POSTPARTUM ELEVATED B-HYDROXYBUTYRATE AND NON-ESTERIFIED FATTY ACIDS TOGETHER OR SEPARATELY AND THEIR ASSOCIATION WITH PLASMA METABOLITES, BODY CONDITION AND REPRODUCTIVE PERFORMANCE IN DAIRY COWS

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Abstract: This study aimed to assess post-partum elevated nonesterified fatty acids (NEFA) and β hydroxybutyrate (BHB), considered either togetheror separately,relative to the estrus cyclicity and first service pregnancy status of cows and their association withbody condition scores and some metabolites.Blood samples from 50 Montbéliarde dairy cowswere collected from 15 to 52 DIMto measure serum BHB, NEFA,glucose, triglycerides, total cholesterol, urea nitrogen, total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), calcium, magnesium, potassium, phosphorus, sodium;and progesterone concentrations.Body condition score (BCS) was assessed at calving and at each time when blood samples were taken.Cows were considered as having post-partum elevated NEFA (H-NEFA) concentration if the concentration was≥0.70 mM and post-partum elevated BHB (H-BHB) concentration if the concentration was≥1.20 mM at 30 DIM. Overall, 93.33 % of cows having an elevated BHB show an elevated of NEFA and 51.61% of cows having an elevated NEFA have not an elevated BHB. Indeed, considering postpartum elevated NEFA as a predictor of sub-clinical ketotic cows can overrateresults. Whereas, considering postpartum elevated BHB as a predictor of cows with NEB can underestimate results. Excessive BCS at calving results in increasing the risk of post-partum elevated BHB. Cholesterol, triglycerides, AST, ALT, and urea were increased in cows having elevated BHB and NEFA compared with those having elevated NEFA only or healthy cows. Further, the risk of estrus cyclicity and pregnancy rate at first insemination (P/AI) was decreased in cows having both elevated BHB and NEFA or NEFA only.

Keywords: BHB; NEAF; Plasma metabolites; BCS; Reproductive performance; Dairy cows

INTRODUCTION

Early lactation is a dynamic period for dairy cattle, during which most dramatic metabolic changes are likely to occur [1]. Cows unableto adapt to this challenging time are more disposed to negativeenergetic balance (NEB)[2] and mobilize more fat reserves[3]releasing more non-esterified fatty acids (NEFA)[4].The liver is responsible for metabolizing circulating NEFA, which can be completelyoxidized for ATP production, exported from the liver as lipoproteins, or partially oxidized intoketone bodies especially β-hydroxybutyrate(BHB)[5]. In

recent literature,NEFA and BHBA are both usedas markers of negative energy balance during the post-partum [6–8]or subclinical ketosis [9, 10] and there isevidence that these two markerscannot be used interchangeably[8].When cows have excessive NEBin terms of depth, duration, and timing [11], they mobilize NEFA with a higher amount than liver capacity [2]inducing a fatty liver[12]. Excessive fat accumulation in the liver impairs normal liver function[5], which may lead to an increasein BHB concentrations[3].

In several studies,elevated NEFA orBHBA concentrations during the pre- and postpartumperiodswere the mainrisk factors for predictors of health disorders [13–15], productive outcomes[16–18] and reproductive performance [19, 20]. In these studies, authors considered elevated one of these biomarkers as subclinical ketosis (SCK) or negative energy balance (BEN). Recently, McCarthy et al. [8] reported a weak relationshipbetween blood concentrations of NEFA andBHB, suggesting that elevated concentrations of one shouldnot be extrapolated to suggest elevated concentrationsof the other metabolite. In a meta-analysis conducted by Abdelli et al. [21], the author reported that cows with elevated post-partum circulating NEFAwere 32% less likely to conceive at first service (PR/IA) than cows classified aspost-partum elevated circulating BHB. In the same study, the authors reported, also, that the inclusion of the test(NEFA/BHB) as a moderator in the meta-regression reduced the heterogeneity by 12% in PR/IA outcomes [21]. In light of the weak correlationsbetween blood concentrations of NEFA andBHBA observed in the study of McCarthy et al. [8],along with the data of Abdelli et al.[21], changing reproductive outcomes with the test (e.g., NEFA/BHB);we hypothesized that cows that failed to have estrus cyclicity or failed to conceive to the first insemination would haveelevated at least either or both postpartum blood NEFA and BHB concentrationsthat indicated greater severity of NEB or SCK [22]. The presentstudy consequently aims to assess how high post-partum bloodNEFA and BHBA levels, considered either separately or together,may improve the prediction of estrus cycling and first servicepregnancy status (P/AI1) in dairy cows.

MATERIAL AND METHODS

The protocols and procedures applied in this study were according to the ethical principles for the use of experimental animals as established by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research (Ethical approval number: 98-11, Law of August 22, 1998).

Animals

Samples were collected during a previously reported study to determinethe effect of metabolic profiles on reproductive performance in post-partum Montbéliarde cows[23]. Briefly, a total of 50Montbéliarde cows from 2farms in the north of Algeria were enrolled from April to September 2014. Their daily average milk production was 25 kg/d. During the period of study, cows receive green fodder, clover in the cold season, and meadow fodder in warm-season with vetch oats hay. According to the production, the basic ration was individually supplemented with commercial

concentrate (18% digestible raw protein), as well as roughly crushed maize grains, soybean meal, barley, and vitamin-mineral mixture.

Blood sampling and measurement of BCS

A diagram of the activities is displayed in Figure 1. The farms were visited fortnightly on the same day and at approximately thesame time.Blood was collected from the coccygeal vein into10 mL vacuum tubes, one containing lithiumheparin and the other with no anticoagulant.Samples were taken before the morning feeding at for times (wk 2, 4, 6, and 8 postpartum). Blood samples were chilled on ice-packs immediately after collection and within 5 h were centrifuged at 1,400 ×gfor 10 min. Harvested sera were frozen at −20°C until analyzed.Simultaneous with the blood collection and at calving, the BCS wasevaluated on a half-point scale, with quarter-point divisions,using the visual technique developed by Edmonsonet al. (1989).Tow variables of fatness were of particular importance: at calving (BCS-calv) and the difference from calving to wk 4 postpartum (dBCS).

Measurement of metabolite and progesterone levels

Heparinized plasma was used for the determination of NEFAs, BHBA, glucose, total cholesterol, triglycerides (TG), urea nitrogen, total protein, aspartate aminotransferase (AST), γ-glutamyltransferase (γGT), alanine aminotransferase (ALT), calcium, magnesium, sodium, potassium, and phosphoruslevels. Allblood metabolites except NEFAs and BHBAwere determined withthe enzymatic method by spectrophotometric assay in anauto-analyzer (Cobas 6000, Roche Hitachi, Mannheim, Germany)in a commercial laboratory, using commercial kits.The intra-and interassaycoefficients of variation were < 5% for each assay.Serum BHBA concentration (mmol/L) was measured using a handheld meter(Precision Xceed, Abbott Laboratories, Abbott Park,IL) at room temperature [2]. Optium Xceed is a hand-held deviceused to test blood BHBA concentrations; there sensitivityand specificity were 85 to 90% and94 to 98%, respectively[24]. Because there were not sufficient reagentsfor NEFAs, plasma NEFAsconcentration was measured one time (at wk 4) using the DVM- NEFA test (VeterinaryDiagnostics, Newburg, Wisconsin, USA). The sensitivity and specificity of the DVM-NEFA testwere 84% and 96%, respectively[25].Progesterone was quantified by ELISA (Elecsys 2010, Roche Diagnostics GmBH, Mannheim,Germany) using human progesterone ECL kit.These human kits can be used to measure P4 in serum bovine and[26], inter- and intraassaycoefficients of variation were 16.6% and 6.7%, respectively.

Determination of estrous cyclicity and pregnancy diagnoses

Pregnancy was diagnosed in all cows on d 30 after AI via transrectal ultrasonography of the uterus and its contents and characterized by visualization of a live embryo. Cows diagnosed as pregnant on d 30 were reexamined by transrectal palpation 35 d later.Progesterone data were dichotomized using a threshold of 1 ng/mL for indicating the presence of an active corpus luteum [27]. Ovulation was considered to have occurred 5 days before the first progesterone measurement >1 ng/mL and was followed by another consecutive sample of luteal concentrations.The resumption of ovarian activity was calculated at 30 DIM which is considered optimal under practical conditions [28].

Statistical analysis

Statistical analyses were performed with SAS (Version 9.1.3; SAS InstituteInc., Cary, NC). Postpartum plasma concentrations ofmetabolites were reported as continuousvariables.Each variable of these metabolites was tested for normal distribution usingthe PROC UNIVARIATE (SAS Inst. Inc.).If the variable does not fit the normal distribution, adjustments such as logarithmic, squared, Square root transformations were possible tools to normalize the data to calculate valid descriptive statistics.Fertility responses of interest were estrous cyclicity ond 52postpartum and P/AI.Normalized results and all other variables wereanalyzed as repeated measures using a mixed modelprocedure (PROC MIXED; SAS Inst., Cary, NC). For each analyzed variable,cows were subjected to 3 covariance structures:compound symmetric (CS), autoregressive order one (ar1), andunstructured covariance (UN). (fixed effects of treatment, day and their interaction, random effect of cows).

Additionally, a mixed general linear model was fitted using the MIXED procedureof SAS (random effect of cows) to evaluate the effect of the test on BCS change from calving to wk 4 postpartum (dBCS).

Diagnostic sensitivity and specificity, with 95 percent confidence intervals (CI) and accuracy of tests based on BHB, NEFA, or both of detecting anoestrus and non-pregnant cows were calculated by using Win Episcope 2.0 (. A Stacked line plot of BCS, and a diagram of BCSchanges (dBCS)was generated using Prism 6.07 (GraphPad Software, Inc. La Jolla, CA USA).

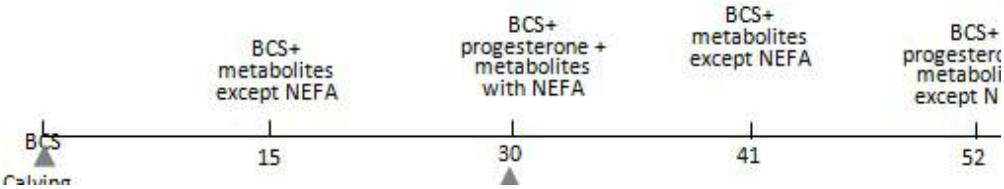

Fig. 1. Illustration of the measurement protocol and pregnancy diagnoses.

RESULTS AND DISCUSSION

The overall incidence of sub-clinical ketosis at 30 DIM was 30% and it was 62% for elevated NEFA (table 1). However, all cows except one who has an elevated BHB show an elevated NEFA (93.33%).

Plasma metabolites

Cows having simultaneously elevated NEFA and BHB tended to have increased circulating cholesterol concentrations from 30 to 52 DIM relative to healthy cows. However, when NEFA was elevated only, there no significant effect on cholesterol and triglycerides compared with healthy cows (table2). Cows having elevated NEFA and BHB togethertended to have increased circulating urea concentrations at 30 and 41 DIM compared with healthy cowsand at 52 DIM compared with healthy cows or cows having elevated NEFA only. Thus, cows having elevated NEFA and BHB together showed a tendency for higher concentrate of ASAT at 15, 30, 41 DIM, and higher ALAT at 41 DIM compared with healthy cows or cows having

elevated NEFA only. There was a significant effect of sampling time on glucose, cholesterol, and alanine aminotransferase. Nevertheless, there were not group-bytime interactions on all plasma metabolites. Furthermore, no significant effects of group, sampling time, and group-by time interactions were found for γGT, albumin, total protein, inorganic phosphorus, calcium, potassium, magnesium, and sodium.

Table 1

Comparison of least square means (LSM) and standard error (SE) of plasma constituents and between cows having elevated NEFA and BHB together (Both), having elevated NEFA (H-NEFA), and healthy cows (None) at 15, 30, 41 and 52 DIM

Metabolites	class	LSM				SE	P*		
		d15	**d30**	**d41**	**d52**		**Group**	**Time**	G *T
Glucose	Both	0.651	0.652	0.657	0.655	0.031	0.951	0.009	0.988
	H-NEFA	0.664	0.666	0.669	0.668	0.029			
	None	0.656	0.660	0.664	0.663	0.026			
Cholesterol	Both	1.743	1.777a	1.792a	1.799a	0.091	0.149	<0.0001	0.657
	H-NEFA	1.589	1.616ab	1.620ab	1.632ab	0.110			
	None	1.495	1.513b	1.525b	1.526b	0.105			
Triglycerides	Both	0.216a	0.214a	0.213a	0.215a	0.016	0.083	0.869	0.885
	H-NEFA	0.175ab	0.176ab	0.175ab	0.176ab	0.015			
	None	0.171b	0.169b	0.168b	0.166b	0.014			
Urea	Both	0.344	0.351a	0.347a	0.354a	0.026	0.171	0.199	0.422
	H-NEFA	0.306	0.305ab	0.305ab	0.308b	0.031			
	None	0.271	0.275b	0.272b	0.271b	0.033			
AST	Both	97.93a	99.21a	104.85a	104.43	5.624	0.519	0.681	0.799
	H-NEFA	85.23b	84.88b	82.14b	96.58	4.646			
	None	91.27ab	84.05b	80.50b	84.39	3.643			
ALT	Both	36.973	37.738	38.932a	38.279	2.858	0.373	<0.0001	0.239
	H-NEFA	34.422	34.904	35.199b	35.315	3.495			
	None	39.459	39.963	40.036b	40.572	3.324			

BCS and BCS change

For BCS, the repeated measure ANOVA in mixed model revealed a tendency toward asignificant effects ofthe group (P =0.01), time (P < 0.0001) and group-bytime interaction (figure 2.A). The BCS at calving was higher in cows experienced an elevated BHB and NEFA together compared with cows experienced an elevated NEFA only or healthy cows. However, BCS at 41 DIM was higher in health cows compared with cows experienced an elevated NEFA and it was higher in healthy cow compared with cows experienced an elevated BHB and NEFA together or with elevated NEFA only.Thus, cows that experienced an elevated NEFA and BHB together or elevated NEFA only lostmore BCS than cows experienced none of the above during the first month of lactation (figure 2.B).

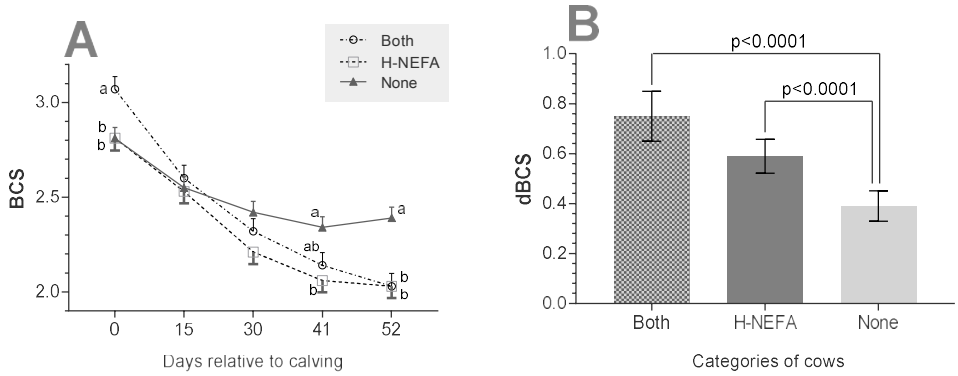

Fig. 2. Least square means ± SEM for BCS at calving, 15, 30, 41, and 52 DIM (A) and BCS change(dBCS, means± SEM) from calving to 30 DIM for cows that showed elevated NEFA, or elevated NEFA and BHB (both).

Estrous Cyclicity

The proportion of cowswith serum P4 >1 ng/mL was 56% (n=28) at 52 DIM. Using BHB thresholds to detect anestrus cows resulted in higher sensitivity (72.4 %) and lesser specificity (33.3%). Though, using NEFA thresholds to detect anestrus cows resulted inlessersensitivity (45.5%) and higher specificity (75.0%). When the threshold was changed to BHB and NEFA to gather, it resulted in a sensitivity of 68.2 % (95% CI = 48.7-87.6), and specificity of 70% (95% CI = 41.6-98.4). The accuracyof tests was 58.0, 66.0, and 69.7 for BHB, NEFA, and both thresholds respectively.

Table 2

Diagnostic sensitivity (%) and specificity (%), with 95 percent confidence intervals (CI) and accuracy of test based on BHB, NEFA, or both detecting anestrus cows.

Test	N° of cows	Anestrus cows	Sensitivity (95%CI)	Specificity (95%CI)	Accuracy
H-BHB	15/50	8/15	36.4% (16.3, 56.5)	75.0% (59.0, 91.0)	58.0%
H-NEFA	31/50	18/31	81.8% (65.7, 97.9)	53.6% (35.1, 72.0)	66.0%
Both	14/50	7/14	70.0% (41.6, 98.4)	69.6% (50.8, 88.4)	69.7%

Table 3

Risk factors for the resumption of estrous cycles by 52 days postpartum in lactating dairy cows

Value	Estimate	SE	OR	95%CI	P-value
Both vs. None	-1.862	1.098	0.155	0.021-1.120	0.064
Elevated NEFA vs. None	-2.234	0.816	0.107	0.021-0.529	0.006
Both vs. Elevated NEFA	0.372	0.953	1.451	0.224-9.411	0.695

Theeffect of having anelevated NEFA only resulted in 84.5 % decreased risk (OR = 0.155, p=0.006)of estrus cyclicity at 52 DIM. However, no significant difference (p=0.064) between healthy cows and cows having elevated NEFA and BHB together was recorded. Thus, cows having elevated NEFA only or with BHB have the same chance of estrus cyclicity at 52 DIM (p=0.695).

Pregnancy to first AI

The PR/AI at first inseminationwas 34.0%. Using BHBthresholds to detect non-pregnant to first AI cows resulted in lesser sensitivity (46.4 %) and higher specificity (90.9%). However, Using NEFA only or NEFA and BHB together thresholds to detect non-pregnant cows resultedin higher sensitivity and specificity.The accuracyof the test was 66.0, 84.0, and 81.8 for BHB, NEFA, and both thresholds respectively.

The PR/AI was reduced by 87% (OR =0.13, P = 0.001) for each one mmol of BHB and NEFA /L increase and by 92.6% (OR =0.074, P = 0.006) for each one mmol of NEFA /L increase. Though, cows having elevated NEFA only or elevated BHB only have the same chance of PR/AI (p=0.647).

Table 4

Diagnostic sensitivity (%) and specificity (%), with 95 percent confidence intervals (CI) and accuracy of test based on BHB, NEFA, or both to detecting non-pregnant cows.

Test	N° of cows	Non-pregnant cows	Sensitivity (95%CI)	Specificity (95%CI)	Accuracy
H-BHB	15/50	13/15	46.4% (28.0,64.9)	90.9% (78.9, 102.9)	66.0%
H-NEFA	31/50	28/31	84.8% (72.6, 97.1)	82.4% (64.2, 100.5)	84.0%
Both	14/50	12/14	75.0% (53.8, 96.2)	88.2% (72.9, 103.6)	81.8%

Table 5

Risk factors for conception rate after AI in lactating dairy cows

Value	Estimate	SE	OR	95%CI	P-value
Both vs. None	-2.038	0.651	0.130	0.036-0.467	0.001
Elevated NEFA vs. None	-2.592	0.950	0.074	0.011-0.482	0.006
Both vs. Elevated NEFA	0.553	1.211	1.739	0.161-18.688	0.647

Several studies have investigated the associationbetween individual transition animals with increased NEFA or BHBA concentrations and detrimental downstream reproductive performance [13, 20, 29]. The analytical approach of thiswas study used to examine the associationbetween elevated both postpartum NEFA and/or BHBA, cycling, and first service pregnancy status of the cow.

In the current study, all cows expect one (93.33%) having elevated BHB at 30 DIM, they had, at the same time, elevated NEFA and 54.83 % of cows how had elevated NEFA, they had not an elevated BHB. In other words, whereas NEFA concentration is elevated, BHB concentration is not necessarily elevated and when

BHB concentration is elevated, NEFA concentration is most likelyelevated.Recent researchers demonstrated that postpartum NEFA concentrations are weakly correlated with BHB concentrations [8]. Thus, studies that have published results of NEFA and BHB concentrations over time in postpartum, the peak of mean NEFA concentration occurs before the peak of the mean BHB [30].In the current study, the correlationcoefficientof the whole cows was significant ($r=0.625$, $p<0.0001$) but this correlation was not significant in healthy cows, cows having elevated NEFA, or both.Correlationcoefficients of these groups were 0.291, 0.448, 0.248 for healthy cows, cows having elevated NEFA, and both respectively.

In early lactation, stored energy from fat ismobilized as NEFA, some of which are taken up by theliver[13].Uptake of NEFA from plasma by the liver is proportional to the plasma concentration [31]. According to Grummer etal. [32], the increase of plasma NEFA concentration led to the increase of ketogenesis by hepatocytes which explain that almost all cowswho had elevated BHB, had elevated NEFA. Though,over half of the cows having elevated NEFA, they didn't have elevated BHB. This may be partially explained by an individual capacity to adapt against excessive fat mobilization[12, 16] and more accurately, liver capacity[31, 33]. Hence, the homoerotic changes required to achieve this fat mobilization appear to be primarily under genetic control [34].

Indeed, hepatic uptake of NEFA is limited by the capacity of thetricarboxylic acid cycle (TCA) [4]and when NEFA exceeds this capacity, there is increased production of ketone bodiesand deposition of triacylglycerol[35] causing fatty liver disease [30].

However, inthe study of Reynolds et al. [36], the authors observed that the contributionof NEFA carbon to BHB synthesis cannot account forall of the carbon used in ketone body synthesis in theliver. Thus,Vernon et al. [37] demonstrated that postpartum ketosis in cows is not due to significant changes in the activity of liver enzymes involved in fatty acid metabolism.In the current study, higher concentrations of Cholesterol, triglycerides, and AST were also found in ketotic cows whichmight have elicited increased fat mobilization. This is similar to findings reported by Harpøth et al [38]; those authors suggested the elevated plasma cholesterol and triglycerides concentrations being a result of the increased secretion of very-low-density lipoprotein by the liver by incomplete oxidation through ketogenesis.Further, AST is an enzyme that becomes elevated with celldamage and may be elevated in cows with fatty liver disease[4]. Thus, Jorritsma et al. [39] concludedthat high serum NEFA and low serum glucose and ureain early lactating dairy cows are significant indicatorsof hepatic lipidosis.

Interestingly, there was no difference for glucose between cows having elevated NEFA and BHB compared with healthy cows which in contrast to previous findingswhere glucose is negatively correlated with BHB and NEFA [10]. In other studies, high NEFA or BHB concentrations did not influence blood glucose concentration but they induced insulin resistance [3].

The body condition score (BCS)is still an object of research forthe prevention of excessive negative metabolic changes indairy cows, as it is a parameter which shows good correlationwith increased concentrations of NEFA and BHB[12].Similar to the present study, others reported associationof BCS at calving, or change in BCS early in

lactation with elevated BHB [40] and elevated NEFA [12, 20]. In the current study, cows having both elevated NEFA-BHB tended to a higher BCS at calvingcompared with cows having elevated NEFA only but this difference was not evident for BCS loss during the first month of lactation. That means cows of two groups mobilized the same amount of fatty acids during the first month of lactationbut cows having higher BCS at calving had lower hepatic capacity against exceeding NEFA [41]to limit the production of ketone bodies.

Combined with a lower DMI associated with greater BCS [42] and an increase in lactose requirements for milk production (and therefore hepatic gluconeogenesis), hepatic oxaloacetate would likely become limiting for NEFA oxidation, and ketone bodies would accumulate[43]. Consistent with this hypothesis, Compton et al. [44] and shin et al [45]reported that cows having higher BCS at calving were more likely to develop SCK or CK.

Although there has been considerable focus on thestudy of post-partum elevated NEFA or BHB and their association with reproductive outcomes [13, 14, 46].In this study, postpartum elevated NEFA presentedmore reliability than BHB alone and almost the same fiability with elevated both NEFA and BHB when they used as predictors to detect anestrual and non-pregnant cows. Elevated NEFA has been found to be a more accuratemeasure of NEB than ketone bodies[2].

It is also a stronger indicator of postpartum disease andmilk production than elevated BHBA [6].Cows with elevated both NEFA-BHH and elevated only NEFA had a decreasedrisk of estrus cyclicity at 52 DIM and pregnancy to first AI (Tables 3 and 5).

In contrast, we observed no significantchange between cows with elevated both postpartum NEFA-BHBA and only elevated NEFA. Studies by Ospina et al.[13]found that,whenboth postpartum NEFA and BHBA were in the samemodel to predict reproductive performance, BHBA was not as strong a predictor as NEFA. The same results were reported by Dubuc et al [14], the authors found thatNEFA is more closely associatedwith early ovulation than hyperketonemia.

The negative effect of NEFA and BHB on reproductive performance may be indirect via the physiological relationship between these biomarkers and NEB[19]or directly. Indeed, several studies reported that elevated BHB may affect follicular cell function [47], oocyte maturation ([48], corpus luteum function [49]and early embryo development [50]. Whereas, the direct effect of elevated NEFA has received less attention[7].

CONCLUSIONS

Considering postpartum elevated NEFA as a predictor of sub-clinical ketotic cows can overrate results. Whereas, considering postpartum elevated BHB as a predictor of cows with NEB can underestimate results.Excessive BCS at calving results in increasing the risk of post-partum elevated BHB. In addition, as the concentrationof BHBAincreased, cholesterol, triglycerides, AST, ALT, and urea were increased. However, post-partum elevated NEFA concentration was not affected by these metabolites. Further, cows with post-partum elevated BHB and NEFA or NEFA only a decreased risk of estrus cyclicity and pregnancy at first insemination. Further large field-scale studies are required to comprehensively evaluate the herd circumstances

under which a negative effect of elevated NEFA or BHB may be obtained separately or combined to gather.

DECLARATION OF INTEREST. The authors declare that there is no conflict of interest that could be perceived asprejudicing the impartiality of this article.

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