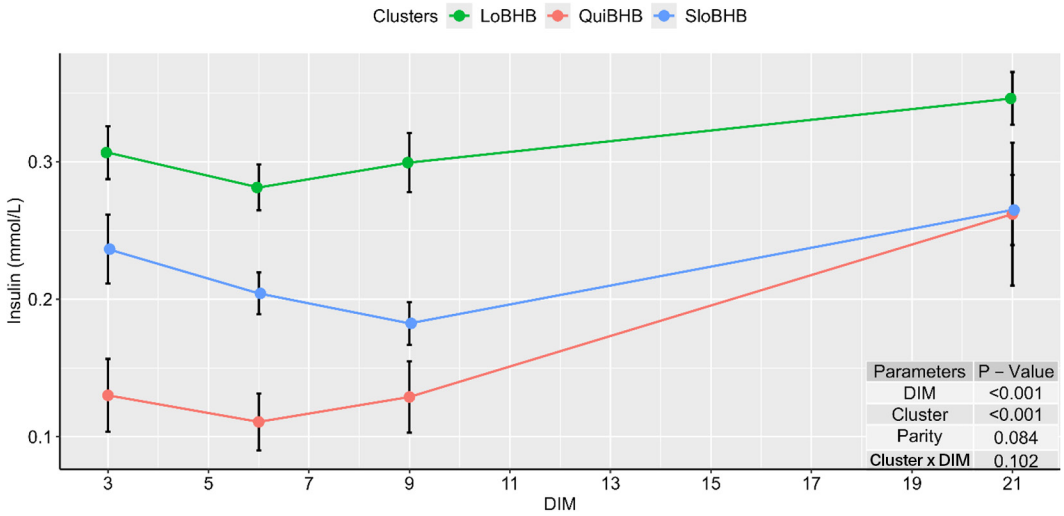

Figure 3. Mean and SE of the time profile of blood insulin concentration for metabolic clusters identified by growth mixture models using the trajectories of BHB. Mixed model analysis was done to describe the association between DIM, clusters, parity, and their interaction with milk yield per day. QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

Table 2. Summary of milk fat and protein content and fat-to-protein ratio for the 3 metabolic clusters identified using the trajectories of blood BHB concentration[1,2]

	Metabolic cluster			*P*-value		
Parameter	QuiBHB	SloBHB	LoBHB	Cluster	Week	Parity
Fat (%)	5.44 (0.203)	5.20 (0.133)	5.06 (0.079)	0.201	<0.001	0.897
Protein (%)	3.39 (0.063)[b]	3.52 (0.041)[b]	3.63 (0.025)[a]	<0.001	<0.001	0.624
F:P	1.59 (0.056)[a]	1.48 (0.037)[ab]	1.40 (0.022)[b]	0.004	0.925	0.729
C4:0 (g/kg milk)	2.12 (0.090)	2.07 (0.059)	1.99 (0.035)	0.278	<0.001	0.873
C16:0 (g/kg milk)	13.1 (0.658)	13.3 (0.258)	13.3 (0.426)	0.972	0.003	0.708

[a,b]Within a row, mean values with a different superscript among metabolic clusters differ significantly ($P < 0.05$).

[1]All parameters were quantified by FTIR. Clusters were compared for all parameters using mixed model analysis. Averages over the 3 wk in milk are reported, as no interaction effect between metabolic cluster and week in milk were observed. Mean and SE (in parentheses) of parameters per cluster are reported.

[2]QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving; F:P = fat-to-protein ratio.

with the other 2 clusters. However, in week 2 and week 3, the UFA and MUFA proportions in milk fat were not different between QuiBHB and SloBHB cows. During week 2, we found a tendency for higher UFA proportion ($P = 0.086$) in QuiBHB cows, and across all weeks, the QuiBHB cows tended to have greater proportions of MUFA compared with SloBHB cows ($P = 0.098$, 0.066, and 0.093 for weeks 1, 2, and 3, respectively). The concentration of total C18:1 FA was highest among QuiBHB cows during week 1, but no difference was observed between SloBHB and LoBHB cows. In week 2, the LoBHB cows displayed the lowest proportion of total C18:1, whereas no variation was observed between the other 2 clusters. In week 3, the QuiBHB cows exhibited higher total C18:1 concentrations compared with the LoBHB cows. Additionally, during the same period, we found a tendency for SloBHB cows to have higher total C18:1 concentrations ($P = 0.092$) in comparison to the LoBHB group.

Dry Matter Intake

Figure 5 illustrates the daily DMI of cows in the 3 metabolic clusters for the first 30 DIM, along with the

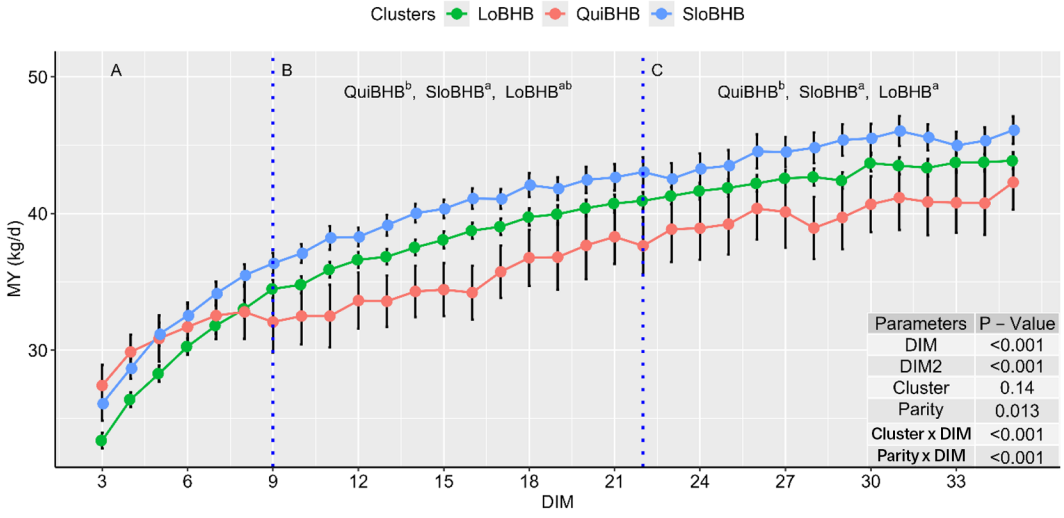

Figure 4. Mean and SE of the daily milk yield of cows in the 3 metabolic clusters identified by growth mixture models using the trajectories of BHB. A mixed model analysis was done to describe the association between DIM, clusters, parity, and their interaction. Clusters were compared for milk yield following a spotlight analysis, and different superscripts (a,b) represent differences between clusters in milk yield per day ($P < 0.05$). In this respect, 3 periods could be distinguished: A (3–15 DIM) without significant cluster differences, B (16–24 DIM), and C (25–35 DIM), each with differences between clusters as indicated by different superscript letters associated with the cluster names. The blue dots separate periods A, B, and C. QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

Table 3. Summary of milk parameters for metabolic clusters identified using the trajectories of blood BHB concentration[1,2]

Parameter	Week 1 (3–9 DIM)			Week 2 (10–16 DIM)			Week 3 (17–24 DIM)			*P*-value			
	QuiBHB	SloBHB	LoBHB	QuiBHB	SloBHB	LoBHB	QuiBHB	SloBHB	LoBHB	C	W	P	C × W
MY (kg/d)	32.4 (0.65)	31.2 (1.10)	30.3 (0.65)	34.4 (1.68)^{b}	39.4 (1.09)^{a}	37.8 (0.65)^{ab}	36.6 (1.68)^{b}	41.3 (1.10)^{a}	40.4 (0.65)^{ab}	0.272	<0.001	0.148	<0.001
FPCM (kg/d)	42.0 (2.04)^{a}	38.1 (1.35)^{ab}	36.4 (0.81)^{b}	40.0 (2.08)	44.3 (1.35)	42.3 (2.04)	37.9 (2.12)^{b}	45.3 (1.36)^{a}	43.7 (0.81)^{a}	0.310	<0.001	0.205	<0.001
C18:0 (g/kg milk)	9.13 (0.410)	7.09 (0.270)	6.69 (0.163)	6.89 (0.421)	6.46 (0.273)	5.75 (0.158)	6.11 (0.421)	5.09 (0.273)	4.82 (0.162)	<0.001	<0.001	0.734	0.073
UFA (g/kg milk)	26.6 (1.13)^{a}	21.1 (0.75)^{b}	19.7 (0.45)^{b}	20.5 (1.16)^{a}	18.3 (0.75)^{ab}	16.7 (0.44)^{b}	17.9 (1.16)^{a}	16.6 (0.75)^{ab}	15.2 (0.45)^{b}	<0.001	<0.001	0.287	0.043
MUFA (g/kg milk)	24.5^{a}	19.4	18.0^{b}	18.9	16.8	15.1	16.4	15.3	13.8	<0.001	<0.001	0.260	0.040
Total C18:1 (g/kg milk)	(1.05) 22.3 (0.96)^{a}	(0.69)^{b} 17.3 (0.63)^{b}	(0.41) 16.0 (0.38)^{b}	(1.07)^{a} 17.1 (0.97)^{a}	(0.69)^{ab} 15.1 (0.63)^{a}	(0.40)^{b} 13.4 (0.63)^{b}	(1.07)^{a} 14.8 (0.97)^{a}	(0.69)^{ab} 13.4 (0.63)^{ab}	(0.41)^{b} 12.1 (0.38)^{b}	<0.001	<0.001	0.221	0.035

[a,b]Mean values bearing a different superscript within the same week differ significantly ($P < 0.05$).
[1]Milk composition parameters are quantified by FTIR. Clusters were compared using the mixed model analysis. Mean and SE (in parentheses) of parameters per cluster are reported.
[2]QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving; MY = milk yield; FPCM = fat- and protein-corrected milk yield; C = metabolic clusters; W = week; P = parity.

results of the mixed effect model analysis, which considered DIM, parity, and their interaction as explanatory factors for DMI. As expected, DMI per day increased in all metabolic groups. However, QuiBHB cows had a lower DMI during the first 35 DIM, whereas SloBHB and LoBHB cows showed comparable DMI. The interaction effect between cluster and DIM was observed in the case of LoBHB and SloBHB cows, with the latter gradually exhibiting slightly higher DMI after the fourth week of lactation.

Prediction of Metabolic Clusters

The predictions of metabolic clusters were explored for each cluster separately and for all clusters together following a one-versus-all and multiclass classification approaches respectively (Figure 6). The models built to predict the QuiBHB cows had higher accuracy in terms of area under the receiver operating characteristic curve (**ROC_{AUC}**) than the other 2 clusters at all weeks of sampling. The models built using week 1 data performed better than the other 2 wk in QuiBHB and SloBHB cows predictions, whereas in the prediction of LoBHB cows, week 1 and week 2 data models performed equally and better than the week 3 data. The LoBHB cows' prediction models were more sensitive, whereas the QuiBHB cows prediction models were more specific than the other clusters' prediction models. The multiclassifcation model metrics are the average of each class of prediction metrics from the synthetic minority oversampling technique- (**SMOTE**) balanced data modeled using the random forest algorithm. Its overall ROC_{AUC} was better in all weeks than the one-to-all models of LoBHB and SloBHB, whereas it was lower than the QuiBHB models. Furthermore, the multiclass classification model was also least sensitive.

DISCUSSION

The objectives of this research were to investigate both intercow and intracow variations in the response of BHB during early lactation and to determine if intercow variations in the intracow variation (trajectory) of BHB could serve as indicators for identifying distinct metabolic groups. To achieve this, we employed a trajectory clustering method to unveil latent metabolic groups characterized by unique profiles of blood BHB over time. Our findings offer novel insights into the diverse metabolic responses among cows, as manifested by both the trajectory and magnitude of blood BHB concentrations. In particular, we identified 2 specific groups of cows (QuiBHB and SloBHB) that exceeded the blood BHB threshold of 1.2 mmol/L, which is a critical value associated with diagnosing subclinical ketosis (**SCK**) or

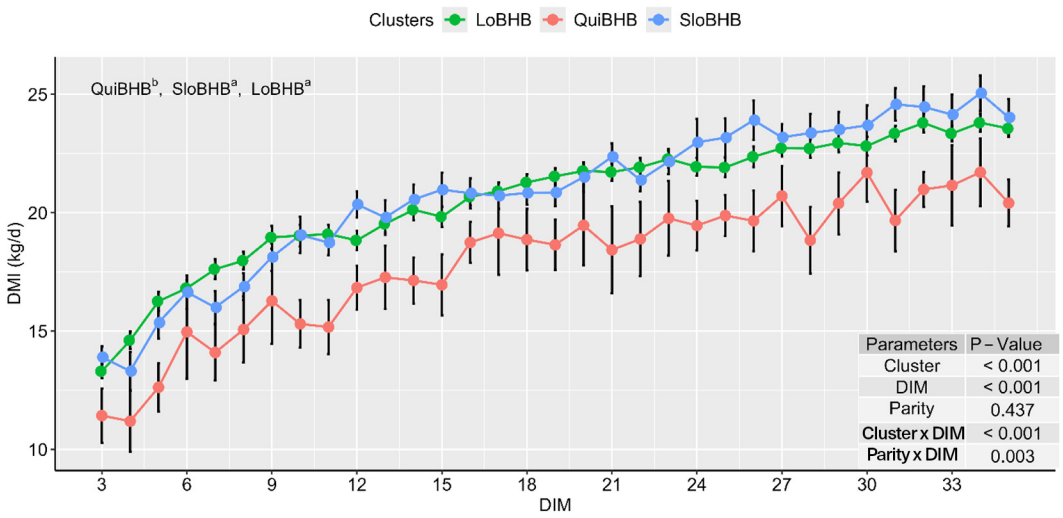

Figure 5. Mean and SE of the daily DMI intake of cows in the 3 metabolic clusters identified by growth mixture models using the trajectories of BHB. A mixed model analysis was done to describe the association between DIM, clusters, parity, and their interaction. Clusters were compared for feed intake following a spotlight analysis, and different superscript letters (a,b) associated with the cluster names represent differences in DMI per day ($P < 0.05$) throughout the entire period. QuiBHB = clusters of cows with quickly increasing BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving.

HYK (LeBlanc et al., 2005; Duffield et al., 2009). It is noteworthy that these groups differed in 2 key aspects: (1) the timing at which they surpassed the threshold, and (2) the extent of their blood BHB elevation. The QuiBHB group demonstrated an immediate elevation of mean BHB concentration that continuously exceeded the threshold in the current sampling period. The mean BHB concentrations at 3, 6, 9, and 21 DIM were 1.26, 2.24, 2.41, and 1.95 mmol/L, respectively. On the other hand, the SloBHB cows only crossed the threshold from 9 DIM onward. The mean BHB concentration at 3, 6, 9, and 21 DIM were 0.88, 1.06, 1.32, and 1.48 mmol/L, respectively. An important implication of this variation is that early sampling (before 6 DIM) would most probably detect QuiBHB cows as having SCK, whereas the condition would remain unnoticed in SloBHB cows. However, somewhat later sampling (e.g., from the second week in lactation onwards) would result in both groups being identified as hyperketonemic, as their mean BHB concentrations would surpass the threshold. In view of this, threshold-based classification using a single measurement of blood BHB might not be efficient in identifying these metabolic groups. This could also be associated with the conflicting reports on the association between HYK status and downstream production and reproduction performance.

Nevertheless, these distinct metabolic groups exhibited discernible physiological differences. The observed variations in blood NEFA and insulin concentrations could potentially mirror the diversity in body reserve mobilization and energy allocation among the distinct metabolic groups. The variation in the insulin concentration among clusters is inversely related to the concentration of ketonemia among the clusters. This is concordant with previous reports (Hove, 1978; Brockman, 1979), but it is in disagreement with studies that reported an association between hyperinsulinemia and hyperketonemia (Holtenius and Holtenius, 1996; Herdt, 2000). Beyond variations in blood metabolites within the initial 9 DIM, intriguingly, they also diverged in terms of milk yield and DMI. Although QuiBHB cows commenced with comparable milk production to the other groups, their rate of increase in milk production seemed to decelerate, which was likely linked to their lower rate of DMI. Although in all groups DMI increased with time after calving, as expected (Bell, 1995), the QuiBHB cows exhibited the lowest DMI per day during the first 35 DIM which aligns with previous reports on a negative association between elevated blood BHB concentrations and DMI (Ingvartsen, 2006; Allen, 2020). However, despite their different blood BHB and NEFA concentrations, the SloBHB and LoBHB clusters did not differ in DMI. These results support the recent argument by Horst et al. (2021) regarding the absence of a direct inhibitory effect of these metabolites on feed intake. Accordingly, the negative association between elevated blood BHB and reduced DMI in the QuiBHB group may be indirect.

The blood BHB-based trajectory analysis allowed us to classify cows into 3 presumably physiologically relevant classes of heterogeneous metabolic adaptation. The

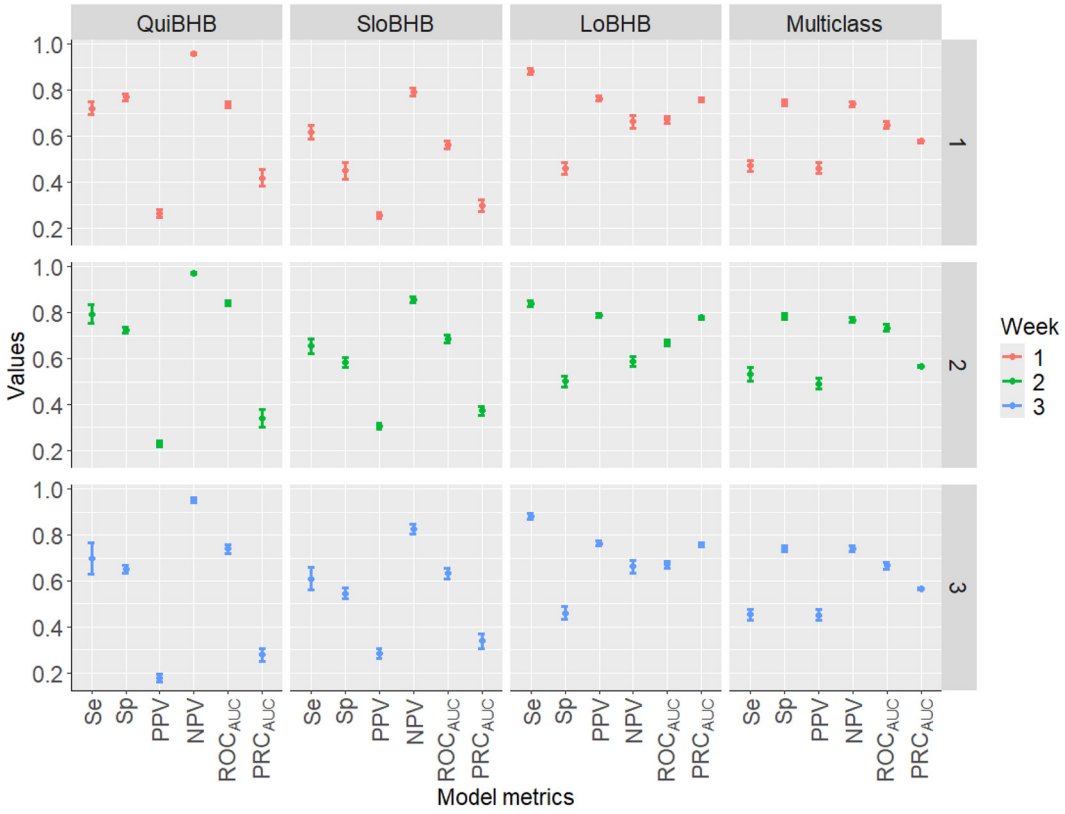

Figure 6. Performance metrics (mean of the 100 times repeated 5-fold cross-validations and across each of the classes for the multiclass prediction model) of metabolic cluster prediction models. These models used milk fat, protein, BHB, and fatty acids quantified based on FTIR, alongside DIM and parity as predictors. Employing a one-to-all approach, separate models were constructed for each cluster. Underbagging with random forest as a weak learner was applied for building prediction models of QuiBHB and SloBHB cows, whereas a random forest algorithm with no imbalanced data management techniques was employed for LoBHB cows. The multiclass prediction model was developed using the random forest algorithm, with the synthetic minority oversampling technique used to balance the data. QuiBHB = clusters of cows with quickly increasing blood BHB after calving which remained elevated for the first 3 wk; SloBHB = clusters of cows with slowly increasing blood BHB observed in the first 3 wk after calving; LoBHB = clusters of cows with low and stable blood BHB in the first 3 wk after calving; Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; PRC_{AUC} = area under the precision receiver curve; ROC_{AUC} = area under the receiver operating characteristic curve. The vertical bars on the dots represent the error bar (SE) of each metric.

metabolic cluster that mobilized high body reserves and had elevated BHB immediately after calving (QuiBHB) could be considered metabolically less adapted or even impaired, as these cows were also characterized by lower DMI. Physiologically, the combination of low DMI and a higher rate of lipid mobilization could lead to limited availability of intermediates of the Krebs cycle (e.g., oxaloacetate) for complete NEFA oxidation in the liver, resulting in elevated ketone levels and probably TAG accumulation in the liver. These factors could have contributed to the lower milk yield in these cows. Interestingly, the LoBHB cows produced a comparable amount of milk per day to the SloBHB cows and even outperformed the QuiBHB cows. This could be attributed to the fact that DMI for these cows was not constrained and was comparable with the SloBHB cows, which mobilized more body reserves as compared with the LoBHB group. This could support the new perspective on the impact of hyperketonemia on the metabolic health status of the cows (Horst et al., 2021) that the (longer term) animal's response in terms of milk yield and DMI could further shed light whether a metabolic profile indicates an impaired or adapted metabolic status. Moreover, despite the fact that the milk yield per day did not differ significantly between the SloBHB and the LoBHB group, the milk yield was consistently numerically ~1 kg/d higher and FPCM yield ~1.5 kg/d higher from the second week in lactation onwards, which could suggest hyperketonemia as an adaptive mechanism in these cows.

Differences among groups in the occurrence of disease may have contributed to the difference in MY and DMI observed among metabolic clusters. Indeed, within the QuiBHB group, 52.6% were diagnosed with 1 or a combination of ailments, in contrast to proportions of 24.4% for SloBHB cows and 17.6% for LoBHB cows. The primary clinical diseases considered in this summary

encompass ketosis, hypocalcemia, displaced abomasum, mastitis, and uterine discharge observed during the first 45 d after calving (Supplemental Table S3, see Notes; Girma et al., 2024a). In a preliminary finding from the pairwise comparison of these metabolic groups concerning inflammatory biomarkers, an elevated systemic inflammatory status was noted in QuiBHB cows compared with the other groups (Supplemental Figure S1, see Notes; Girma et al., 2024b). The figure presents the comparison of the groups with regard to acute-phase proteins, haptoglobin, serum amyloid A, and the ratio of albumin to globulin. Haptoglobin and serum amyloid A in blood were higher in the QuiBHB compared with the SloBHB and LoBHB cows, indicating higher systemic inflammation (Ceciliani et al., 2012; LeBlanc, 2012). On the other hand, in view of the hepatic oxidation theory, the BHB production in the liver of the SloBHB group could have helped to divert NEFA away from the Krebs cycle, thereby preserving glucose for essential metabolic processes and preventing a reduction in DMI. Within his hepatic oxidation theory, Allen (2020) suggested that BHB serves as an adaptive mechanism to prioritize energy allocation in the body, particularly in situations where maintaining DMI is crucial.

Accordingly, the increased blood BHB concentration of the SloBHB group consistently demonstrated higher milk production coupled with increased DMI as compared with the QuiBHB group. These cows seem to be what Baumgard (2023), in a webinar presentation, referred to as "champion" cows, which eat well and produce well. Hence, their higher concentration of BHB (exceeding 1.2 m*M*) could be an adaptive response. However, despite Baumgard's speculation of higher insulin concentrations in metabolically impaired cows compared with those using BHB production as an adaptive response, these cows demonstrated the lowest insulin concentration. In early lactation, cows experience physiological adaptation geared toward preserving glucose for the energy-intensive milk production process. These adaptations coordinate the preference of peripheral tissues for NEFA and BHB as their primary energy sources, a phenomenon rigorously controlled through homeorhetic mechanisms (Bauman, 2000). Previous studies have reported that the effect of high blood BHB concentration on milk yield is more pronounced when HYK is detected during the first week compared with the second week of lactation (Duffield et al., 2009; Chapinal et al., 2012; McArt et al., 2012). Our findings imply that this distinction may be related to the specific metabolic group from which cows were identified as having HYK.

The metabolic clusters also exhibited variations in the concentrations of UFA, MUFA, and total C18:1 FA. Particularly, during the initial week of lactation, the QuiBHB cows displayed the highest concentrations of UFA, MUFA, and total C18:1 FA. In the subsequent second and third weeks, the disparity in milk FA between the QuiBHB and SloBHB groups narrowed, whereas the difference with the LoBHB cows remained consistent. These observed distinctions in UFA, MUFA, and total C18:1 FA could potentially be linked to variations in body fat mobilization, as indicated by blood NEFA concentrations. In early lactation, prominent NEFA released from body fat reserves include C16:0, C18:0, and C18:1 *cis*-9 (Hostens et al., 2012). Furthermore, the possible conversion of C18:0 to C18:1 *cis*-9 within the mammary gland via Δ9-desaturase action might affect the proportion of these FA in milk fat (Leroy et al., 2005). Correspondingly, earlier investigations have demonstrated elevated proportions of long-chain FA, notably C18:1 *cis*-9, in cows diagnosed with subclinical ketosis (Van Haelst et al., 2008; Jorjong et al., 2015).

The difference in milk FA between QuiBHB and other cows in early lactation is of particular interest because, notably, QuiBHB cows seem to exhibit an impaired metabolic status and a diminished adaptive response. Consequently, these cows warrant focused attention, and the identification of such cows through easily accessible milk biomarkers is of paramount importance. Given the potential of milk FA to predict the metabolic status of cows in early lactation, we explored the feasibility of predicting the metabolic clusters using milk fatty acids in conjunction with additional test day variables. It is important to note that the sample size in this study is limited for constructing robust prediction models, and the exploration is primarily intended to assess the identifiability of these clusters using milk biomarkers. The results indicated the potential of milk FA along with test day variables to predict cows with impaired metabolic status (QuiBHB cows). This prediction was achieved with moderately high accuracy (Greiner et al., 2000). The accuracy of the prediction models utilizing data from week 1 and week 2 were superior to models using week 3 data. The accuracies of the SloBHB and LoBHB cows' prediction models were not high for accurate diagnosis. This, apart from the small sample size, could be associated with the comparable milk FA profile of these 2 clusters, suggesting that FA-based models may have limitations in effectively distinguishing between these 2 clusters. Indeed, the performance of LoBHB prediction models might also be affected by the imbalanced proportion in the number of cows between the LoBHB cluster and the other 2 clusters combined. As the imbalanced data management techniques (e.g., underbagging) target minority groups, we did not apply these techniques in the models for LoBHB cows. The multiclass classification model was more specific than the other models but it was less sensitive and have lower overall accuracy. Indeed, this could be associated with the difference in the im-

balanced data management techniques. Particularly, the lower Se and ROC_{AUC} values of the multiclass classification indicated the lower performance of the SMOTE technique as compared with underbagging to balance metabolic groups' data. In our previous report, we also reported models built using underbagging techniques as our preliminary exploration proved its superior performance among available imbalanced data management techniques (Girma et al., 2023).

Obviously, identification of attention cows early in lactation only is of importance if a treatment could be applied. Oral administration of propylene glycol (**PG**) has demonstrated effective resolution of HYK and improved milk yield in certain cases (McArt et al., 2011; Lomander et al., 2012). However, the results of PG administration have not been consistently positive, as evidenced by its lack of effect in other instances (Østergaard et al., 2020; Capel et al., 2021). These contradictory results could be related to the existence of 2 types of HYK animals, as suggested from this experiment. The rationale behind PG administration for HYK cows is that on one hand, it stimulates gluconeogenesis by directly entering the TCA cycle to enhance acetyl CoA oxidation, and on the other hand, the propionate in PG stimulates insulin release, subsequently suppressing fat mobilization and thereby reducing ketone bodies in the bloodstream. In light of this, QuiBHB cows might benefit from PG administration due to their lower feed intake, indicative of potential low blood glucose concentrations, which was further reflected in their reduced milk production rates. However, PG administration might not be advantageous for SloBHB cows; in fact, it even could have negative effects, considering that their HYK status might be an adaptive response aimed at conserving glucose for milk synthesis. Moreover, PG administration could be speculated to include a risk for detrimental effects on intake by SloBHB cows in line with the hepatic oxidation theory (Allen, 2020). According to this theory, adenosine triphosphate production from glucose oxidation in the TCA cycle, potentially increased by PG administration, could lead to decreased feed intake, triggering body fat mobilization and increased ketone bodies.

A limitation of the current study is that the association of precalving BCS with the identified trajectory clusters could not be systematically investigated. During the experiments, BCS was recorded using a camera from DeLaval (Tumba, Sweden). However, a significant amount of data were missing before calving due to logistical challenges, as dry cows did not frequently pass the camera in the stable. However, each cow's mean BCS was computed for the first 3 d after calving, and metabolic clusters were compared for their mean BCS during this period. The mean BCS did not vary among metabolic clusters (Supplemental Table S4, see Notes; Girma et al., 2024a). However, it was apparent that QuiBHB cows were either well-conditioned (BCS >3.0) or over-conditioned (BCS ≥4.0), whereas (in contrast to other clusters) this cluster did include under-conditioned cows (BCS <3.0; Supplemental Figure S2, see Notes; Girma et al., 2024b). This observation aligns with previous reports where higher BCS is associated with hyperketonemia (e.g., Duffield, 2000). Nevertheless, it should be noted that the number of over-conditioned cows was very small (≈3%) in the current experiment.

Overall, our research sheds light on the intercow and intracow variations in metabolic responses during early lactation and demonstrates the potential for clustering cows into different metabolic groups based on these variations. In particular, identified metabolic groups were shown to have variations in milk performance, blood metabolites, DMI, and body reserve mobilization. However, in the context of the current dataset, it is important to note that not only did the trajectories differ between the QuiBHB and SloBHB groups, but also the BHB concentration. Accordingly, this warrants further research to elucidate whether the difference in trajectory serves as a critical factor distinguishing these 2 groups. Because the number of cows observed in this study was relatively low, we were not able to include additional risk factors for elevated BHB to further characterize the metabolic clusters. Our research relied on blood samples collected frequently during the first 9 DIM with additional samples intended to characterize the end of the transition period (21 DIM). Future research might include additional sampling between 9 and 21 DIM and probably before calving to fully elucidate the trajectories of metabolic markers during the transition period. Characterizing the metabolic clusters with disease prevalence and reproductive performance is also worth further consideration using larger datasets.

CONCLUSIONS

In this study, cows in early lactation were clustered into 3 metabolic groups using the time profile of blood BHB concentration. A trajectory clustering approach was employed to cluster cows based on the intercow and intracow variation of blood BHB in repeated measures. The resulting metabolic clusters exhibited notable differences in blood metabolites, milk composition, fatty acid composition in milk fat, milk yield, DMI, and disease occurrence. These variations are indicative of the variation in body fat mobilization and energy partitioning among physiological needs, which play pivotal roles in the adaptive response of high-yielding cows for metabolic stress in early lactation. Beyond the dissimilar trajectories of blood BHB, noteworthy variations were evident in the actual concentrations of blood BHB. Addressing these

distinctions requires further investigation to unravel their individual or combined significance in relation to the health, productivity, and reproductive outcomes of the cows. Identification of metabolic clusters was feasible using milk parameters and test day variables.

NOTES

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Nonstandard abbreviations used: AMS = automatic milking system; DVE-OEB = intestinally digestible protein-rumen undegradable protein balance-system; F:P = fat-to-protein ratio; FA = fatty acid; FPCM = fat- and protein-corrected milk; FTIR = Fourier-transform infrared spectroscopy; GMM = growth mixture model; HYK = hyperketonemia; LoBHB = low BHB; MY = milk yield; NEFA = nonesterified fatty acid; NPV = negative predictive value; PG = propylene glycol; PMR = partial mixed ration; PPV = positive predictive value; PRC_{AUC} = area under the precision receiver curve; QuiBHB = quickly increasing BHB; RIC = roughage intake control; ROC_{AUC} = area under the receiver operating characteristic curve; SCK = subclinical ketosis; Se = sensitivity; SloBHB = slowly increasing BHB; SMOTE = synthetic minority oversampling technique; Sp = specificity; TAG = triglyceride; TCA = tricarboxylic acid cycle.

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