Universitat Autònoma de Barcelona

Facultat de Veterinària

Departament de Ciència Animal i dels Aliments.

Trabajo presentado para la superación de 15 créditos del Módulo "Trabajo Fin de Máster" del Máster Oficial en Calidad de Alimentos de Origen Animal

Milk metabolites for the detection of heat stress in dairy ruminants: Goats

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Inform that,

The research work entitled “Milk metabolites for detection of heat stress in dairy ruminants: Goats” is part of a INIA-RTA competitive project (Ref. RTA2015-00035-C03-00) funded by the Ministerio de Economía, Industria y Competitividad (MINECO) and has been made by the Master’s Student THÂMARA CRYSTINA BRAMBILA-DARÉ BONFIM E SILVA e under our supervision, as a requisite for the completion of the Master’s Degree in Food Quality of Animal Origin of the Universitat Autònoma de Barcelona.

Signed in Bellaterra (Barcelona, Spain), the 1 of September 2019

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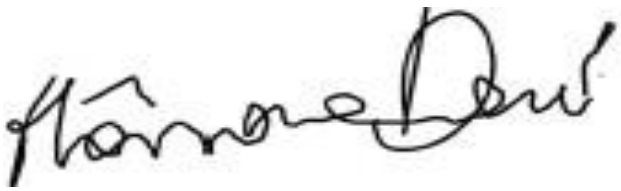

Acknowledgments

First of all, I thank God for the gift of existence and for allowing me to fulfill this dream.

To my parents, who are the best thing I have in this life, I express my profound gratitude for their unconditional support from afar.

To my directors, Gerardo Caja and Elena Albanell thank you for all your patience and for your support and trust, without your help I would not have carried out this work.

Finally, I thank my friends in Brazil who have always supported me during this master, and those who have shared a home and their time with me in Spain.

Thâmara

List of abbreviations

ALP	Alkaline Phosphatase
BHB	β-hydroxybutyrate
CITR	Citrate
CRE	Creatinine
HS	Heat Stress
INS	Insulin
NIRS	Near Infrared Spectroscopy
PRL	Prolactin
RER	Range Error Ratio
RPD	Ratio of Performance Deviation
r^2_{CV}	Coefficient of Determination for Cross- Validation
THI	Temperature and Humidity Index
TN	Thermoneutral
UAB	Universitat Autònoma of Barcelona

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SUMMARY

Milk metabolites for the detection of heat stress in dairy ruminants: Goats

The aim of this study was to analyze a group of milk metabolites used as heat stress (HS) biomarkers: i.e., alkaline phosphatase (ALP), β-hydroxybutyrate (BHB), creatinine (CRE) and insulin (INS), using reference methods and Near Infrared Reflectance Spectroscopy (NIRS) to calibrate and to predict the content of these HS biomarkers in the milk of ruminants. This study focuses on dairy goats. Citrate (CITR) and prolactin (PRL) were also analyzed but data was not available at the deadline of the Thesis.

A total of 367 milk samples were obtained from 3 commercial farms (n = 311) and from the experimental group (n = 56) of the Universitat Autònoma of Barcelona (UAB). A subset of milk samples (n = 98) was used for analysis by standard laboratory methods and by NIRS (1,100 to 2,500 nm). Calibration and cross validation procedures were carried out for optimization with the UAB samples. Prediction accuracy for ALP and INS, according to their ratio of performance deviation (RPD) and range error ratio (RER) values, were acceptable (> 2 and >10, respectively). Additionally, values of the coefficient of determination for calibration (R^2) were relatively good (0.97 and 0.96), indicating that they may be used for predicting ALP and INS in the milk of dairy goats. In conclusion, results from the biomarkers in milk demonstrated the reduction of ALP and INS in milk by effect of HS, thereby validating their use as HS indicators in the milk of dairy goats.

Keywords: heat stress, Near Infrared Spectroscopy, dairy goat, milk bioindicator

RESUMEN

Metabolitos en leche para la detección del estrés por calor en rumiantes lecheros: Cabras

El objetivo de este estudio fue analizar un grupo de metabolitos de la leche utilizados como biomarcadores de estrés térmico (HS): es decir, fosfatasa alcalina (ALP), β-hidroxibutirato (BHB), creatinina (CRE) e insulina (INS), empleando métodos de referencia y Espectroscopia en el Infrarrojo Cercano (NIRS) para calibrar y para predecir el contenido de estos biomarcadores de HS en la leche de rumiantes. Este estudio se centra en cabras lecheras. También se analizaron citrato y prolactina, pero los datos no estaban disponibles en la fecha límite de la tesis.

Se obtuvieron un total de 367 muestras de leche de 3 granjas comerciales (n= 311) y del grupo experimental (n= 56) de la Universitat Autònoma de Barcelona (UAB). Se utilizó un subconjunto de muestras de leche (n= 98) para el análisis por métodos de laboratorio estándar y por NIRS (1.100 a 2.500 nm). Se realizaron procedimientos de calibración y validación cruzada para la optimización con las muestras de la UAB. La precisión de predicción para ALP e INS, de acuerdo con su relación entre los valores de desviación de rendimiento (RPD) y relación de error de rango (RER), fueron aceptables (> 2 y >10, respectivamente). Además, los valores del coeficiente de determinación para la calibración (R^2) eran relativamente buenos (0,97 y 0,96), indicando que se puede utilizar para predecir ALP e INS en la leche de cabras lecheras. En conclusión, los resultados de los biomarcadores en la leche demostraron la reducción de ALP e INS en la leche por efecto del HS, validando su uso como indicadores del HS en la leche de las cabras lecheras.

Palabras clave: estrés térmico**,** Espectroscopia del Infrarrojo Cercano, cabra lechera, bioindicador de la leche

RESUMO

Metabólitos lácteos para detecção de estresse térmico em ruminantes leiteiros: Cabras

O objetivo deste estudo foi analisar um grupo de metabólitos lácteos utilizados como biomarcadores de (HS) estresse térmico: isto é, fosfatase alcalina (ALP), β-hidroxibutirato (BHB), creatinina (CRE) e insulina (INS), por métodos de referência e Espectroscopia no Infravermelho Próximo (NIRS) para calibrar e prever o conteúdo desses biomarcadores do HS no leite de ruminantes. Este estudo centra-se em cabras leiteiras. Citrato e prolactina também foram analisados, mas os dados não estavam disponíveis na data limite da tese.

Um total de 367 mostras de leite foram obtidas de 3 fazendas comerciais (n= 311) e do grupo experimental (n= 56) da Universitat Autònoma de Barcelona (UAB). Um subconjunto de amostras de leite (n= 98) foi usado para análise por métodos laboratoriais e por NIRS (1.100 a 2.500 nm). Procedimentos de calibração e validação cruzada foram realizados para a otimização com as amostras da UAB. A precisão da previsão para ALP e INS, de acordo com os valores de relação de desempenho do desvio (RPD) e razão de intervalo de erro (RER), foram aceitáveis (> 2 y >10, respectivamente). Além disso, os valores do coeficiente de determinação da calibração (R^2) foram relativamente bons (0,97 e 0,96) indicando que ele pode ser utilizado para predizer ALP e INS no leite de cabras leiteiras. Em conclusão, os resultados dos biomarcadores no leite demostraram a redução de ALP e INS sob efeito do HS, e validando seu uso como indicadores de HS em cabras leiteiras.

Palavras chaves: estresse térmico**,** Espectroscopia de Infravermelho Próximo, cabra leiteira, bioindicador de leite

1. INTRODUCTION

1.1. Climate Change

Most of the information collected in the 5th Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) has made it possible to obtain evidence of direct human influence on global warming since the 20th century due to the continued production of greenhouse gases (IPCC, 2014). Climate change is not limited to arid, tropical or Mediterranean zones. Furthermore, weather patterns in northern and central Europe (EEA-JRC-WHO, 2008) and the USA (Adams et al., 1990) have shown noticeable changes over the past several decades and demonstrated seasonal changes and outstanding events, such as unpredictable rainy seasons, heat waves and warmer summers.

De Castro et al. (2009) reported an increase in temperatures between 5 and 7ºC in summer for the last third of this century in the south-central areas of the Iberian Peninsula. This has had an impact on goat milk production in these areas (MAGRAMA, 2018).

Effects of climate change on dairy animal welfare and performance are known and occasionally are quantitatively predictable (St-Pierre et al., 2003). Thus, it is of extreme concern to farmers and researchers to understand heat stress (**HS**), its signs and the adequate management practices that can reduce its negative effects on livestock (IPCC, 2014).

1.2. Heat Stress

HS has an adverse impact on milk yield and composition as well as on feed intake (Silanikove, 2000a,b; Baumgard and Rhoads, 2012, 2013). Milk production losses are justified by metabolic adaptations to HS which can be explained by a milk-born negative feed-back system that down regulates milk synthesis, milk secretion and blood flow to the mammary glandand glucose uptake by the mammary gland (Silanikove, 2000a,b; Silanikove et al., 2009; Baumgard and Rhoads, 2012, 2013; Rhoads et al., 2013). This consequently affects the quality of products and production potential, causing immeasurable economic losses (St-Pierre et al., 2003).

The effects of HS are correlated with the adaptation of certain species and breeds and the physiological stage of their life cycle. Therefore, compared with monogastrics, ruminants are less susceptible to HS conditions. However, those destined to dairy production are more susceptible and have an antagonistic relationship with milk production traits and resistance to HS, as they have to deal with higher temperatures associated with a higher level of nutrition and their need to regulate their body temperature (Hansen, 2007).

Goat breeds living in desert and tropical environments are one of the most efficient ruminants (Silanikove, 2000a). However, some species have an adaptive capacity determined by their ability to develop strategies when exposed to climate change (Silanikove, 2000a,b). These adaptation strategies can be ordered into six categories: 1) anatomical, 2) morphological, 3) physiological, 4) feeding behavior, 5) metabolism and 6) performances (Devendra, 1987; Silanikove, 2000b).

Goats are more resilient compared to other ruminants (Devendra, 1987). According to Williams el al. (2008), resilience is the capacity of a species to recover and survive a perturbation. Thus, both resilience and adaptive capacities are influenced by ecology, species, genetic diversity and physiology (Silanikove, 2000a). In areas with warm and arid climatic conditions, goats have a high sweating rate, greater water conservation capability, higher skin temperature, and lower metabolic weight (Hamzaoui et al., 2013). In that regard, goats are more resilient and adapted to HS if compared to cows (Silanikove, 2000a,b; Escareno et al., 2013). Measuring when dairy animals enter in HS conditions is complex because it is not only related with energy balance but also with metabolism of water, sodium and potassium (Sevi et al., 2001).

The temperature and humidity index (**THI**) remains traditionally an indicator of HS in ruminants (Bernabucci et al., 2014) and is usually calculated using the formula:

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$$

Where T_{db} is dry-bulb temperature (ºC) and RH is relative humidity (%), respectively (NRC, 1971). A THI value of 72 is employed as the threshold of HS onset in dairy cows (Hammani et al., 2013) and is not a straight biomarker of metabolic alterations (Wheelock et al., 2010).

According to Brown et al. (1988), there was a diminution of milk yield in Alpine dairy goats exposed to moderate HS conditions for about 5 wk (34ºC and 25% humidity; THI = 79), but not in Nubian dairy goats. However, another study reported that Saannen lactating goats exposed to moderate or severe conditions of HS for 4 d (THI = 81 or 89) had a loss in milk yield by 3 or 13%, respectively (Sano et al., 1985). Therefore, the response to HS is correlated with breeds that are more adapted or affected by HS (Lucena et al., 2013).

Metabolomics can be used as a powerful platform for the determination of low-molecular weight metabolites in animals, humans and plants related with pathophysiological alterations caused by exposure to specific environmental factors including HS (Sreekumar, 2009; Miguel et.al., 2014; Contreras-Jodar et al., 2018).

Metabolic profiling can be useful for the detection of biomarkers and advancement of measures to diagnose and help with HS-induced disorders (Tian et al., 2015). Critical

concentration thresholds are employed to define the concentration range for each metabolite used in a metabolic profile test. Thus, values which are not within this range may indicate disorders or production losses (Ospina et al., 2010).

Additionally, milk is a useful fluid for monitoring physiological alterations and its natural daily production allows a non-invasive sample collection as opposed to that of blood (Tian et al., 2016; Contreras-Jodar, 2019).

1.3. Near Infrared Spectroscopy

Near infrared spectroscopy **(NIRS)** is a rapid, robust, non-destructive analytical methodology which has shown high efficiency in simultaneous determination of milk components (Melfsen el al., 2012). This spectroscopy is based on the analysis of the interaction between electromagnetic waves and matter, and different regions within the electromagnetic radiation spectrum can be used depending on the component to be analyzed (O'Donnell et al., 2014).

NIRS has been applied in the dairy industry with a spectral range from 800 to 2,500 nm (De Marchi et al., 2018). Several studies have shown NIRS to be efficient in the analysis of raw milk (Albanell et al., 1999, 2003; Tsenkova et al., 1999, 2001, 2006; Chen et al., 2002; Kawasaki et al., 2008).

2. OBJECTIVES

Although milk metabolites of dairy animals and humans have been recently studied (Hu et al., 2004; Smilowitz et al., 2013; Tian et al., 2016; Contreras-Jodar, 2019), there is limited information about the metabolomic response to HS in dairy goats and further study using NIRS for metabolite detection is needed.

The general aim of this study was to detect metabolites of HS in the milk of dairy goats using reference laboratory methods and NIRS.

The specific objectives were to:

1. Analyze a group of previously selected milk metabolites used as HS biomarkers: Alkaline phosphatase (**ALP**), β-hydroxybutyrate (**BHB**), Creatinine (**CRE**) and Insulin (**INS**) using reference methods. Citrate (**CITR**) and Prolactin (**PRL**) were also included but the analytical results were not available at the deadline of the presentation of this Thesis.
2. Collect the NIR spectrum of the same milk samples in the reflectance mode within a spectral range of 1,100 – 2,500 nm.

3. Determine if NIRS calibration could be used to predict the concentration of the previously selected HS biomarkers (ALP, BHB, CITR, CRE, INS, and PRL) in milk.
4. Allow an early identification of individuals showing the typical milk metabolic profile of HS in dairy goats for developing effective strategies to minimize productivity loss and the occurrence of diseases, as well as warrant that they are kept in accordance with the principles of animal wellbeing.

3. MATERIAL AND METHODS

The samples collected for this experimental study came from 2 different sources: 1) from on-farm animals managed under standard commercial farm conditions, and 2**)** from an experimental group under controlled experimental conditions. The animal care conditions and management practices of this study meet the requirements for livestock wellbeing of the Ministry of Agriculture, Food and Environment of Spain and, in the case of the experimental farm conditions, were approved by the CEEAH (Ethical Committee of Animal and Human Experimentation) of the **UAB** (Universitat Autònoma of Barcelona) with the reference CEEAH- 2019/3142.

3.1. On-farm Animals under Standard Commercial Farm Conditions

With the aim of obtaining milk under thermoneutral (**TN**) and hot environmental conditions, a total of 311 milk samples were collected from 3 different dairy goat farms placed in Chillón (Ciudad Real), Alcolea del Rio and La Campana (Sevilla) in Spain on 3 dates during early- and mid-summer of 2018 (**Table 1**). All the goats were lactating and had similar range of days in milk (60 to 100 d). Milk samples were collected in standard plastic containers (45mL) with caps used for milk composition recording.

All milk samples were cooled (4ºC) after collection and frozen (–20ºC) upon processing at the laboratory. No preservative was added to the milk samples to avoid analytical interferences and they were sent frozen to the UAB using CO_2 dry ice.

Table 1. Description of the on-farm samples of milk of dairy goats

Farm code	Village (Province)	Samples 1 (dd/mm/aaaa)	Samples 2 (dd/mm/aaaa)	Samples 3 (dd/mm/aaaa)	Total
SC	Alcolea del Rio (Sevilla)	33 (21/6/2019)	32 (5/7/2019)	34 (19/7/2019)	99
PZ	Chillón (Ciudad Real)	40 (20/6/2019)	33 (4/7/2019)	34 (18/7/2019)	107
VR	La Campana (Sevilla)	35 (18/6/2019)	35 (2/7/2019)	35 (16/7/2019)	105
Total	3	108	100	103	311

3.1.2. Experimental Group under Controlled Experimental Conditions

Animals and Treatments. Fourteen multiparous Murciano-Granadina dairy goats from the herd of the SGCE (Servei de Granges i Camps Experimentals) of the UAB in Bellaterra (Barcelona, Spain) at late-lactation (149 ± 3 d) and machine milked once a day at a vacuum of 42 kPa, 90 pulses/min and 66% pulsation ratio (Westfalia-Surge Ibérica, Granollers, Spain), were adapted to wood chips bedded pens since the start of the experiment. The goats were divided in 2 balanced groups and randomly allocated to 2 ambient conditions according to a 2×2 (treatment × period) crossover design, with periods of 21 d.

Environmental treatments were: 1) Thermal-neutral indoors (TN; 15 to 20ºC; 45 to 50% humidity) and heat stress in climatic chamber (HS; day 37ºC, night 30ºC; 45 to 50% humidity) conditions. Day-night length was set to 12–12 h. The THI ranges, calculated according to NRC (1971), were: TN = 59 to 66, and HS = 78 to 88. Milk samples (n = 56; 50 mL per sample) were collected and stored at –20ºC in plastic containers for further analysis, with or without the use of an antimicrobial preservative tablet (Bronopol, Broad Spectrum Microtabs II; D & F Control Systems USA Inc., San Ramon, CA, USA) in 3 groups of animals on 2 different days as shown in **Table 2**.

Table 2. Description of experimental farm samples of milk of dairy goats

Treatment	Preservative	Group 1 (dd/mm/aa)	Group 2 (dd/mm/aa)	Group 3 (dd/mm/aa)	Total
TN	Yes	5 (18/1/2019)	5 (10/2/2019)	4 (3/3/2019)	14
TN	No	5 (18/1/2019)	5 (10/2/2019)	4 (3/3/2019)	14
HS	Yes	5 (3/2/2019)	5 (24/2/2019)	4 (17/3/2019)	14
HS	No	5 (3/2/2019)	5 (24/2/2019)	4 (17/3/2019)	14
Total		20	20	16	56

3.2. Sample Selection and Laboratory Processing

A total of 367 milk samples were finally obtained from the goats belonging to commercial farms managed under natural weather conditions (n = 311) and from the animals under controlled experimental conditions (n = 56). A subset of milk samples (n = 98) was selected based on milk quality at processing (i.e., coagulated samples were discarded) and availability for extreme climate conditions.

Milk samples were thawed at room temperature (20ºC) and immediately analyzed using NIRS, as later described. For milk metabolites, the thawed raw milk samples were centrifuged (Hettich Zentrifugen, D-78632 Tuttlingen, Germany) at 3,000 rpm (1500 × g) for 15 min at 4ºC to obtain skim milk. The skim milk was separated from the fat and precipitates by creating a

small perforate in the central side of the centrifuge tube and draining the clear liquid portion into 4 separate eppendorf tubes (1.5 mL) that were conserved frozen (−20ºC) until further analysis.

3.3. Analysis of Milk Biomarkers

3.3.1. Alkaline phosphatase. The ALP was measured by a kinetic rate method following the procedure designed in the manufacturer manual. Prior to analysis, eppendorf samples were thawed, vortexed and centrifuged at 1,500 rpm (150 × *g*) for 3 min (Micro centrifuge sigma 112, Bristol, UK) and the reagents stored in the fridge were carried to room temperature.

Activity was analyzed using an Olympus AU400 autoanalyzer (Olympus Europa, Hamburg, Germany) using p-nitrophenyl phosphate (15.8 mM in 0.9 M 2-amino-2-methyl-1-propanol buffer at pH 10.3).

3.3.2. β-hydroxybutyrate. The BHB was determined by the kinetic enzymatic method as designed in the manufacturer manual. Prior to analysis, eppendorf samples were thawed, vortexed and centrifuged as previously indicated.

Activity was analyzed using the same Olympus autoanalyzer using commercially available kits RANBUT (Randox Laboratories Ltd, Crumlin, UK), with the presence of 3- hydroxybutyrate dehydrogenase, BHB was oxidized by NAD giving acetoacetate (AcAc) and NADH.

3.3.3. Creatinine. The CRE was measured by the colorimetric method based on the Jaffe method and using a commercial kit. Prior to analysis, eppendorf samples were thawed, vortexed and centrifuged as previously indicated. Activity was analyzed using the Olympus AU400 autoanalyzer.

3.3.4. Insulin. The INS of the goat milk was determined by using Mercodia bovine insulin enzyme-linked immunoassay sandwich (ELISA) kits (Mercodia AB, Uppsala, Sweden). Eppendorf samples were thawed, vortexed and centrifuged as previously indicated.
Reacted ELISA plates were analyzed for INS in an automatic reader (iEMS Reader MF V.2.9-0, Labsytsems España, Barcelona, Spain) at an optical density of 450 nm to calculate the concentration

3.4. NIR Spectroscopy

3.4.1. NIR Spectra Collection. A NIRSystems 5000 (Foss, Hillerod, Denmark), with a scanning range of 1,100 to 2,500 nm was utilized for predicting milk metabolites (ALP, BHB, CRE and INS) in goats. A total of 367 goats milk samples were used for spectra collection. Frozen goat milk samples were thawed at room temperature and then conditioned in a water

bath (40ºC). Approximately 30 mL of each milk sample were placed in a quartz glass measurement cup containing a gold reflectant cell (path length, 0.5 mm). Milk samples were softly stirred to avoid additional scattering effect, which may arise as a result of fat flocculation, and positioned in the cell holder for scanning. Scanning of milk samples was done in duplicate.

Reflectance (R) data was obtained every 2 nm (R = log [1/transmittance]) and calibration equations were developed by the software WinISI III (v.1.6, FOSS NIRSystems/Tecator Infrasoft International, Sylver Spring, MD, USA).

3.4.2. NIR Calibration and Validation. For the improvement of calibration models, the original spectra were subjected to different pre-treatment methods in order to enhance the signal-to-noise ratio and, as a result, to maximize the signal intensity for the milk component of interest (Heise and Winson, 2002).

Spectral correction algorithms – detrend (DT), multiplicate scatter correction (MSC), standard normal variate (SNV) – were employed to reduce fat flocculation or the effects of light scattering due to particle size. Then, different mathematical treatments using first and second derivatives, with different subtraction gaps and smoothing intervals were also tried. Varying spectral models for each predicted parameter were obtained, resulting from the evaluation of four scatter correction techniques (SNV, DT, SNV+ DT and Standard MSC) and eight scatter correction techniques (1.4.4.1; 2.4.4.1; 1.5.5.1; 2.5.5.1; 1.8.8.1; 2.8.8.1; 1.10.10.1; 2.10.10.1 -
derivate number, subtraction gap, first smooth and second smooth).

Modified partial least square (MPLS) regression was used to elaborate the calibration models (Martens and Martens, 2001) and ´leave-n-out´ cross-validation (Shao, 1993) was later performed for model optimization. Cross-validation was employed to design samples as Spectral
(H) or chemical (t) outliers (about 7% of the samples). The t outliers are samples that have a relationship between their reference values and spectra that is dissimilar to the relationship of other sets and with sizable residuals (t values >2).

Quality and predictive ability of the calibration equations were estimated by using the following statistical parameters: coefficient of determination for calibration (R^2), minimum standard error of calibration (SEC), coefficient of determination for cross-validation ($\mathbf{r^2_{CV}}$), minimum standard error of cross-validation (SECV), range error ratio (**RER**), which were calculated by dividing the range in the reference data used in the validation set by the SECV and the ratio of performance deviation (**RPD**), which was calculated by dividing the standard deviation of the reference values in the validation set by the SECV.

The final evaluation of the calibration models was established on the RPD and RER. RPD,

which is a nondimensional statistic, has been generally employed for quick evaluations of infrared calibrations (Williams, 2014) and established according to the error between the reference and the predicted values, and the standard deviation of the reference data (De Marchi et al., 2018).

3.5. Statistical Analysis

3.5.1. Milk Metabolite Analysis. A primary analysis was applied to all the milk metabolites and milk components in order to know if there were some explanatory effects. All of them were checked for normality of distribution and the assumption of homogeneity of the variances was also tested. Explanatory effects and possible interactions were modelized and no significant variables were removed from the models. The final model selection was based on the Akaike Information Criterion corrected (Burnham et al., 2002) where the lower the value, the better the model fits. R 3.0.2 software (R Core Team, 2013). The models were performed using the nlme package (Pinheiro et al., 2014) of R v.3.0.2 software (R Core Team, 2013). Significant statistical differences were considered in all cases when *P* value were lower than 0.05, otherwise indicated.

4. RESULTS AND DISCUSSION

Major components of the milk samples analyzed correlated among themselves and were accurately predicted by NIRS, as expected and according to the previous studies developed in different types of milk (Albanell et al., 1999, 2003). For this reason, they will not be discussed here.

4.1. Reference Data for NIRS Calibration

This study investigated the potential of predicting the metabolite ALP, BHB, CRE and INS contents in the milk of dairy goats as HS biomarkers using the NIRS technique. Results of the standard methods used as reference data for the calibration set for each milk sample are displayed in **Table 3**.

Even though some studies have investigated the use of mid infrared spectroscopy **(MIR)** to predict milk serum fatty acids concentrations and BHB, to the best of our knowledge this is the first study to investigate the use of NIR spectral data to predict milk metabolites in dairy goats. Accordingly, the values obtained in the present analysis cannot be corroborated with other studies, and values of BHB data did not present any significance.

Table 3. Sample size and range values of biomarkers in the milk of dairy goats used for the NIRS calibration set

Item	Samples, n	Range	Mean	SD
All samples:				
Alkaline Phosphatase	98	1.7-137.8	34.6	28.7
Creatinine	98	0.94-2.72	1.49	0.35
Insulin	98	0.06-3.07	0.81	0.52
UAB experimental farm:				
Alkaline Phosphatase	40	1.7-83.4	25.3	24.6
Creatinine	40	0.94-2.46	1.51	0.39
Insulin	40	0.06-2.28	0.74	0.46

An important range of variation was lost when the number of samples was reduced from 98 to 40. Therefore, an external validation could not be made, and cross validation was necessary. Additionally, the variation range within each metabolite due to environmental conditions (TN vs. HS groups) were similar between all samples when compared to UAB samples.

4.2. NIR Calibration and Cross-Validation

The statistic models for predicting milk biomarkers (ALP, CRE and INS) are shown in **Table 4.** Spectra pre-treatment methods that showed the best prediction equation models were chosen for statistical analysis. Therefore, the most appropriate mathematical treatment was 2.4.4.1.

In all cases, the results were better when the UAB samples were used, if compared to all samples, since reducing the number of samples led to improved homogeneity of the results. This can be owed to the fact that the animals used in the UAB experiment were under extreme environmental conditions, as well as the sample quality being easily maintained from collection to arrival at the laboratory.

The best predictions achieved and the highest coefficient of determination for calibration (R^2) were for ALP **(**R^2 = 0.97; **Figure 1a)** and INS **(**R^2 = 0.96; **Figure 1b)**. The r_{cv}^2 values used for model optimization were r_{cv}^2 = 0.88 and r_{cv}^2 = 0.67 for ALP and INS, respectively.

Williams and Sobering (1996) demonstrated that RER and RPD should have values greater than 3 and 10, respectively. On the other hand, Saeys et al. (2005) reported that RPD values ranging from 2.0 to 2.5 may be used to make considerable quantitative prediction for some compounds, while asserting that good prediction is achieved with RPD greater values than 2.5. RPD values lower than 2.0 are commonly regarded as very poor and are not recommended.

In this study, the RPD and RER values in the UAB samples were 3.1 and 10.2 for ALP, and

2.4 and 11.9 for INS content **(Table 4)**, further establishing the accuracy of NIRS in predicting these metabolites which does not occur with the whole number of samples collected.

The low accuracy obtained for CRE may be related to the conventional standard methods of laboratory analysis, because as more steps are taken, laboratory errors increase, directly affecting the accuracy of the prediction models. This observation corroborates the fact that even with reductions in the number of samples, the values change insignificantly, thereby obtaining insignificant prediction values. Additionally, Kennedy et al. (1996) explained that such low values can be attributed to either a narrow variation range of reference values in the validation set (giving small standard deviation; SD) or to large NIR prediction error compared to SD values.

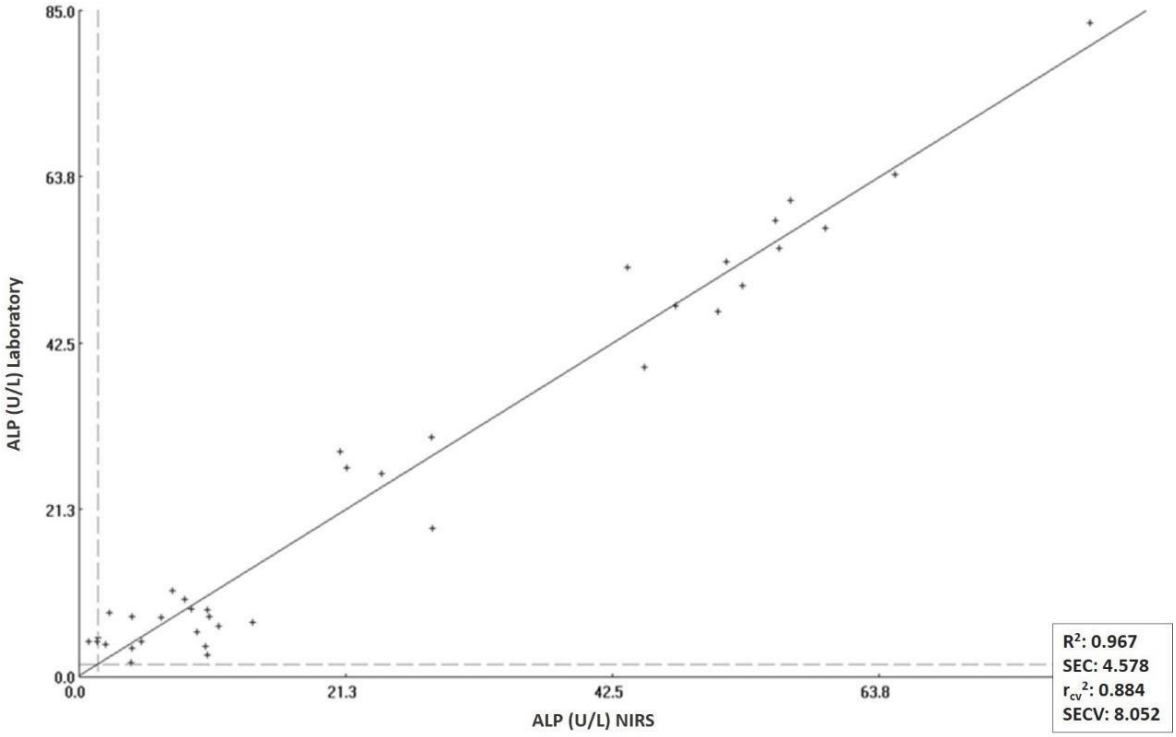
Table 4. Calibration and cross validation statistics for predicting the concentration of biomarkers (alkaline phosphatase, creatinine and insulin) in the milk of dairy goats es by NIR analysis

Item	Calibration				Cross validation			
	Mat treatment*	Scatter correction	R^2	SEC	r_{cv}^2	SECV	RPD	RER
All samples								
Alkaline Phosphatase	2.4.4.1	SNV + DT	0.75	11.0	0.205	19.1	1.5	7.2
Creatinine	2.4.4.1	SNV+DT	0.07	0.226	0.034	0.241	1.3	11.3
Insulin	2.4.4.1	SNV	0.56	0.212	0.233	0.277	2.2	11.1
UAB experimental farm:								
Alkaline Phosphatase	2.4.4.1	MSC	0.97	4.6	0.88	8.1	3.1	10.2
Creatinine	2.4.4.1	SNV+DT	0.41	0.297	0.351	0.305	1.2	8.1
Insulin	2.4.4.1	SNV	0.96	0.073	0.67	0.191	2.4	11.9

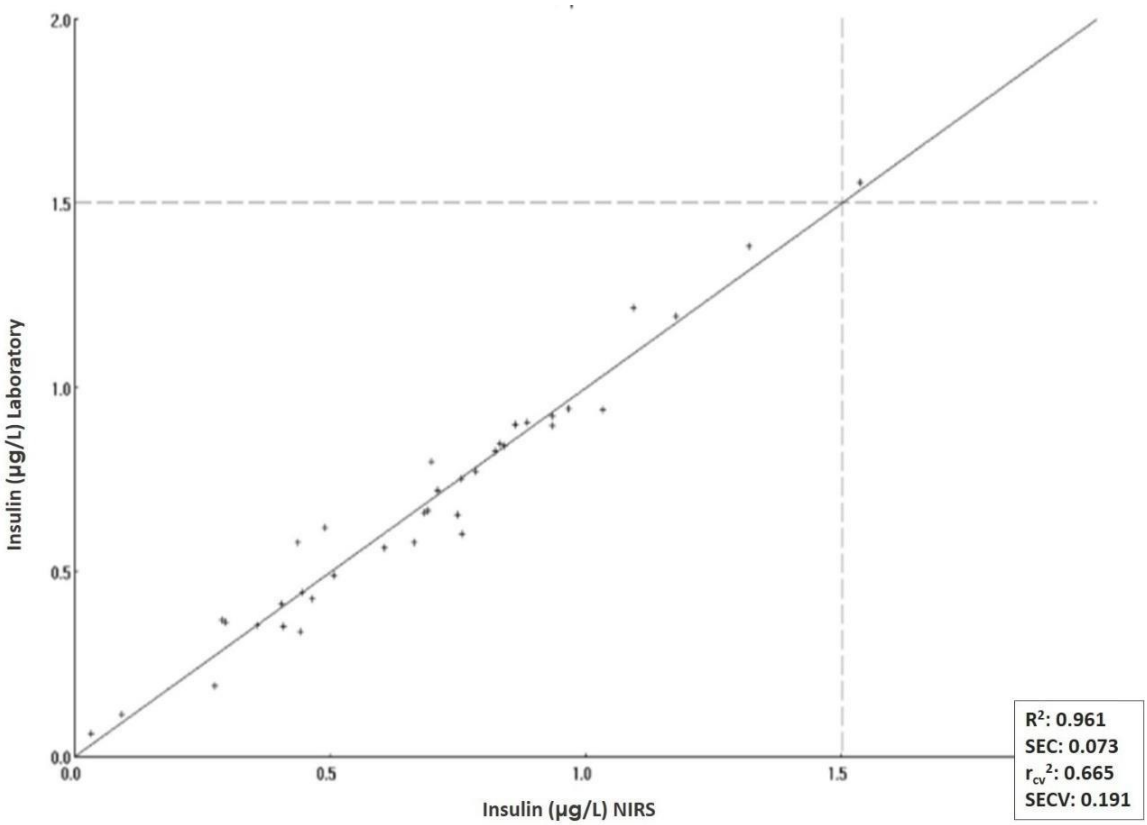
*Mathematic treatment: derivate order, subtraction gap, first smoothing, second smoothing.
SNV, standard normal variate; DT, detrend; MSC, multiple scatter correction; R^2, coefficient of determination for calibration; SEC, standard error of calibration; r^2_{CV}, coefficient of determination for cross validation; SECV, standard error of cross validation; RER, rang error ratio; RPD, ratio of performance to deviation.

Figure 1. Relationship between NIRS predicted data and metabolite values by reference analytical methods of Alkaline Phosphatase (ALP) and Insulin (INS) for the calibration set of the milk samples from the UAB dairy goats under experimental conditions

a) ALP



b) INS



In conclusion, a calibration model with low levels of accuracy and precision could not be recommended for monitoring CRE levels in the milk of dairy goats, whereas calibration models for ALP and INS in the UAB samples can be used for monitoring their values.

4.3. Impact of heat stress on milk biomarkers in Experimental Group under Controlled Conditions

HS causes unfavorable consequences for animal productivity and welfare, as well as economic losses for the global dairy industry. Decreased feed intake is a common response among HS animals, supposedly in an attempt to reduce metabolic heat production (Fuquay, 1981; Beede and Collier, 1986; West, 2003).

Dairy goats are considered to be resistant to THI values lower than 75, and THI higher than 80, 85 and 90 reflect the potential for modest, severe and fatal HS levels, respectively (Silanikove et al., 2015). However, Vitalino et al. (2012) indicate that THI > 79 values presents dangerous conditions for Saanen goats. On the other hand, Salama et al. (2016) indicated that upper critical THI values, though not previously established, should correspond to those causing rectal temperature to rise above 39.0ºC. Furthermore, Caja et al. (2019) reported in Murciano- Granadina goats rectal temperatures of 39.4ºC when THI = 74, the goats showing alterations in thermophysiological indicators (i.e., increased respiratory rate and water intake) but without impact on feed intake or milk production. These results confirm that there is not a simple relationship between THI and HS in dairy goats.

According to the previous observation, it can be stated that there are differences between studies and limited information on the effect of environmental conditions on different breeds, invalidating the generalized use of the THI values in dairy ruminants.

Metabolomics may be a holistic approach for the identification of biomarkers and to help in the development of strategies to detect and mitigate the metabolic disorders induced by HS (Tian et al., 2015; Contreras-Jodar et al., 2019). Accordingly, the metabolic variables analyzed in this study showed differences when TN and HS groups were compared, as shown in **Table 5.**

Table 5. Milk biomarkers of dairy goats under experimental environmental conditions (TN, Thermo neutral; HS, heat stress).

Milk metabolite	Treatment	THI[1]	Mean	SE
Alkaline phosphatase (U/L)	TN	65	30.1[a]	4.0
	HS	86	20.4[b]	3.5
Insulin (ng/mL)	TN	66	0.90[a]	0.08
	HS	85	0.60[b]	0.05

[1]Temperature and humidity index (NRC, 1971); [a, b]Different superscripts in the same metabolite indicates differences at $P < 0.001$.

Milk values of ALP decreased 32% by effect of HS in our dairy goats. Previous studies reported similar effects in dairy cows under HS (Vazhapilly et al., 1992). Moreover, the reduction of ALP was also reported in cows exposed to high temperatures by Ronchi et al. (1997) and Abeni et al. (2007). ALP has a relevant physiological role in the regulation of cell division and growth and acts on the transport of metabolites across the cell membrane (Swarup et al., 1981). Different ALP isoenzymes, which are a group known as zinc metal-enzymes, may be found predominantly (in descending order) in the placenta, ileal mucosa, kidney, bone and liver. The short half-life of intestinal or renal ALP partly explains its absence in serum (Kramer and Hoffmann, 1997) and its low values in milk, as demonstrated in this study. Furthermore, in males and non-pregnant females, serum ALP originates mainly from liver and bone tissues (Tennant 1997).

ALP is induced by glucocorticoids, carrying out the initial response of the non-specific ALP gene, expressed in kidney, liver, placenta and bone and, to a lesser extent, in other organs. Ronchi et al. (1999), correlated the endocrine acclimation response of cattle to hot environmental conditions and the alterations in energy metabolism, with decreased bowel and liver activity. In the same study, Ronchi et al. (1999) also observed a decrease in plasma ALP activity and a different hepatic activity index in heifers under HS. On the contrary, when pair- feed conditions were used (restricted feed) under TN conditions, plasma ALP activity increased, which correlates with an increase in liver activity and decreased metabolic rate due to the reduced dry matter intake in the heifers.

Similarly to ALP, values of INS decreased 33% in the HS group. This result is contrary to that reported by Baumgard et al. (2012) and Vernon (1992) in HS dairy cows which increased their INS basal levels and their sensitivity to INS, having a potent antilipolytic effect which may be responsible of the reduced body fat mobilization observed in HS dairy cows. Nevertheless, this is not observed in small ruminants under HS conditions, these being capable of controlling similar blood glucose levels compared with TN animals, with no change in blood INS concentration as observed in non-lactating ewes (Sano et al., 1985; Achmadi et al., 1993) and lactating dairy goats (Sano et al., 1985; Hamzaoui et al., 2013). According to this, making adipose tissue insensitive to INS seems to be key in enhancing the HS adaptation of dairy cows, but not in small ruminants, such as the dairy goats in the present study.

From these findings, it can be said that the decrease of ALP and INS values observed in this study can be employed, at least, as biomarkers of HS in dairy goats.

5. CONCLUSIONS

The results obtained in the present study show that NIRS can be a useful tool for predicting metabolites in the milk of dairy goats. Prediction accuracy of ALP and INS was acceptable when reducing the number of samples, due to environmental conditions of TN and HS group, and was also acceptable with regard to RPD and RER values achieved for those milk metabolites.

BHB did not present significant values in NIR Spectroscopy and cannot be used as a biomarker in dairy goats. The CRE value was not satisfactory when reducing the number of samples and had low accuracy, which may be a consequence of errors in the conventional standard methods of laboratory analysis, directly affecting the accuracy of the prediction models.

From the milk samples analyzed there was not a significant correlation between THI and HS for the dairy goats and environmental conditions used, demonstrating the fact that further studies are necessary to elucidate breed-specific characteristics and the equation of THI developed for dairy cows.

Despite the previous observations, the goats submitted to HS conditions exhibited significant changes in the values of some milk biomarkers, particularly ALP and INS, which can be interesting metabolites for studying individual responses to HS. Nevertheless, due to limited information previous to this study, further research is needed to investigate the thermophysiological mechanisms involved in the decrease of metabolite concentration in dairy goats under lactating HS conditions.

Nonetheless, HS causes productivity losses and can contribute to the occurrence of diseases, compromising animal wellbeing, thus being necessary to put in place early detection procedures, as suggested in the present study based on the use of typical milk metabolites, with the aim of implementing effective mitigation strategies in dairy goats.

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