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ORIGINAL ARTICLE

Identification of genetic markers associated with hyperketonemia patterns in early lactation Holstein cows

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Abstract

Ketosis, evidenced by hyperketonemia with elevated blood β-hydroxybutyrate (BHB) levels, is a significant metabolic disorder of dairy cattle, typically diagnosed within the first 6 weeks post-calving when high energy levels are essential to milk production. Our study aimed to identify genetic markers linked to hyperketonemia (HYK) patterns in Holstein cows during early lactation and compare these to HYK-negative cows. We screened 964 cows for HYK using a threshold of BHB ≥1.2 mmol/L during the first 2 weeks postpartum (screening period, SP). Cows that tested negative initially were retested the following week. Cows were deemed HYK-negative (CON group) if BHB levels were below 1.2 mmol/L in both tests, while those with BHB levels exceeding this threshold at any test were treated and classified as HYK-positive (HYK+). Post-treatment, HYK+ cows were monitored for two-week follow-up period (FP) and classified based on their recovery: cured (CUR; consistently low BHB), recurrent (REC; fluctuating BHB levels), severe (SEV; high initial BHB that decreased), or chronic (CHR; persistently high BHB). Using 489 cows that were genotyped, a GWAS was conducted using GCTA software, revealing significant associations of several SNPs across different HYK patterns when compared to the CON group. These SNPs were primarily linked to genes affecting milk traits and were enriched in biological pathways relevant to protein glycosylation, inflammatory response, glucose homeostasis, and fatty acid synthesis. Our findings highlight genomic regions, potential candidate genes, and biological pathways related to ketosis, underscoring potential targets for improving health management in dairy cattle. These insights could lead to better strategies for managing ketosis through genetic selection, ultimately enhancing dairy cattle welfare and productivity. Further research with a larger number of cows is recommended to validate these findings and help confirm the implicated SNPs and genes.

Flavio Schramm Schenkel and E. James Squires share the senior authorship.

KEYWORDS
dairy cattle, metabolic disorder, SNP, β-Hydroxybutyrate

1 | INTRODUCTION

Ketosis, evidenced by hyperketonemia with elevated blood β-hydroxybutyrate (BHB) concentration, is an important metabolic disorder of dairy cows that may present clinically or subclinically. It arises from a metabolic disorder most likely to occur within the first 6 weeks after calving (Dobbelaar et al., 1996) when cows require high-energy levels to satisfy milk production requirements (Gordon et al., 2013). To meet the energy requirements for milk production, cows can use two energy sources: feed intake and body reserves. During early lactation, the energy intake is insufficient to meet the energy output for milk production. Although considered part of a normal metabolic adaptation to lactation, high-yielding cows face energy deficit in early lactation (Civiero et al., 2021). As part of the homeorhetic adaptation to lactation, cows respond to the energetic deficits by mobilizing adipose tissue through lipolysis, which increases circulating fatty acid concentrations that can overload the oxidative capacity of the liver (Loiklung et al., 2022). This process results in the formation of ketone bodies that, despite being an important source of energy, in excess can result in hyperketonemia (HYK, blood β-Hydroxybutyrate (BHB) concentration ≥1.2 mmol/L) (Capel et al., 2021). Ketosis has been a concern in dairy production, due to its negative association with cow health and productivity, leading to significant economic losses (Soares, Vargas, Duffield, et al., 2021; Walsh et al., 2007). Studies have reported an average incidence of 43% of HYK (defined as BHB concentration ≥1.2 mmol/L with concurrent clinical signs) in North American herds within the first 2 weeks of lactation. This can result in losses of $289 per ketosis case (McArt et al., 2015) due to reduced milk production and greater risk of displaced abomasum, reduced fertility, and increased culling risk (Duffield et al., 2009; Mostert et al., 2018; Roberts et al., 2012).

Hyperketonemia is a multifactorial condition, it is potentially influenced by genetic factors (Ghavi, 2022; Heringstad et al., 2005; Soares, Vargas, Muniz, et al., 2021), and also linked to nutritional management and environmental stress (e.g., adverse temperatures, feed or water access, stress, poor feed) (Antanaitis et al., 2021; Serrenho et al., 2023; Shahzad et al., 2019). Within the same environment and management, some cows adapt better than others to the great energy demand experienced in early lactation (Kessel et al., 2008). Genetic differences may explain the distinct ability to maintain high milk yield without detrimental outcomes (Brito et al., 2021). Previous studies reported clinical ketosis heritability estimates ranging from 0.02 to 0.16 (Heringstad et al., 2005; Mäntysaari et al., 1991; Oliveira-Junior et al., 2021), and 0.17 to 0.36 for concentrations of plasma (van der Drift et al., 2012) or blood BHB (Belay et al., 2017; Benedet et al., 2020) as a lowly to moderately heritable trait; this suggests that the risk of this disease can be decreased by genetic selection. Furthermore, clinical ketosis (defined by BHB ≥1.2 mmol/L with concurrent clinical signs) shows a moderate genetic correlation (0.47–0.48) with blood BHB concentration trait (Belay et al., 2017; Koeck et al., 2014) and high correlation (0.71 to 0.99) with milk BHB (Klein et al., 2020). These estimates suggest that the incidence of HYK can be reduced by genetic selection, using blood or milk BHB concentration as an indicator trait for HYK (Gebreyesus et al., 2020; Ježek et al., 2017).

While the broad range of heritability estimates underscores the challenge of accurately assessing phenotypes due to variability in diagnostic protocols, protocol compliance, recording procedures, and generalization of phenotypes (Pralle et al., 2020), our study is specifically focused on investigating genetic markers associated with hyperketonemia (HYK) patterns in dairy cows. By understanding the transition period challenges and genetic mechanisms related to cows' response to HYK treatment during early lactation. Therefore, the specific objectives of this study were (1) to observe the dynamic of BHB concentration (mmol/L) among HYK-positive (HYK^+; cows with BHB concentration ≥1.2 mmol/L at the first or second SP assessments) groups in the two-weeks *postpartum* and two-weeks after HYK diagnosis and treatment; and (2) to investigate genetic markers associated with different HYK patterns in treated cows compared to HYK-negative cows. We hypothesized that genetic markers are associated with different patterns of HYK and blood BHB concentration.

2 | MATERIALS AND METHODS

2.1 | Data set description and analytical approach

In the current study, we used a dataset that was generated through a randomized controlled trial (RCT; May to July 2019) aiming at evaluating the effect of adding dextrose to a HYK treatment protocol, which used propylene glycol (PG) solution (details are provided in Capel et al., 2021). Briefly explaining the RCT, a total of 964 dairy cows

(Table 1) from four commercial farms located in New York, USA, were screened for blood BHB concentration in the first 2 weeks postpartum (3–16 DIM). Blood samples were collected from the coccygeal vein and tested cow-side with a hand-held ketone meter (Nova Vet Ketone/Glucose Meter; Nova Biomedical, Waltham, MA, USA). All HYK-positive cows (HYK^+; defined as a blood BHB concentration ≥1.2 mmol/L) were assigned to 1 of 3 treatment groups following a RCT (Capel et al., 2021), where groups received one of the 3 treatments: (1) 300 mL of a 100% PG solution (100% propylene glycol, USP/EP; Dow, Midland, MI) administered orally once daily for 3 days; (2) 300 mL of a 100% PG solution administered orally once daily for 3 days + 500 mL dextrose solution (dextrose 50% injection, VetOne, Boise, ID) administered i.v. on day 1 only; or (3) 300 mL oral propylene glycol once a day for 3 days +500 mL 50% dextrose once a day for 3 days. HYK^+ cows had their BHB concentration reassessed in the two subsequent weeks after diagnosis/treatment (one assessment per week). At the end of the trail, no significant differences among treatments were observed for the average BHB concentration of HYK^+ cows (Capel et al., 2021).

In the current study, cows that were screened as negative for ketosis, evidenced by no HYK in both the first (3–9 DIM) and second week of lactation (10–16 DIM) of the Screening Period (SP) in Capel et al. (2021), i.e., with blood BHB concentration <1.2 mmol/L, were categorized as controls (CON; HYK-negative cows). As described above, when diagnosed as HYK positive (HYK^+; concentration ≥1.2 mmol/L), cows were treated with one of the 3 treatment protocols and had their BHB concentration reassessed in the two subsequent weeks after diagnosis/treatment (follow-up period, FP). As there were no significant differences between the HYK treatments at the end of the trail, in the current study individual patterns of HYK were created for all HYK^+ cows, regardless their treatment, based on the presence of HYK in the 2 weeks of the FP. HYK^+ cows were then classified as cured (CUR; BHB <1.2 mmol/L in both FP tests), recurrent (REC; BHB <1.2 mmol/L in first FP test and ≥1.2 mmol/L in the second FP test), severe (SEV; BHB ≥1.2 mmol/L in first FP test and <1.2 mmol/L in the second FP test), or chronic (CHR, BHB ≥1.2 mmol/L in both FP tests; Table 1), which were used in the analyses.

Descriptive statistics for BHB concentrations across HYK pattern groups (CUR, SEV, CHR, REC) and control (CON) were performed in R (version 4.3.1) using "CAR" (Fox & Weisberg, 2019) and "FSA" packages. Data normality was verified through the Shapiro test ($\alpha = 0.05$) and QQ-plot, which showed significant violation of normality of the data ($p < 0.05$). Therefore, the Kruskal–Wallis test (Kruskal & Wallis, 1952), a nonparametric one-way ANOVA on ranks, was used to test for differences in BHB concentrations across HYK pattern groups and specific group pairs were compared by Dunn's test (Dunn, 1964) adjusting for multiple comparisons via false discovery rate (FDR) (Benjamini & Hochberg, 1995) at a 5% level. The formula to calculate the z-test statistic for the difference between two groups is (Dunn, 1964)

$$z_i = y_i / \sigma_i$$

where i is one of the 1 to m comparisons, $y_i = W_A - W_B$ (where W_A is the average of the sum of the ranks for the ith group) and σ_i is calculated as:

$$\sigma_i = \sqrt{\left(\frac{N(N+1)}{12}\right) - \left(\sum T_s^3 - \frac{T_s}{(12(N-1))} / \left(\frac{1}{n_A}\right) + \left(\frac{1}{n_B}\right)\right)}$$

TABLE 1 Holstein cows were included in an observational study to assess hyperketonemia (HYK) patterns in the 2 weeks followed by diagnosis and treatment of the condition and their association with genetic markers.

Patterns	Cows *n* (%)	Timing of HYK diagnosis (%)		No. and proportion of genotyped animals
		1st week postpartum	2st week postpartum	
HYK-positive[a]	354 (36.7%)	232 (65.5%)	122 (34.5%)	280 (79.1%)
CUR[b]	145 (41.0%)	90 (62.0%)	55 (38.0%)	109 (75.1%)
REC[c]	40 (11.3%)	25 (62.5%)	15 (37.5%)	32 (80.0%)
SEV[d]	65 (18.3%)	42 (64.6%)	23 (35.4%)	56 (86.1%)
CHR[e]	104 (29.4%)	75 (72.1%)	29 (27.9%)	83 (79.8%)
HYK-Negative[f]	610 (63.3%)	–	–	209 (34.3%)
Total	964	–	–	489

[a]Cows that presented blood β-hydroxybutyrate (BHB) concentration ≥1.2 mmol/L in the first 2 weeks postpartum (3–9 and 10–16 DIM; screening period, SP).

[b]Cured (BHB <1.2 mmol/L in both follow-up (FP) tests).

[c]Recurrent (BHB <1.2 mmol/L in first FP test and ≥1.2 mmol/L in the second FP test).

[d]Severe (BHB ≥1.2 mmol/L in first FP test and <1.2 mmol/L in the second FP test).

[e]Chronic (BHB ≥1.2 mmol/L in both FP tests).

[f]Cows that presented BHB concentration <1.2 mmol/L in both SP assessments, and were categorized as the control group (CON).

where N is the total number of observations across all groups, n_A and n_B are the number of observations in the two groups being compared, and T_s is the number of observations tied at the sth specific tied value.

2.2 | Genotypic data, imputation, and quality control

Hair samples were collected for DNA extraction from 638 (54.2% HYK$^+$; 45.8% CON) of the 964 (36.7% HYK$^+$; 63.3% CON) phenotyped cows (Table 1). Although all HYK$^+$ cows had their hair samples collected at the time of RCT allocation, CON cows' samples were collected 18 months later for genotyping. The 638 hair samples were genotyped using an Illumina Bovine 50 K BeadChip array (57,798 SNPs, Zoetis, Kalamazoo-USA) and, out of those, 489 (65.7% HYK$^+$; 34.3% CON) passed the Zoetis genotyping quality control and were used for the further analyses.

The genotypes of another 652 non-phenotyped Canadian Holstein cows were available (Soares, Vargas, Muniz, et al., 2021) for this study. These 652 cows were twice genotyped, firstly with a low-density panel (998 SNPs) (Kroezen et al., 2018), and then using an Illumina Bovine 60 K BeadChip array (57,798 SNPs). Both genotypes were combined, merging their information into 61 K file (Soares, Vargas, Muniz, et al., 2021) to be used as a reference population in imputation analysis. The SNPs in the low-density panel (998 SNPs) were previously selected based on their location and functional consequences on genes related to metabolic mechanisms that may be key to the development of ketosis conditions (Kroezen et al., 2018; Soares, Vargas, Muniz, et al., 2021). Thus, the genotypes of the 489 cows (50 K BeadChip array) were imputed to 61 K SNPs using Beagle 5.4 software (Browning et al., 2021).

The genotype quality control was performed for the imputed genotypes using the Plink 1.9 software (Chang et al., 2015), which filtered out markers with minor allele frequency (MAF) lower than 1% and individual SNP or cows with call rates <90%. In addition, SNPs located on sex chromosomes and with undetermined map positions in the reference bovine genome (ARS.UCD1.2) were excluded from the final dataset. A total of 47,343 SNPs remained for GWAS analysis.

2.3 | Genome-wide association analyses

The GCTA (v1.91.4 beta3) software was used to conduct a mixed linear model-based association analysis (MLMA) as in as Yang et al. (2014) and Yang, Lee, et al. (2011) using the following model:

$$y = \mu + f + sy + l + bx + g + e$$

where y is the cow binary phenotypes, which were assigned a value of 0 for CON cows and a value of 1 for either CUR, SEV, CHR or REC cows; μ is the overall mean; f, sy, and l are the fixed effects of farm, season-year of calving, and lactation numbers, respectively; b is the fixed additive genetic effect of the SNP to be tested for the association with y; x is the coded SNP genotype (0, 1 or 2 copies of the reference allele); g is the random polygenic additive genetic effect of the cow, and e is the random error term. The covariance of random polygenic additive genetic effects was assumed to be equal to GRM*σ^2_g, where GRM is the genomic relationship matrix and σ^2_g is the polygenic additive genetic variance. The GRM was calculated following method 1 in VanRaden (2008). For ease of computation, σ^2_g was estimated based on the null model, i.e., $y = \mu + f + sy + l + g + e$, and then kept constant while testing for the association between each SNP and the trait (VanRaden, 2008).

GWAS was performed on binary phenotypes, which were assigned a value of 0 for CON cows and a value of 1 for either CUR, SEV, CHR or REC cows in the following four subsets of data: (1) CUR-CON, (2) SEV-CON, (3) CHR-CON; and (4) REC-CON. For instance, in the case of CUR-CON, CUR cows were set to phenotype 1 and cows CON were set to 0; other pattern cows (REC, CHR, SEV) were not included in the analysis. A 5% FDR threshold for each chromosome was calculated following Benjamini & Yekutieli (2001).

2.4 | Searching for "Linkage Disequilibrium friends"

GCTA (v1.91.4 beta3) software was used to find "Linkage Disequilibrium (LD) friends" (defined as SNPs in significant LD with each of the significant SNPs identified in the GWAS analyses) considering that if a variant is in LD with a causal variant (i.e., they are genetically linked), it may also exhibit an association with the causal. The significance in test statistics is directly related to the strength of LD between the variants and the causal variant (Yang et al., 2011).

A simple regression was used to search for SNPs that were in significant LD with significant SNP in GWAS analysis within a 2.5 Mb genomic window in either direction (Yang et al., 2011). A SNP was accepted as in LD with the targeted SNP if regression coefficient was significant at $p < 0.05$ and model $R^2 > 0.30$, i.e., the SNP

explained more than 30% of the variation of the significant SNP.

2.5 | In silico functional annotation of genomic variation and functional enrichment analysis

The Ensembl Variant Effect Predictor (VEP) software was used to annotate and analyse the predicted effects of SNPs in coding and non-coding regions of the genome. This tool makes use of a machine learning approach to predict the effect of variants based on several factors including protein multiple sequence alignment, sequence- and structure-based features, and conservation across available homologous sequences (Thormann et al., 2019). In addition, we searched for QTL and Gene Ontology (GO) terms associated with our targeted SNPs, and genes that may be locally associated with statistically significant SNPs and their "LD friends" through VEP-Ensembl (https://useast.ensembl.org/info/docs/tools/vep/index.html), considering a 200 kb genomic window (100 kb up and downstream of the variant).

The PANTHER Overrepresentation Test (Released 20,220,712), a GO tool (http://geneontology.org/), was used to perform pathways analyses for gene lists identified for each of the HYK pattern contrasts, as well as for a total gene list identified among all four contrasts using the Reactome pathways database and applying the Fisher's exact statistical test for a threshold $p < 0.05$. However, when no pathways were found using the Reactome database, which is implemented in the PANTHER Classification System, we used the ToppGene suite database, applying a probability density and a 5% FDR threshold. The reason why we chose the GO tool in the first place was that allows us to search for species-specific "Bos Taurus" and ToppGene as the second option because it searches pathways in a larger number of annotations (e.g., Canonical, KEEG, PID, Wiki, Reactome and BioCarta pathways), but applies human genome as main reference. In addition, STRING: functional protein association network (https://string-db.org/) was used to perform protein–protein interaction (PPI) analyses.

3 | RESULTS

3.1 | Hyperketonemia patterns and blood BHB concentration

The population included in our study ($n = 964$) presented an overall 36.7% (HYK $n = 354$) incidence of HYK in the first 2 weeks postpartum, with 65.5% of HYK cows diagnosed in the first week of lactation (3–9 DIM) and 34.5% in the second week (10–16 DIM; Table 1). Overall, the most frequent pattern was CUR (41%). Cows of all HYK patterns were more likely to be diagnosed in the first week postpartum, however, the CHR group had the greatest proportion of cows diagnosed in the first week postpartum (72.1%; Table 1).

Figure 1 shows boxplots for blood BHB concentration (mmol/L) for cows screened (Figure 1a) and reassessed after HYK treatment (Figure 1b) and File S1: Tables S1 and S2 show further data dispersion analysis for both

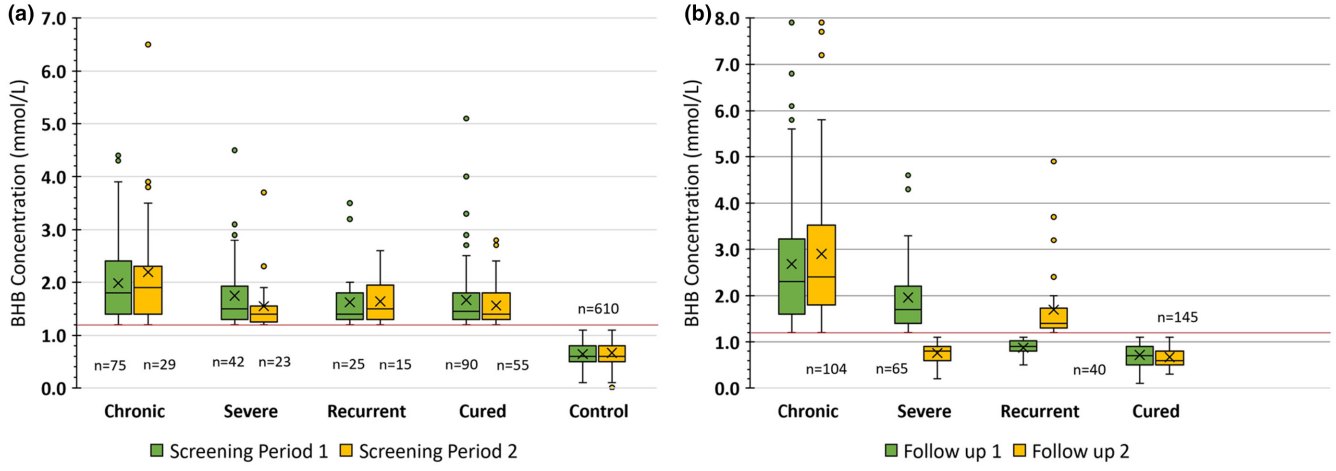

FIGURE 1 Blood BHB concentration dynamics in Holstein cows included in an observational study to assess hyperketonemia (HYK) patterns in the 2 weeks postpartum followed by diagnosis and treatment of the condition. (a) Cows were diagnosed in the Screening Period (SP), SP1(3–9 DIM) or SP2 (10–16 DIM). (b) Following treatment, HYK^+ cows had their blood BHB concentrations reassessed in the 2 weeks post-treatment, Follow-up Period (FP), FP1 (1 week post initial diagnosis; 10–16 or 17–23 DIM) and FP2 (2 weeks post initial diagnosis;17–23 or 24–30 DIM). The red line represents the HYK threshold BHB concentration (BHB ≥ 1.2 mmol/L) and the "x" on each boxplot represents the means for BHB concentration.

TABLE 2 Results indicated differences in blood BHB concentration (mmol/L) among HYK patterns in Holstein cows that were included in an observational study to assess their hyperketonemia patterns in the 2 weeks postpartum followed by diagnosis and treatment of the condition.

	Screening period-1st week postpartum (3–9 DIM)		Screening period-2nd week postpartum (10–16 DIM)	
Comparisons	***Z*-score[a]**	***p* value[b]**	***Z*-score[a]**	***p* value[b]**
Control-Chronic	15.02	5.53E−50***	9.74	1.01E−21***
Control-Cured	14.96	6.34E−50***	12.2	3.16E−33***
Control-Recurrent	8.19	6.51E−16***	6.7	4.96E−11***
Control-Severe	−10.86	5.84E−27***	−8.01	3.60E−15***
Chronic-Cured	3.43	3.68E−03**	3.48	3.01E−03**
Chronic-Recurrent	2.61	2.74E−02*	1.84	1.30E−01[#]
Chronic-Severe	1.97	9.77E−02[#]	3.17	4.59E−03**
Cured-Recurrent	0.29	7.69E−01[ns]	−0.73	5.60E−01[ns]
Cured-Severe	−0.83	4.85E−01[ns]	0.35	7.29E−01[ns]
Recurrent-Severe	−0.88	5.68E−01[ns]	0.9	5.53E−01[ns]
	Follow-up Period-1st week post-treatment (10–23 DIM)		**Follow-up Period-2nd week post-treatment (17–30 DIM)**	
Comparisons	***Z*-score[a]**	***p* value[b]**	***Z*-score[a]**	***p* value[b]**
Chronic-Cured	14.92	1.42E−49***	15.03	2.94E−50***
Chronic-Recurrent	8.39	9.74E−17***	2.3	2.60E−02*
Chronic-Severe	1.78	7.59E−02[#]	10.64	5.54E−26***
Cured-Recurrent	−2.0	5.51E−02[#]	−8.42	7.59E−17***
Cured-Severe	−10.97	1.68E−27***	−1.66	9.68E−02[#]
Recurrent-Severe	−6.37	2.82E−10***	6.25	6.20E−10***

Note: [#]$p < 0.1$ and ns no significant difference.

[a]Dunn's *z*-test statistic for calculate the difference between two groups.

[b]Dunn's test adjusting for multiple comparisons via false discovery rate (FDR) at a 5% level ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

periods. The average BHB concentration at HYK diagnosis (SP; 3–16 DIM) was 2.1 mmol/L ± 0.9 SD for CHR, 1.6 mmol/L ± 0.5 SD for SEV, REC and CUR patterns, respectively, and control cows had an average BHB concentration of 0.6 mmol/L ± 0.2 SD mmol/L (Figure 1a; File S1: Table S1).

Cows classified as CUR, REC, and SEV did not present statistical differences (FDR > 0.05) in BHB concentration (mmol/L) in the SP; however, differences were detected in the FP (Figure 1b; Table 2). Most cows in these groups had BHB concentrations close to the blood BHB threshold used to define HYK (BHB ≥1.2 mmol/L; Figure 1) showing medians that varied between 1.4 to 1.5 mmol/L in the SP and 0.6 to 1.7 mmol/L in the FP (File S1: Table S2). Nevertheless, CHR cows presented BHB concentration (mmol/L) statistical differences from CUR, REC, and SEV cows (FDR ≤ 0.05; Table 2) in the SP. Blood BHB concentration in CHR cows was considerably greater than the threshold (BHB ≥1.2 mmol/L); Median (FP1 = 2.3, FP2 = 2.4) and comparatively greater than the BHB concentration observed in the other groups (Figure 1b; File S1: Table S2). A progressive increase in BHB concentration from the SP (Figure 1a) to the FP (Figure 1b) was observed in CHR cows; the same trend was not observed in other HYK patterns.

3.2 | Genome-wide association study (GWAS) for HYK patterns

After quality control of imputed genotypes, the analysed dataset containing 489 cows (50.7% of the phenotyped cows; of those 57.3% HYK$^+$; 42.7% CON cows; Table 1) and 47,343 SNPs, of which 450 SNPs came from the 1 K customized panel. Cows of each HYK pattern were analysed along with the CON cows (i.e., CUR-CON, SEV-CON, CHR-CON, and REC-CON). Cows HYK negative (CON) had a lower proportion of genotypic information available than HYK$^+$ cows (34% vs. 79%, respectively, Table 1).

In the analysis of CUR-CON, a total of 9 statistically significant SNPs (FDR ≤ 0.05) were detected (Table 3). These SNPs were "SNP Friends" of 44 other SNPs and were classified as intergenic or intron variants located across chromosomes 4, 6, 7, and 14. These SNPs were hosted or nearby the genes *FRAS1* (Fraser Extracellular Matrix Complex Subunit 1), *SNTG1* (Syntrophin Gamma 1), *PRSS38* (Serine Protease 38), *SNAP47* (Synaptosome Associated Protein 47), *NKAIN3*(Sodium/Potassium Transporting ATPase Interacting 3), *COL28A1* (Collagen Type XXVIII Alpha 1 Chain), *ANXA3* (Annexin A2), *XKR4* (XK Related 4), *TMEM71*(Transmembrane Protein 71), *DNAAF11*(Dynein Axonemal Assembly Factor 11) and *ENSBTAG00000027760* (Fibronectin type-III domain) genes. In the File S2: Spreadsheet S1, the functional consequences of all statistically significant SNPs identified in the contrast CUR-CON as well as for their "LD Friends" are shown. The functional consequences of these variants were reported in relation to 48 coding protein genes, 4 lncRNA, 2 miRNA, 1pseudogene, and 1 snoRNA with a maximum distance of 100 kb up or downstream of them.

For the SEV-CON contrast, 3 statistically significant SNPs (*FDR* ≤ 0.05) were found (Table 4) and were in intergenic regions of chromosome 17. These SNPs were "LD Friends" of 30 other SNPs. The VEP analysis reported the functional consequences of these SNPs in relation to 20 coding protein genes, 3 lncRNA, 3 snoRNA, and 4 snRNA, which was located at a maximum distance of 100 kb from the targeted SNPs (File S2: Spreadsheet S2). The *ARFIP1*(ADP Ribosylation Factor Interacting Protein 1) and *ENSBTAT00000083487*.1 were the nearest associated genes with statistically significant SNPs identified for this comparison.

For the CHR-CON contrast, 3 statistically significant SNPs (FDR ≤ 0.05), located in intergenic regions of chromosomes 7 and 27, were detected (Table 5). These SNPs were "LD Friends" of 8 other SNPs. The VEP analysis reported the functional consequences of these SNPs in relation to 17 coding protein genes and one rRNA, located at a maximum distance of 100 kb from the targeted SNPs (File S2: Spreadsheet S3). The *PRSS38*, *SNAP47*, and *ENSBTAG00000033381* genes were nearest associated with the statistically significant SNPs identified for this comparison.

For the REC-CON contrast, a total of 2 SNPs "rs110950216" and "rs110815185" were detected (FDR ≤ 0.05) (Table 6). The VEP analysis reported the functional consequences of these SNPs in relation to 13 coding protein genes and two miRNA, which were located at a maximum distance of 100 kb from the targeted SNPs (File S2: Spreadsheet S4). The "rs110950216" SNP is an intronic SNP located in the *CRYZL1* (Crystallin Zeta Like 1) and was an "LD Friend" of 2 other SNPs. Considering a maximum distance of 100 kb up or downstream, nearby genes included *SON* (SON DNA and RNA Binding Protein), *DONSON* (DNA Replication Fork Stabilization Factor DONSON), *GART* (Phosphoribosylglycinamide Formyltransferase, Phosphoribosylglycinamide Synthetase, Phosphoribosylaminoimidazole Synthetase), and *ITSN1* (intersectin 1). The "rs110815185" SNP is an intergenic variant, and it had no "LD Friends" identified. However, this SNP was located 61.1 kb from the *ADGRF1* (Adhesion G Protein-Coupled Receptor F1) gene and 15.88 kb from the *ADGRF5* (Adhesion G Protein-Coupled Receptor F5) gene.

All statistically significant SNPs detected in this study were uniquely identified for a single contrast analysis, as well as their "LD Friends", except for "rs110142844" and "rs109467980" which were "LD Friends" commonly identified for the CHR-CON and CUR-CON contrasts. Most genes associated with these SNPs were uniquely found in one of the investigated contrasts, except for the *PRSS38* and *SNAP47* genes that were commonly found between the contrasts CUR-CON and CHR-CON. Figure 2 shows the summary of all structural and functional consequences predicted for SNPs found in each one of the four HYK pattern contrasts. Most variants identified were in intergenic regions, up or downstream of a gene, regardless of the analysed contrast, and the variants located within genes were in intronic regions.

The total number of 26, 10, and 11 ($p < 0.05$) pathways was identified in the CUR-CON, CHR-CON, and REC-CON contrasts, respectively (File S3: Spreadsheet S1, S3, S4). For the SEV-CON contrast, statistically significant Reactome pathways were not identified, but one pathway "Regulation of TLR by endogenous ligand" was detected ($P < 0.05$) through the ToppGene Suite database (File S3: Spreadsheet S2). The Surfactant metabolism pathway was commonly found in the CUR-CON and REC-CON contrasts, and it was the most statistically significant pathway identified when analysing the full list of genes detected in this study. Among the genes found in four contrasts evaluated ($N = 96$), a total of 19 pathways were statistically significant (File S3: Spreadsheet S5). In all four contrasts investigated, SNPs previously associated with phenotypes and QTLs related to milk traits (such as fat and protein %, milk and protein yields, milk vitamin B12 and palmitoleic acid content, and milking speed), and cow fertility traits (e.g., calving ease maternal, stillbirth, and teat length) were detected. In addition, gene ontology (GO) terms, such as protein glycosylation, beta-galactoside (CMP) alpha-2-3-sialyltransferase activity, positive regulation of inflammatory response, glucose homeostasis, hormone biosynthetic process, lipid catabolic process, negative regulation of the fatty acid biosynthetic process, and lipid binding were associated with significant SNPs and their "LD Friends"

TABLE 3 Statistically significant identified SNPs (FDR ≤0.05) within or very close (±100 kb) to potential candidate genes for the Cured (CUR; BHB <1.2 mmol/L in both FP tests) vs. Control (CON; HYK-negative) contrast.

Rs dbSNP	Position	Effect[a]	SE[b]	*p*-value[c]	Variant type[d]	MAF[e]	Mean r^2[f]	LD Friend[g]	Nearest transcript[h]
rs29022916	6:93233475	0.39	0.08	2.67×10^{-06}	Intron	0.05	0.35	rs43473503	FRAS1, ANXA3
rs109467980	7:3356618	−0.19	0.04	2.25×10^{-05}	Intergenic	0.22	0.52	rs110142844, rs110316234	PRSS38, SNAP47
rs3423093104	14:27675777	0.17	0.04	3.00×10^{-05}	Intergenic	0.28	0.52	rs41639945, rs42458532, rs29024888, rs109908675, rs110753260, rs41735007, rs41722033	NKAIN3
rs111003765	4:15495171	−0.13	0.03	4.34×10^{-05}	Intron	0.42	0.58	rs136175724, rs137049526, rs110936013	COL28A1
rs110553458	14:27855587	0.15	0.04	1.23×10^{-04}	Intron	0.26	0.58	rs41639945, rs29024888, rs110753260, rs41735007, rs109908675, rs109292290, rs41722033	NKAIN3
rs41665025	14:20348300	0.17	0.05	2.56×10^{-04}	Intergenic	0.19	0.54	rs110508367, rs41629249, rs109699724, rs42018872, rs110206280	ENSBTAG00000027760, SNTG1
rs109172384	14:28861801	0.15	0.04	3.08×10^{-04}	Intergenic	0.25	0.55	rs41657755, rs41722033, rs41607044, rs41627964, rs109416537	
rs29020689	14:22710032	0.13	0.04	3.22×10^{-04}	Intron	0.41	0.38	rs41657755, rs41722033, rs41607044, rs41627964, rs109416537	XKR4
rs109383111	14:8551277	−0.14	0.04	3.64×10^{-04}	Intergenic	0.33	0.47	rs110456580, rs41632874, rs109937736, rs109087490, rs109500445, rs109667881, rs29021775, rs110108948, rs109418348	TMEM71, DNAAF11

[a]The estimated allele substitution effect.

[b]Standard error.

[c]The significance level is reported for chromosome-wise false discovery rate (FDR) ≤0.05.

[d](1) intron: a sequence variant occurring within an intron, (2) Intergenic: A sequence variant located in the intergenic region, between genes.

[e]The frequency of the minor allele.

[f]Mean LD r^2 between the target SNP and all other SNPs in the 2.5 Mb window in either direction.

[g]Names of all SNPs in LD ≥0.3 with the target SNP (GWAS – FDR <0.05) within a 2.5 Mb window in either direction.

[h]Genes harbouring or nearby significant SNPs.

TABLE 4 Statistically significant identified SNPs (FDR ≤0.05) within or very close (±100 kb) to potential candidate genes for the severe (SEV; BHB ≥1.2 mmol/L in the first FP test and <1.2 mmol/L in the second FP test) vs. control (CON; HYK-negative) contrast.

Rs dbSNP	Position	Effect[a]	SE[b]	p-value[c]	Variant type[d]	MAF[e]	Mean r^2[f]	LD Friend[g]	Nearest transcript[h]
rs109867202	17:5505632	0.13	0.03	9.66×10^{-05}	Intergenic	0.37	0.44	rs41578840, rs41583523, rs29026096, rs41572252, rs41634393, rs41838044, rs41835908, rs41634367, rs41837899, rs41663967, rs42834114, rs109451050, rs41833536, rs109886225	
rs109710004	17:5529898	0.13	0.03	9.66×10^{-05}	Intergenic	0.37	0.44	rs41578840, rs41583523, rs41831603, rs29026096, rs41572252, rs41634393, rs41838044, rs41835908, rs41634367, rs41837899, rs41663967, rs42834114, rs109451050, rs41833536	U6*
rs109367134	17:4948606	−0.13	0.03	9.88×10^{-05}	Intergenic	0.44	0.39	rs109987749, rs41641144, rs43499108, rs41830879, rs43095916, rs110629803, rs110123517, rs110686512, rs41830512, rs41835125, rs41620215	ARFIP, ENSBTAT00000083487.1, ENSBTAT00000080790.1*

[a]The estimated allele substitution effect.

[b]Standard error.

[c]The significance level is reported for chromosome-wise false discovery rate (FDR) ≤0.05.

[d](1) intron: a sequence variant occurring within an intron, (2) Intergenic: A sequence variant located in the intergenic region, between genes.

[e]The frequency of the minor allele.

[f]Mean LD r^2 between the target SNP and all other SNPs in the 2.5 Mb window in either direction.

[g]Names of all SNPs in LD ≥0.3 with the target SNP (GWAS – FDR <0.05) within a 2.5 Mb window in either direction.

[h]Genes harbouring or nearby statistically significant SNPs.

*These are long non-coding RNA (lncRNA) transcripts; otherwise, they are protein-coding transcripts.

TABLE 5 Statistically significant identified SNPs (FDR ≤0.05) within or very close (±100 kb) to potential candidate genes for the chronic (CHR, BHB ≥1.2 mmol/L) in both FP tests vs. control (CON; HYK-negative) contrast.

Rs dbSNP	Position	Effect[a]	SE[b]	*p*-value[c]	Variant type[d]	MAF[e]	Mean r^2[f]	LD Friend[g]	Nearest transcript[h]
rs109467980	7:3356618	−0.15	0.04	4.30×10^{-05}	Intergenic	0.23	0.77	rs110142844	PRSS38, SNAP47
rs41623800	27:32727582	−0.13	0.03	1.16×10^{-04}	Intergenic	0.44	0.34	rs109321915, rs42908330, rs41570143	ENSBTAG00000033381
rs42556412	27:31666441	−0.12	0.03	1.29×10^{-04}	Intergenic	0.42	0.45	rs42908330, rs41615000, rs42754559, rs42752486, rs43733149	–

[a]The estimated allele substitution effect.
[b]Standard error.
[c]The significance level is reported for chromosome-wise false discovery rate (FDR) ≤0.05.
[d](1) intron: a sequence variant occurring within an intron, (2) Intergenic: A sequence variant located in the intergenic region, between genes.
[e]The frequency of the minor allele.
[f]Mean LD r^2 between the target SNP and all other SNPs in the 2.5 Mb window in either direction.
[g]Names of all SNPs in LD ≥0.3 with the target SNP (GWAS – FDR <0.05) within a 2.5 Mb window in either direction.
[h]Genes harbouring or nearby statistically significant SNPs.

(File S4: Spreadsheet S1–S4). The protein–protein interaction (PPI) analysis was statistically significant, showing a $p < 9.99e^{-16}$ (Figure 3; File S3: Spreadsheet S6).

4 | DISCUSSION

4.1 | Hyperketonemia patterns and blood BHB concentration

The overall objective of this study was to better understand the transition period challenges and adaption mechanisms of dairy cows diagnosed with HYK in contrast with HYK-negative cows in the first 2 weeks postpartum.

Among HYK studied groups, we observed a considerable variation in BHB concentration post treatment, which may be due to distinct homeorhetic mechanisms of nutrient partitioning. During the SP, blood BHB concentrations (mmol/L) were not different among CUR, REC, and SEV groups. However, in the FP, we detected differences among them. We hypothesize that these HYK groups are associated with specific metabolic pathways of developing HYK. The CHR pattern cows had greater blood BHB concentration than CUR, REC, and SEV pattern cows. This suggests that excessive BHB concentration in CHR cows may have been triggered by a distinct mechanism.

We speculate that HYK in the CUR, REC, and SEV groups might be secondary to fat mobilization and energy deficit triggered by inadequate dietary intake level throughout the transition period (Ceciliani et al., 2018; Roberts et al., 2012). These cows seemed to better recruit gluconeogenic precursors from external sources (e.g., propylene glycol (PG)). Their ability to control NEFA and subsequently beta-hydroxybutyrate (BHB) concentrations in the blood, allow for a better metabolic adaptation to the high-energy requirement (El-Kasrawy et al., 2020; Sauer et al., 1973). During the screening period, SEV cows had similar BHB concentrations to the CUR cows. However, the SEV cows required a longer period to recover once treatment was initiated. The biological cause behind REC pattern is intriguing. After recovering from their initial HYK event, their BHB concentration increased. It is unclear if the second BHB increase is part of or an independent event from the initial HYK trigger. The effects and efficacy of additional PG treatment to REC cows are unknown. However, we emphasize the importance of further investigating the mechanisms that differs REC cows from the other patterns in FP.

Our results led to the hypothesis that HYK in CHR pattern cows originated from excessive accumulation of liver lipids and incomplete β-oxidation of mobilized fats (Geisler & Renquist, 2017). Concentration of BHB seems to be a promising trait to be used for genetic selection of

TABLE 6 Statistically significant identified SNPs (FDR ≤0.05) within or very close (±100 kb) to potential candidate genes for the recurrent (REC; BHB <1.2 mmol/L in the first FP test and ≥1.2 mmol/L in the second FP test) vs. control (CON; HYK-negative) contrast.

Rs dbSNP	Position	Effect[a]	SE[b]	*p*-value[c]	Variant type[d]	MAF[e]	Mean r^2[f]	LD Friend[g]	Nearest transcript[h]
rs110950216	1:1909740	0.33	0.07	7.95×10^{-06}	Intron	0.03	0.57	rs108994381, rs110257324	CRYZL1, ITNS1, SON, DONSON, GART, bta-mir-6501*
rs110815185	23:20152875	0.51	0.13	9.98×10^{-05}	Intergenic	0.01	0.00	–	ADGR5, ADGRF1

[a]The estimated allele substitution effect.
[b]Standard error.
[c]The significance level is reported for chromosome-wise false discovery rate (FDR) ≤0.05.
[d](1) intron: a sequence variant occurring within an intron, (2) Intergenic: A sequence variant located in the intergenic region, between genes.
[e]The frequency of the minor allele.
[f]Mean LD r^2 between the target SNP and all other SNPs in the 2.5 Mb window in either direction.
[g]Names of all SNPs in LD ≥0.3 with the target SNP (GWAS–FDR <0.05) within a 2.5 Mb window in either direction.
[h]Genes harbouring or nearby statistically significant SNPs.
*These are long non-coding RNA (lncRNA) transcripts; otherwise, they are protein-coding transcripts.

cows less susceptible to developing HYK. Moderate to high genetic correlation between clinical ketosis (BHB ≥1.2 mmol/L with clinical symptoms) and BHB concentration (Ježek et al., 2017; Koeck et al., 2014) and moderate heritability for BHB concentration (Belay et al., 2017; Benedet et al., 2020) have been demonstrated in previous work. Concentrations of BHB in blood or milk are commonly used to diagnose ketosis, especially in subclinical conditions (Duffield, 2000; Soares, Vargas, Muniz, et al., 2021). Classification of HYK cows based on the defined HYK patterns might generate better phenotypes to be used in genetic studies to better understand the risk of developing HYK. However, further studies with a larger database should be performed to explore and confirm these hypotheses.

4.2 | Functional genomic analysis for HYK patterns

The pathogenesis of bovine HYK is not completely understood. An imbalance between glucose demand and the rate of glucose replenishment via gluconeogenesis is the most recognized pathway (Aschenbach et al., 2010). This imbalance may be caused mainly by an impairment in the ability to adapt to endocrine and immune changes that initiate and sustain lactation by altering nutrient metabolism in adipose tissue, liver, and skeletal muscle (Hartmann et al., 1998; Holtenius & Holtenius, 1996). However, the contribution of genetic factors and molecular mechanisms that control individual differences for adaptation to lactation and HYK are poorly understood. Therefore, there is a need to identify SNPs and genes that can be used to genetically distinguish different HYK patterns and select animals based on their susceptibility to HYK and response to treatment. The genome-wide association study explored genetic differences among different HYK patterns compared to the HYK-negative cows. We aimed to assess the association between SNPs and different HYK^+ patterns (i.e., CUR, REC, SEV or CHR) compared with CON cows, exploring the persistence and severity of HYK and individual variability.

PRSS38 and *SNAP47* genes were the only genes identified in more than one of the four contrasts analyses (HYK pattern–CON). When evaluating the CUR-CON and CHR-CON contrasts, these genes were located close to the SNP "rs109467980". *PRSS38* encodes the serine protease 38 protein, which is related to serine-type endopeptidase activity, predicted to be involved in proteolysis and is an important paralog of the gene *CTRL* (Chymotrypsin Like). The *CTRL* encodes zymogen, a proenzyme expressed specifically in the pancreas and likely functions as a digestive enzyme. Some studies associated serine protease with

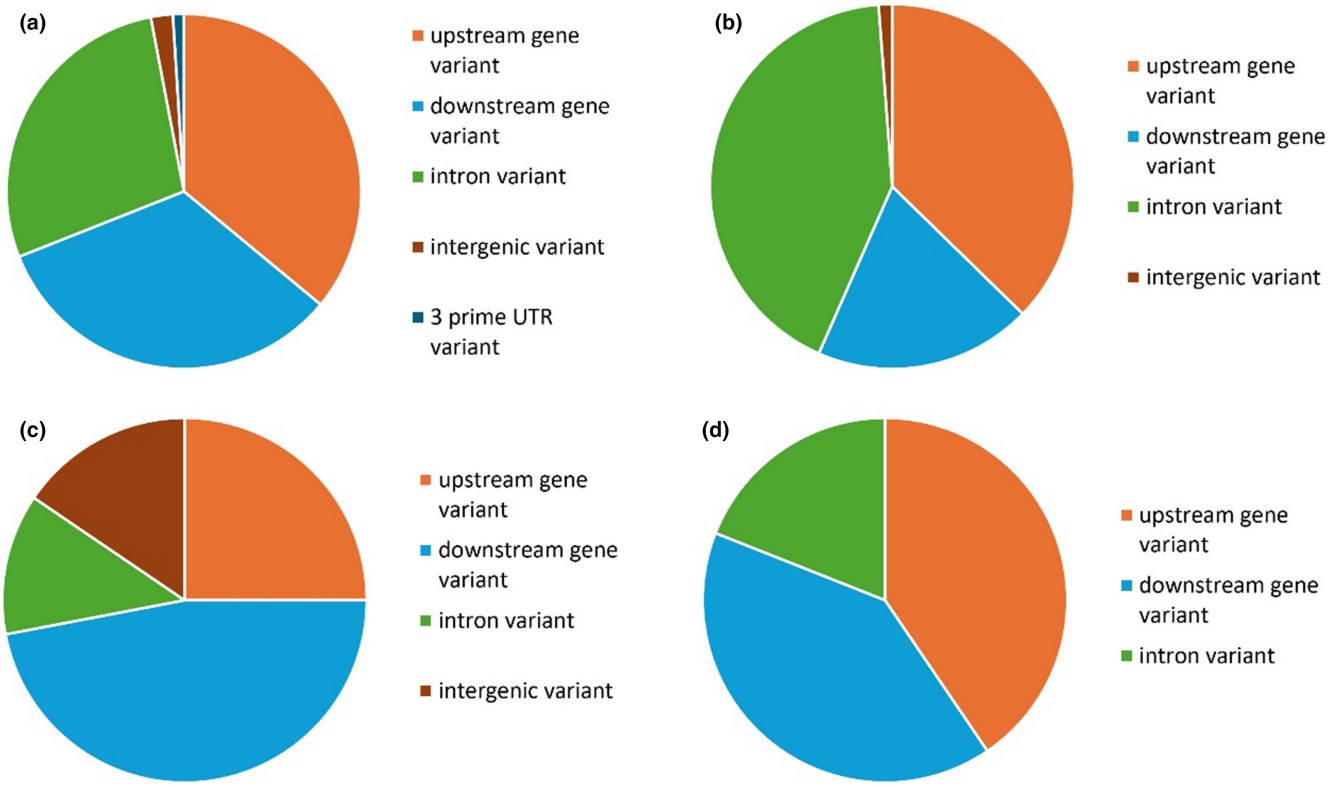

FIGURE 2 Summary statistics of predicted variant consequences for statistically significant SNPs (FDR ≤ 0.05) and their "LD Friends" identified in each contrast between HYK-positive (HYK^+) pattern cows and control (CON) using Variant Effect Predictor (VEP). (a) cured vs. control (CUR-CON), (b) severe vs. control (SEV-CON), (c) chronic vs. control (CHR-CON), and (d) recurrent vs. control (REC-CON).

inflammation and an increase in the severity of pancreatitis in mice (Huang et al., 2020) and correlated severe fatty liver disease and acute pancreatitis in humans (Qi et al., 2016). The *SNAP47*, also known as *C1orf142* or *C7H1orf142*, is predicted to enable SNAP receptor activity and syntaxin binding activity, regulates brain-derived neurotrophic factor (BDNF) and may play a role in intracellular membrane fusion (Sap et al., 2021). This gene is involved in glutamatergic synapse cellular components and has barely been studied. We hypothesize that these genes might have an important role in HYK development. Future research should explore the *PRSS38* and *SNAP47* genes and their contribution to the risk of ketosis and pattern of HYK.

The CUR-CON contrast showed a greater number of statistically significant SNPs, genes, and pathways uniquely identified among HYK patterns. Genes such as *FRAS1*, *SNTG1*, *ANXA3*, *NKAIN3*, *COL28A1*, *XKR4*, *TMEM71*, and *DNAAF11* were highlighted. The *FRAS1* hosted an intronic SNP "rs29022916" that also was located upstream of the *ANXA3* gene, and is related to GO terms, such as phospholipase inhibitor activity, calcium-dependent phospholipid binding, cell communication and negative regulation of catalytic activity. The *FRAS1* gene encodes an extracellular matrix protein that aids in the regulation of epidermal-basement membrane adhesion and organogenesis during development; it has also been related to fertility in cattle (Cai et al., 2019). Similarly, a study investigating subclinical ketosis (BHB ≥ 1.2 mmol/L and no clinical signs) in primiparous cows identified two statistically significant SNP close to the *FRAS1* gene (Nayeri et al., 2019). The *ANXA3* gene belongs to a family of calcium-dependent phospholipid-binding proteins (involved in inositol phosphate metabolism) and in a study investigating conjugated linoleic acid synthesis in the bovine mammary gland it was found to be downregulated in cultured epithelial cell lines treated with trans-11 C18:1 (Wang et al., 2015). The SNP "rs111003765" is an intronic variant in the *COL28A1* gene and was associated with serine-type endopeptidase inhibitor activity and negative regulation of endopeptidase activity. The *COL28A1* gene, a collagen encoder, was also identified by Soares, Vargas, Muniz, et al. (2021) when studying a similar SNP set used in this study. These authors detected this gene in a genomic window that explained 0.66% of the clinical ketosis genetic variance in second to fifth lactation cows. Park et al. (2021) investigated nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) in mice and suggested that collagen mRNAs may be used as biomarkers to identify NAFLD. It might be a potential genetic

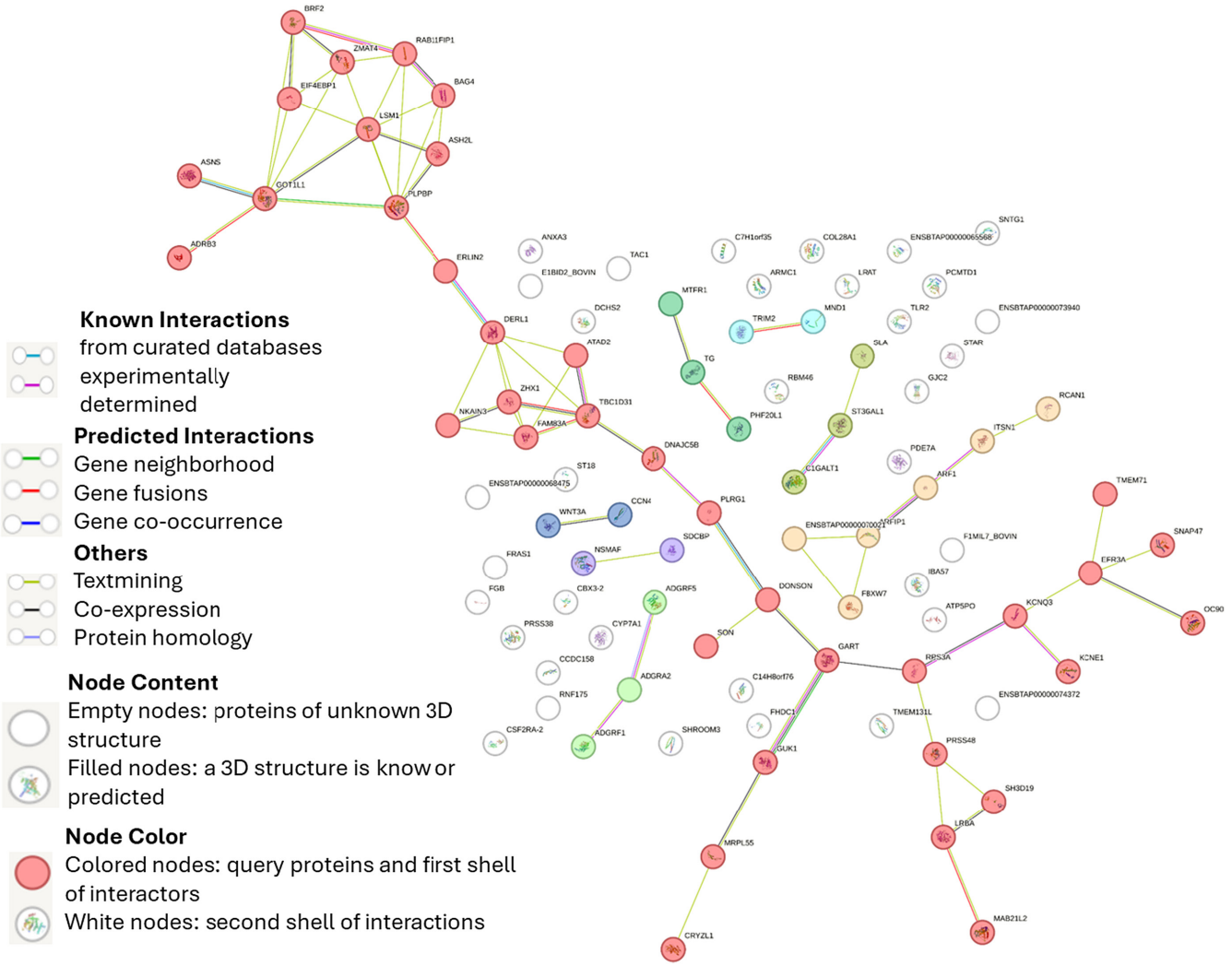

FIGURE 3 Visualization of protein–protein interaction (PPI) enrichment for genes associated with Hyperketonemia (HYK) patterns in Holstein cows. The different node colours showed the *K*-means clustering ($N = 8$ groups) that split proteins into clusters per similarities, for more details File S3: Spreadsheet S6. Edges represent protein–protein associations; the largest and solid link gives a 0.900 edge confidence while the weakest link gives an edge confidence of 0.015, which were represented by the lightest lines.

marker to select dairy cattle less susceptible to ketosis, encouraging further investigations of collagen encoders.

The SNP "rs29020689" relates to milking speed and reproductive traits (e.g., stillbirth and calving ease QTLs) and was in an intron of the *XKR4* gene. The *XKR4* gene is involved in phosphatidylserine exposure on the apoptotic cell surface and has been associated with residual feed intake, average daily feed intake, and average daily gain in cattle (de las Heras-Saldana et al., 2019; Lindholm-Perry et al., 2012), important features in energy balance. Within the *XKR4* gene, Bastin et al. (2016) detected a statistically significant SNP "rs42646708" associated with serum prolactin concentrations, explaining 2.45% of the phenotypic variance of this trait. Prolactin plays an essential role in metabolism, regulation of the immune system, pancreatic development, and milk production (Nappi et al., 2021). These results suggest *XKR4* as an important gene for the study of HYK given its involvement in crucial aspects, such as milk production residual feed intake, and average daily feed intake. In addition, genes *TMEM71* and *DNAAF11* (known as *LRRC6*) were located near a statistically significant SNP "rs109383111". The *TMEM71* gene encodes a transmembrane protein that was associated with the heat stress response of saturated fatty acids in dairy cattle (Bohlouli et al., 2022). Soares, Vargas, Muniz, et al. (2021) found *TMEM35B* (Transmembrane Protein 35B), which belongs to the transmembrane family, in a genomic window on chromosome 3 that explained 0.48% of genetic variance for clinical ketosis. Further studies would be necessary to better understand the link between these genes and HYK patterns.

For the contrast SEV-CON, the gene *ARFIP1* was highlighted for hosting two intronic SNPs ("rs41830879" and "rs41830512") and for being located near the SNP

"rs109367134". These SNPs were related to QTL such as milk stearic acid content, milk protein percentage, somatic cell score, and dairy form, as well as reproductive traits (e.g., daughter pregnancy rate, teat placement-front, length of productive life, and calving ease maternal). The *ARFIP1* gene enables phosphatidylinositol-4-phosphate binding activity and acts upstream of or within intracellular protein transport and regulation of protein secretion. Lee et al. (2016) reported *ARFIP1* as a candidate gene hosting statistically significant SNPs associated with milk-related traits (e.g., milk yield, fat, and protein) in Holstein, suggesting that SNPs within this gene may be important for selection purposes. In addition, we identified the SNPs "rs109367134" and "rs41572252" located near the *ENSBTAT00000083487.1* gene. This novel annotated gene is predicted to encode a transmembrane protein 154 (*TMEM154*) and reported to be involved in type 2 diabetes in humans (Harder et al., 2015). Type 2 diabetes, like HYK, is a metabolic disease with origins in energy metabolism.

The *TLR2* (Toll-like receptor 2) and *FGB* (Fibrinogen beta chain) genes were enriching the "Regulation of TLR by endogenous ligands" pathway (unique pathway (FDR < 0.05) associated with the list of genes in the SEV-CON contrast). Toll-like receptors play a crucial role in innate and adaptive immunity. Possibly endogenous ligand mediated TLR signalling is involved in pathological conditions, such as tissue injury, repair, and regeneration and autoimmune diseases and tumourigenesis (Yu et al., 2010). The *FGB* gene encodes a β component of fibrinogen, a blood-borne glycoprotein composed of three pairs of non-identical polypeptide chains. Zhang et al. (2018) reported that in response to malnutrition or high-fat diet feeding, fibrinogen is synthesized by hepatocytes and secreted into the blood in humans and mice. High fibrinogen levels may contribute to the development of thrombosis (mechanism linking fatty liver with cardiovascular disease). A study in *Notomys Fuscus* (a rodent species) demonstrated the development of a unique energy metabolism strategy in a hypoxic environment, which promotes oxygen transport by increasing haemoglobin synthesis (Li et al., 2021). This strategy reduces the risk of thrombosis through cooperative regulation of genes, including *FGB* and genes such as *ACS16*, *GPAT4* and *NDUFB7* that are involved in regulating lipid synthesis, and fatty acid β-oxidation, thereby maintaining a normal energy supply in hypoxic conditions (Li et al., 2021). Therefore, SNPs affecting the genes *TLR2* and *FGB* might be affecting or contributing to cows' adaptive process to transition through regulating lipid synthesis and fatty acid β-oxidation. These genes should be further investigated to characterize their functional implications for HYK in dairy cattle.

For the CHR-CON contrast, the SNP "rs41623800" was downstream of the gene *ENSBTAG00000033381*, which is a novel annotated gene predicted to encode a Solute carrier family 35 member F2 (SLC35F2) protein. This protein is involved in all kinds of substrate transport, such as metabolites, cofactors, vitamins, nutrients, ions and drugs. He et al. (2017) reported that SCL gene superfamily is essential to control physiological functions and its disturbance may result in diseases. Some authors found *SLC35F2* gene upregulated in high-fat/high-sugar diet-induced and Non-alcoholic fatty liver disease (NAFLD) groups of mice and leptin receptor-deficient obese groups of mice (Xiang et al., 2022). The SNP "rs43733149" is an intron variant in the gene *EIF4EBP1* (Eukaryotic Translation Initiation Factor 4E Binding Protein 1). This gene encodes one member of a family of translation repressor proteins and is phosphorylated in response to various signals including insulin signalling (Wang et al., 2019). Salama et al. (2019) observed that phosphorylated *EIF4EBP1* was lower during heat stress in dairy cows, which can contribute to diminished translation initiation and reduced protein synthesis affecting mammary metabolism. The SNP "rs41570143" was an "LD Friend" of a statistically significant SNP "rs41623800" for CHR-CON contrast and is located on an intron of the *ZMAT4* (Zinc Finger Matrin-Type 4) gene. This gene is an important paralog of the *ZNF346* gene, which is associated with elevated apoptosis and chronic inflammation (Peng et al., 2023). A zinc finger encoder gene, *ZMYM6* (Zinc finger MYM-type) also was identified in a genomic window on chromosome 3 that explained 0.48% of genetic variance for clinical ketosis lactation (Soares, Vargas, Muniz, et al., 2021). These findings highlight the important of further investigations regarding zinc finger gene's family for the development of chronic cases of HYK in dairy cows.

Some genes were highlighted to CHR-CON contrast for being associated with the immune mechanisms, such as *ADGRA2* (adhesion G protein-coupled receptor A2, *RAB11FIP1* (RAB11 family interacting protein 1), *LSM1* (LSM1 Homologue, MRNA Degradation Associated)) (Hamann et al., 2016; Ming et al., 2022; Rahman et al., 2022) and *BAG4* (Molecular chaperone regulator 4), which was related apoptosis-related genes and immune response (Safdar et al., 2020). Furthermore, the genes *GOT1L1* (glutamic-oxaloacetic transaminase 1 like 1), *ERLIN2* (ER lipid-raft-associated 2 isoform 2), *PLPBP* (pyridoxal 5′-phosphate binding protein), and *ADRB3* (Adrenoceptor Beta 3) were highlighted to be involved in the endocrine system, fat deposition and cytosolic lipid content, liver disease, regulation of lipolysis and thermogenesis, and transport of cholesterol (Engel & Chen, 1975; Martins-de-Souza et al., 2012; Mitchell et al., 1976; Wang et al., 2023; Yonggang, 2010; Zheng et al., 2018). In bovine, *PLPBP* is associated with inhibiting and inactivating bovine liver glutamate

dehydrogenase (Engel & Chen, 1975). *ADRB3* encodes a protein that belongs to the family of beta-adrenergic receptors, which mediate catecholamine-induced activation of adenylate cyclase through the action of G proteins and is involved in the regulation of lipolysis and thermogenesis (Wang et al., 2012). *ADRB3* is located mainly in the adipose tissue and was found to be downregulated in back subcutaneous fat tissue of beef cattle (Wang et al., 2023); thus, it may be a candidate gene for better understanding individual differences in fat metabolism.

Two SNP were statistically significant for REC-CON contrast. The SNP "rs110815185" is an intergenic variant located near the genes *ADGR5* (Adhesion G Protein-Coupled Receptor E5) and *ADGRF1* (Adhesion G Protein-Coupled Receptor F1), which are genes from the same family of *ADRB3* gene that was identified in the contrast CHR-CON. SNP "rs110950216" is an intron variant of the gene *CRYZL1* (Crystallin Zeta Like 1). This gene encodes a protein that has sequence similarity to zeta crystallin, also known as quinone oxidoreductase and is involved in oxidoreductase activity and NADP binding (Kim et al., 1999). The *ADGR5* gene enriched the "Surfactant metabolism" pathway. Surfactant is composed primarily of phospholipids enriched in phosphatidylcholine (PC) and phosphatidylglycerol (PG). They influence surfactant homeostasis, contributing to the physical structures of lipids in the alveoli and to the regulation of surfactant function and metabolism (Agassandian & Mallampali, 2013). Moreover, surfactant phospholipids are produced by "de novo" pathway that is highly dependent on the availability of fatty acids (FA) in type II cells that are supplied by the circulation in the form of free FA or triacylglycerols within lipoproteins (Agassandian & Mallampali, 2013). Those are genes and mechanisms that can be involved in recurrent cases of HYK in dairy cows and must be further investigated.

In addition, the other genes such as *ITSN1*, *GART* (Phosphoribosylglycinamide Formyltransferase), *KCNE1* (Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1) and *ATP5PO* (ATP Synthase Peripheral Stalk Subunit OSCP) were found in REC-CON contrast. They were related to disease severity increase, elevated inflammation, and poor survival in septic patients (Dixit et al., 2020; Zeng et al., 2019), LPS-induced release of pro-inflammatory factors (Zhang et al., 2014) and ATP synthesis (Zech et al., 2022). The genes identified in this contrast were mostly related to cell maintenance, inflammation, energy production and fatty acid oxidation, suggesting that those genes may play an important role in the production and correct functioning of cells related to the adaptive immune system, such as T cells. T cells require a massive increase in energy and amino acids for activation, proliferation, and differentiation; thus, this overhaul of the cellular transcriptome and proteome results in substantial remodelling of multiple pathways involved in cellular metabolism and protein synthesis, including key energy production pathways governing mitochondrial, glycolytic and lipid metabolism (Marchingo et al., 2022). Abuajamieh et al. (2016) reported that increased biomarkers of inflammation appear to be closely associated with ketosis in transition dairy cows. These results highlight potential genes and pathways in the pathogenesis of ketosis, which need to be further investigated.

Most SNP and genes identified in this study were uniquely found for a particular HYK pattern contrast and when analysing all genes jointly, a significant ($p < 9.99^{e-16}$) protein–protein interaction (PPI) network was found (File S3: Spreadsheet S6). This means that the set of genes investigated had more interactions among themselves than what would be expected for a random set of proteins of the same size and nodes drawn from the genome (Doncheva et al., 2019). In addition, QTL, biological processes, and pathways were commonly identified for more than one HYK pattern contrast, which was expected since we are looking at different patterns of a HYK greater condition, which might be better explained by the integration of biological findings of these patterns. Among the biological terms shared by one or more HYK patterns, were found: negative regulation of the fatty acid biosynthetic process and positive regulation of inflammatory response, glucose homeostasis, lipid catabolic, hormone biosynthetic, phospholipid metabolic process, and oxidoreductase activity. These biological mechanisms were related to energy metabolism in studies investigating subclinical and clinical ketosis, where authors demonstrated associations between the development of this disorder and deficiency or limitation in metabolic processes (Duffield, 2000; Ježek et al., 2017; Lou et al., 2022; Nayeri et al., 2019; Roberts et al., 2012; Soares, Vargas, Duffield, et al., 2021; Soares, Vargas, Muniz, et al., 2021), such as energy metabolism.

4.3 | Challenges and limitations

Due to logistical constraints, biological samples from CON and HYK+ cows for genotyping were not collected simultaneously. Hair samples from CON cows were collected 18 months later, with some cows having left the herd by then, which may have introduced a potential survival bias in our results. The CON cows that remained in the herd might represent an even more resilient subgroup, which may have influenced genomic results but, if there was any bias, it should be small. Genomic data were obtained in approximately half of the phenotyped cows, with

most samples being from HYK^+ cows. Proportion of missing genomic information was unequal among CON and HYK^+ cows. Even though appropriate statistical methods were used to account for this imbalance, we acknowledge the potential influence this limitation in the observed genetic differences among various HYK patterns compared to CON cows. Additional studies are required to confirm and further explore the genetic component of different HYK patterns.

5 | CONCLUSIONS

This study looked at the SNP profile of HYK^+ cows diagnosed within first 2 weeks postpartum, treated and followed up in the next 2 weeks subsequent to treatment in comparison with HYK-negative via GWAS, which allowed for identifying genomic regions, candidate genes and biological pathways related to HYK. Despite the limited sample size, important genomic regions associated previously with HYK were identified, and a list of candidate functional genes associated with fatty acid synthesis, lipid binding, oxidoreductase, cell homeostasis and inflammatory response was created, which may facilitate the identification of causal genetic variants underlying HYK in dairy cows. Further studies with a larger number of cows are warranted to improve statistical power and confirm the SNPs and candidate genes identified.

AUTHOR CONTRIBUTIONS

M.M.M.M., R.C.S., F.S.S., E.J.S., J.A.A.M., C.F.B. and T.D. designed research; G.A.O.J. performed data imputation; M.M.M.M. performed genomic analysis and wrote the first draft of the manuscript; R.C.S. and J.A.A.M. worked on data collection; resources, C.F.B., T.D., F.S.S., and E.J.S.; all authors contributed and reviewed the final version of the manuscript; all the authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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