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# Serum metabolomics assessment of etiological processes predisposing ketosis in water buffalo during early lactation

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## ABSTRACT

Metabolic disorders as ketosis are manifestations of the animal's inability to manage the increase in energy requirement during early lactation. Generally, buffaloes show a different response to higher metabolic demands than other ruminants with a lower incidence of metabolic problems, although ketosis is one of the major diseases that may decrease the productivity in buffaloes. The aim of this study was to characterize the metabolic profile of Mediterranean buffaloes (MB) associated with 2 different levels of β-hydroxybutyrate (BHB). Sixty-two MB within 50 days in milk (DIM) were enrolled and divided into 2 groups according to serum BHB concentration: healthy group (37 MB; BHB <0.70 mmol/L; body condition score: 5.00; parity: 3.78; and DIM: 30.70) and group at risk of hyperketonemia (25 MB; BHB ≥0.70 mmol/L; body condition score: 4.50; parity: 3.76; and DIM: 33.20). The statistical analysis was conducted by one-way ANOVA and unpaired 2-sample Wilcoxon tests. Fifty-seven metabolites were identified and among them, 12 were significant or tended to be significant. These metabolites were related to different metabolic changes such as mobilization of body resources, ruminal fermentations, urea cycle, thyroid hormone synthesis, inflammation, and oxidative stress status. These findings are suggestive of metabolic changes related to subclinical ketosis status that should be further investigated to better characterize this disease in the MB.

**Key words:** metabolomics, negative energy balance, Mediterranean buffaloes, $^1$H-NMR, ketosis

## INTRODUCTION

The transition period is defined as the period between 3 wk before to 3 wk after parturition, and it is critically important to the health and profitability of dairy cows as well as in buffaloes (Fiore et al., 2017). This phase is characterized by major physiological, nutritional, metabolic, and immunological changes (Raphael and Sordillo, 2013; Lisuzzo et al., 2022c). Buffaloes adjust their metabolism to deal with the considerable increase of energy and nutrient requirements needed for milk production which makes them susceptible to negative energy balance (**NEB**; Purohit et al., 2013). The metabolic adaptation to NEB requires interactions with different energy resources, and its failure may occur in various tissues such as the liver, adipose tissue, and others (Herdt, 2000).

Metabolic disorders in clinical or subclinical form are manifestations of the animal's inability to manage the greater metabolic demands (Sundrum, 2015). The metabolic response to lactation of buffaloes shows a different pattern compared with other ruminants, as demonstrated by the low incidence of metabolic disorders (Fiore et al., 2017). However, as reported for different buffalo species, a NEB is still one of the major concerns that may decrease the productivity in these ruminants and predispose to other pathologies and fertility disorders (Ghanem and El-Deeb, 2010; Youssef et al., 2010; Sundrum, 2015). The energy deficit is characterized by elevated concentrations of the ketone bodies BHB, acetoacetate, and acetone in blood (hyperketonemia), urine, and milk. Similar to cattle, the disorder could have a clinical and subclinical exhibition of diseases or production decreases in dairy buffaloes (Youssef et al., 2010) and represents the inadequate metabolic adaptation to contribute in the development of ketosis (Herdt, 2000). The gold-standard test to diagnose an energy deficit is the measurement of serum BHB concentration. Nevertheless, a specific BHB threshold for dairy

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buffaloes has not been established and dairy cow reference ranges are often used (Youssef et al., 2010; Purohit et al., 2013). The early diagnosis of metabolic disorders can be performed by different metabolism analyses, and it is essential to properly treat ongoing disorders (Youssef et al., 2010; Gianesella et al., 2019).

The metabolic processes can be investigated using the metabolomics approach, which reflects the animals health status (Sun et al., 2017). Metabolomics is an important branch of system biology that studies endogenous metabolism stimulated by internal and external factors (Nicholson et al., 1999). In recent years, the metabolomic approach has been used in other ruminants as cows and sheep to study metabolic alterations associated with ketosis. The differences found between healthy and diseased animals concern different metabolites belonging to diverse metabolic classes with differences depending on the type of technique used (Zhang et al., 2017; Fiore et al., 2021; Lisuzzo et al., 2022b). The $^{1}$H-nuclear magnetic resonance ($^{1}$**H-NMR**) spectroscopy is one of the main platforms of metabolomics because the very simple sample preparation and highly reproducible molecule quantification counter-balance a sensitivity lower than the one granted by other platforms such as mass spectrometry (Jones and Cheung, 2007).

The serum metabolic profiling of buffaloes related to ketone bodies has not been investigated, to the best of the authors' knowledge. For all these reasons, the investigation has been performed in this dairy species, hypothesizing that different etiological processes predispose buffalo to ketosis at the start of lactation compared with other ruminant species. Based on the statements, the aim of the current study was to use $^{1}$H-NMR to assess the metabolomic profile of Mediterranean buffaloes (**MB**) at milking resumption to investigate the metabolic changes associated with different levels of energy deficit.

## MATERIALS AND METHODS

### Animals and Farm

The current investigation received an institutional approval by the Ethical Animal Care and Use Committee of the University of Naples Federico II (n.PG/2017/0099607). All the clinical procedures were performed by the investigators abide by the common good clinical practices (European Medicines Association, 2015). Moreover, the farmer was previously informed and in agreement with purposes as well as methods used. Finally, the protocol of this study was carried out according to the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU.

Sixty-two Italian MB were selected from an artificially induced seasonal calving herd (late winter–springtime) consisting of 400 dairy MB and located in Caserta district (Campania, Italy), between January 2019 and April 2019. The sample size was calculated according to Friedman (1982): assuming an effect size of 0.40, a correlation analysis with 0.90 power level, and a 2-tailed significant level of 0.05. All the animals were randomly selected within the entire group of fresh buffaloes (<50 DIM) available during the considered period. All MB were characterized by an average: BCS of 4.87 ± 1.03 points (9-point scale), parity of 3.77 ± 2.13, daily milk production of 14.50 ± 3.27 L, and 31.69 ± 11.90 DIM.

Regarding the farm, it was casually extracted within a group of 10 (n°5 placed in Caserta district and n°5 in Salerno one) regularly requesting consultancy services at the Veterinary Teaching Hospital – Didactic Mobile Clinic Service of the Department of Veterinary Medicine and Animal Production of Napoli (Italy). Moreover, the group of farms met the following eligibility criteria: (1) a similar herd size (~400 buffaloes, consistent along the year); (2) a feeding system TMR based, given 2 times/d; (3) the absence of a regular monitoring program for metabolic diseases; (4) housing and overall management system respecting the minimum welfare standard for buffalo (De Rosa et al., 2015).

On the farm, milking buffaloes were kept in a separate group up to 60 DIM, so all trial subjects were selected from one group. The animals were housed in a roofed area consisting of solid-grooved concrete floors in the walking and feeding alleys. The lying area was represented by elevated cubicles covered with rubber mattresses. As reported by De Rosa et al. (2015), a mean space allowance >16 m$^2$/head was guaranteed, as well as a minimum space of 0.75 m/head, and 0.08 m/head at manger and drinking frontage, respectively. Milking MB were milked twice a day by means of a herringbone parlor. Animals enrolled were fed 2 times/d with a TMR including as ingredients: dry-hay, ryegrass silage (plastic-wrapped baled) and corn silage, buffalo cake (Stick-Florido, Fusco, characterized by 23% of CP originating mainly from legumes and cereals, crude fat 5.5%, crude fiber 7.5%, ash 6.9%, sodium 0.32%), and sodium bicarbonate.

The chemical composition of the diets used during dry and early lactation periods are reported in Table 1. The composition of TMR was determined using a portable analyzer based on near infrared reflectance spectroscopy (AgriNIR Analyzer, Dinamica Generale s.p.a.).

### Clinical Procedures

This study used a cross-sectional experimental design. Mediterranean buffalo selected for enrollment were in-

**Table 1.** Feed chemical composition of TMR used for dry and milking Mediterranean buffaloes (MB)[1]

| Item | Milking MB | Dried-off MB |
|---|---|---|
| DM (%) | 57 | 70 |
| Percentage of DM | | |
| Starch | 22.6 | 7.50 |
| NFC | 37.0 | 18.0 |
| CP | 13.0 | 7.50 |
| ADF | 22.0 | 28.0 |
| NDF | 39.0 | 64.0 |
| Ash | 6.50 | 8.20 |
| Ether extract | 3.80 | 2.40 |

[1]Results obtained by portable analyzer based on near-infrared reflectance spectroscopy.

dividually confined to a trimming chute and submitted to complete a clinical examination to rule out organic or systemic diseases, as well as foot disorders recognized as causing a reduction of feed intake in MB (Guccione et al., 2016, 2018). A complete examination of the gastrointestinal systems (including forestomaches, small intestine, and large intestine) was performed according to Jackson and Cockcroft (2002), to assess the presence of ongoing clinically obvious pathologies, potentially resulting from a NEB, and the udder was examined for clinical mastitis. During the exam, the BCS of the animals was performed using a 9-point scoring system (score 4.5 = ideal BCS), according to De Rosa et al. (2005) and slightly modified by the authors, using visual inspection and manual palpation of 4 areas of the body where MB store fat: ribs, spine, hips, and base of the tail as reported also by Guccione et al. (2016). At the end of the examination procedures, blood sampling was performed by jugular venipuncture with a 10-mL monouse syringe (Becton Dickinson Hypodermic Syringe equipped with 21-gauge needle). Some drops of blood (obtained directly by the syringe) were immediately used for a BHB test in field (FreeStyle, Abbott), while the remaining amount of blood was placed into serum clot activator tubes (Vacutainer, Becton and Dickinson) and centrifuged (908 × *g* for 15 min at room temperature; centrifuge model DMO412, Giorgio-Bormac s.r.l.) to obtain the serum in field.

### Experimental Design and Biochemical Analysis

Samples were placed in a cool box (4°C) and brought at the same temperature to the reference laboratory of the University of Naples within 1 h of collection for further investigations. The obtained sera were immediately transferred to Eppendorf tubes (1mL of serum/tube) to obtain 2 aliquots for each animal. These aliquots were immediately sent on dry ice to the Department of Animal Medicine, Production, and Health at the University of Padua (Italy) arriving within 24 h. One aliquot of serum was stored at −18°C until biochemical analysis, and the other one was sent at the same temperature using a portable freezer (CoolFreeze CFX65 W professional, Dometic; minimum temperature −22°C) to the University of Bologna where it was stored at −80°C until metabolomic analysis by $^1$H-NMR.

Biochemical analysis was performed using an automatic analyzer (BT3500 Biotecnica Instruments s.p.a.). β-hydroxybutyrate concentration was determined by a RANBUT RX Monza test (Randox). Nonesterified fatty acid (**NEFA**) concentration was determined by a colorimetric method (NEFA RX Monza test; Randox). Glucose concentration was determined by Glucose Monoreagent, LR (Gesan s.r.l.).

Considering that there is no specific BHB cut-off for buffaloes to identify animals as healthy and hyperketonemic, we selected a subjective cut-off. Specifically, we used the mean BHB value of 0.4 mmol/L plus 3 standard deviations of 0.1 described in previous work of healthy buffaloes around 30 DIM (Fiore et al., 2017) to establish a subjective BHB cut-off of 0.7 mmol/L. According to serum BHB concentration obtained in the laboratory, MB were divided into 2 groups: healthy group (**group H**) enrolled 37 MB with level of BHB <0.70 mmol/L and group at risk of hyperketonemia (**group K**) enrolled 25 MB with level BHB ≥0.70 mmol/L.

### Metabolomics Analysis

An NMR analysis solution was created with 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt 10 m*M* in non-deuterium oxide, set at pH 7.00 ± 0.02 by means of 1 *M* phosphate buffer, also containing 10 μL of $NaN_3$ 2 m*M*. We employed 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt as the NMR chemical-shift reference, while $NaN_3$ avoided microbial proliferation as suggested by Zhu et al. (2020b). Serum samples were prepared for $^1$H-NMR by thawing them. This was done by placing the still-sealed Eppendorf tubes, each containing 1 mL of serum, under a gentle flux of air at room temperature for 10 min. After centrifuging the same Eppendorf tubes for 15 min at 18,630 × *g* and 4°C, 700 μL of supernatant was transferred to a new tube where 100 μL of NMR analysis solution was added. Finally, each of the so-obtained samples were centrifuged again at the above conditions (15 min at 18,630 × *g* and 4°C) immediately before analysis.

The $^1$H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker) operating at a frequency of 600.13 MHz, equipped with the software Topspin 3.5. Following Zhu et al. (2020a), the signals from broad resonances originating from large molecules were suppressed by a CPMG-filter composed by 400

echoes with a $\tau$ of 400 μs and a 180° pulse of 24 μs, for a total filter of 330 ms. The oxide of deuterium and protium residual signal was suppressed by means of presaturation. This was done by employing the cpmgpr1d sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7,184 Hz spectral window, with an acquisition time of 2.28 s.

Differences in water content among samples were taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006), more reliable than the once more common normalization on creatinine. Spectra phase was manually adjusted in Topspin, whereas the subsequent adjustments were performed in R computational language by means of script developed in-house (R Core Team, 2018). After the removal of the residual water signal, $^{1}$H-NMR spectra were baseline-corrected by means of peak detection, according to the "rolling ball" principle (Kneen and Annegarn, 1996), implemented in the baseline R package (Liland et al., 2010). The signals were assigned by comparing their chemical shift and multiplicity with the Chenomx software library (ver. 8.3, Chenomx Inc.). According to the Metabolomics Standard Initiative for metabolites annotation (Salek et al., 2013), this allowed the confidence in the identification of each metabolite to be of level 1. To apply NMR as a quantitative technique (Zhu et al., 2019), the recycle delay was set to 5 s, by considering the relaxation time of the protons under investigation. The molecules of the first serum sample analyzed were quantified by means of an external standard, by taking advantage of the principle of reciprocity (Hoult, 2011).

Molecules' quantification was performed by means of rectangular integration, considering one of the corresponding signals, free from interferences (Foschi et al., 2018).

### Statistical Analysis

Statistical analysis was performed with R software ver. 4.0.3 (R Core Team, 2018). Normal distributions of data were assessed using the Shapiro-Wilk test. The comparison between the 2 groups was conducted by one-way ANOVA for normally distributed data and the unpaired 2-samples Wilcoxon test for not normally distributed data. Data were expressed as least squares means and standard error of the mean. The significance threshold was set at $P$-value $\leq 0.05$. Parameters that presented a $P$-value between 0.05 and 0.1 were considered as a trend to significance.

The MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca) was used to assess metabolite fold change expressed as the ratio between group K and group H concentration of each metabolite. A volcano plot was generated using the fold changes and $P$-values of all identified metabolites. A principal component analysis (**PCA**), a partial least squares-discriminant analysis (**PLS-DA**), and an orthogonal partial least squares-discriminant analysis (**OPLS-DA**) were generated using autoscaling data (mean-centered and divided by the standard deviation of each variable) to highlight the trends of serum metabolome according to BHB value. The variable importance in projection (**VIP**) scores was applied to PLS-DA and OPLS-DA analyses to identify the metabolites contributing the most to variance between groups. A hierarchical clustering heatmap was then generated to identify trends of significant metabolites between groups. Using the MetaboAnalyst 5.0 software, the website of PubChem (https://pubchem.ncbi.nlm.nih.gov/), Human Metabolome Database (https://hmdb.ca/metabolites/), and Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/) were consulted to assess the function of significant and not significant metabolites (Shi et al., 2021). The software's function "Enrichment Analysis" was used to assess the metabolic pathways influenced by the increase of BHB concentration.

## RESULTS

No MB showed clinical signs of diseases at examination time. Mean animal demographic and biochemical data are provided in Table 2 for healthy animals and animals at risk of hyperketonemia. The only significant differences between groups were BHB ($P$-value $< 0.0001$) and aspartate transaminase (**AST**; $P$-value $= 0.034$) values.

A total of 57 molecules were characterized in MB serum samples: 27 AA and derivates, 9 organic acids, 5 alcohols, 4 carbohydrates, 3 amines and derivates, 2 fatty acids, 2 ketone bodies, 1 sulfone, 1 vitamin, 1 imidazole, 1 nucleoside, and 1 guanidine. Six of the quantified metabolites were significantly different between groups: glycerol, taurine, creatinine, acetone, acetate, and 3-hydroxybutyrate. Six of the quantified metabolites tended to be significant: methanol, formate, citrate, Glu, Pro, and Gly. The metabolite concentrations that differed between the groups or tended to differ and the fold changes are listed in Table 3, whereas the concentrations and fold changes of the remaining metabolites that did not differ are shown in Supplemental Table S1 (https://data.mendeley.com/datasets/c5h6wpv856; Lisuzzo et al., 2023a). The volcano plot displayed the association between the base-2 logarithm of fold change (x-axis) and the base-10 negative logarithm of $P$-value (y-axis; Supplemental Figure S1, https://data.mendeley.com/datasets/crx6hygbf8; Lisuzzo et al., 2023b). The PCA analysis was first conducted as an overview with

**Table 2.** Characterization of Mediterranean buffaloes (MB) categorized as healthy with low BHB (group H) or at risk of hyperketonemia group with high BHB (group K; BHB ≥0.70 mmol/L)[1]

| Parameter[2] | Group H (37 MB) | Group K (25 MB) | SEM | *P*-value |
|---|---|---|---|---|
| BHB (mmol/L) | 0.47 | 0.75 | 0.02 | <0.0001 |
| BCS | 5.00 | 4.50 | 0.18 | 0.112 |
| Parity | 3.78 | 3.76 | 0.39 | 0.966 |
| DIM | 30.7 | 33.2 | 2.18 | 0.447 |
| Milk yield (L/d) | 14.1 | 14.6 | 0.60 | 0.892 |
| Glucose (mg/dL) | 64.7 | 62.1 | 1.25 | 0.154 |
| NEFA (mmol/L) | 0.25 | 0.24 | 0.02 | 0.460 |
| CHO (mg/dL) | 77.2 | 83.0 | 5.77 | 0.486 |
| TGR (mg/dL) | 9.45 | 10.0 | 0.49 | 0.435 |
| AST (units/L) | 142.0 | 164.0 | 6.93 | 0.034 |
| ALT (units/L) | 48.3 | 48.9 | 2.08 | 0.837 |
| GGT (units/L) | 20.0 | 21.5 | 1.07 | 0.333 |

[1]Results obtained by automatic analyzer.

[2]NEFA = nonesterified fatty acids; CHO = cholesterol; TGR = triacylglycerol; AST = aspartate amino transferase; ALT = alanine amino transferase; GGT = γ-glutamyl-transferase.

an unsupervised method to identify the difference between groups. However, the groups were not well clustered (Figure 1A). Then, PLS-DA and OPLS-DA analyses were performed to maximize the separation between groups (Figure 1B and 1D). According to VIP score >1.5, the most important metabolites for this separation were acetate, BHB, acetone, and glycerol (Figure 1C and 1E). Two heatmaps were generated: the first one was generated for each MB enrolled (Figure 2A), whereas the second one reflects the trends within the group for significant metabolites (Figure 2B).

The identified metabolites were used to perform a quantitative enrichment analysis (Figure 3) to understand the metabolic pathways influenced by the increase of BHB concentration. Five metabolic pathways were influenced in group K: glyoxylate and dicarboxylate metabolism, pyruvate metabolism, glycolysis or gluconeogenesis, glycerolipid metabolism, and taurine and hypotaurine metabolism (Table 4).

## DISCUSSION

A specific BHB cut-point for hyperketonemia in dairy buffaloes has not been established and dairy cow cut-points (BHB ≥1.0–1.4 mmol/L) are often used (Youssef et al., 2010; Purohit et al., 2013; Lisuzzo et al., 2022b). According to the study of Fiore et al. (2017), BHB concentration has a tendency to increase from the prepartum to postpartum period in dairy buffaloes. In the current study, buffaloes had a BHB concentration under 0.50 mmol/L after 20 DIM. In this study, the BHB concentrations of both groups were under the threshold value for dairy cows. For this reason, animals enrolled in group H were considered healthy (BHB = 0.47 mmol/L; DIM = 31), whereas animals enrolled in group K were considered at risk of hyperketonemia (BHB = 0.75 mmol/L; DIM = 33).

### *Evaluation of Biochemical Analysis*

Ketosis is associated with lower glycemia and milk production (Youssef et al., 2010; Fiore et al., 2018). The increase of the energy requirement and fat for milk yield around the peak of lactation around 20–30 DIM can lead to a reduction of triacylglycerols which normally range between 14 and 25 mg/dL (Ciaramella 2014; Fiore et al., 2017). We detected no significant difference between groups in triacylglycerols concentration, though both groups had values below the normal reference range (9.45 and 10.0 mg/dL in group H and K, respectively) suggesting a state of energy deficiency at the peak of lactation. The peripartum period is generally associated with higher lipolysis rate due to

**Table 3.** Representative metabolites of Mediterranean buffaloes (MB) expressed as μmol/L of serum categorized as healthy with low BHB (group H) or at risk of hyperketonemia group with high BHB (group K; BHB ≥0.70 mmol/L)[1]

| Class | Metabolite | Group H | Group K | SEM | FC[2] | $Log_2FC$[3] | *P*-value |
|---|---|---|---|---|---|---|---|
| AA and derivates | Creatinine | 12.6 | 11.0 | 0.52 | 0.91 | −0.14 | 0.047 |
| (5 of 27) | Glycine | 138.0 | 127.0 | 4.13 | 0.92 | −0.12 | 0.067 |
| | Glutamate | 86.5 | 91.1 | 2.74 | 1.05 | 0.08 | 0.058 |
| | Proline | 29.1 | 27.8 | 0.61 | 0.96 | −0.06 | 0.068 |
| | Taurine | 10.7 | 9.56 | 0.37 | 0.90 | −0.16 | 0.040 |
| Organic acids | Acetate | 198.0 | 310.0 | 14.1 | 1.57 | 0.65 | <0.0001 |
| (3 of 9) | Citrate | 27.0 | 30.4 | 1.26 | 1.13 | 0.17 | 0.063 |
| | Formate | 7.52 | 8.20 | 0.25 | 1.09 | 0.12 | 0.064 |
| Alcohols | Glycerol | 17.7 | 15.6 | 0.63 | 0.88 | −0.18 | 0.022 |
| (2 of 5) | Methanol | 3.25 | 2.86 | 0.15 | 0.88 | −0.19 | 0.096 |
| Ketone bodies | 3-Hydroxybutyrate | 58.3 | 76.0 | 2.23 | 1.30 | 0.38 | <0.0001 |
| (2 of 2) | Acetone | 2.76 | 3.65 | 0.21 | 1.32 | 0.41 | 0.003 |

[1]Results obtained by $^1$H-nuclear magnetic resonance spectroscopy.

[2]Fold change expressed as ratio between group K on group H.

[3]Base-2 logarithm of fold change.

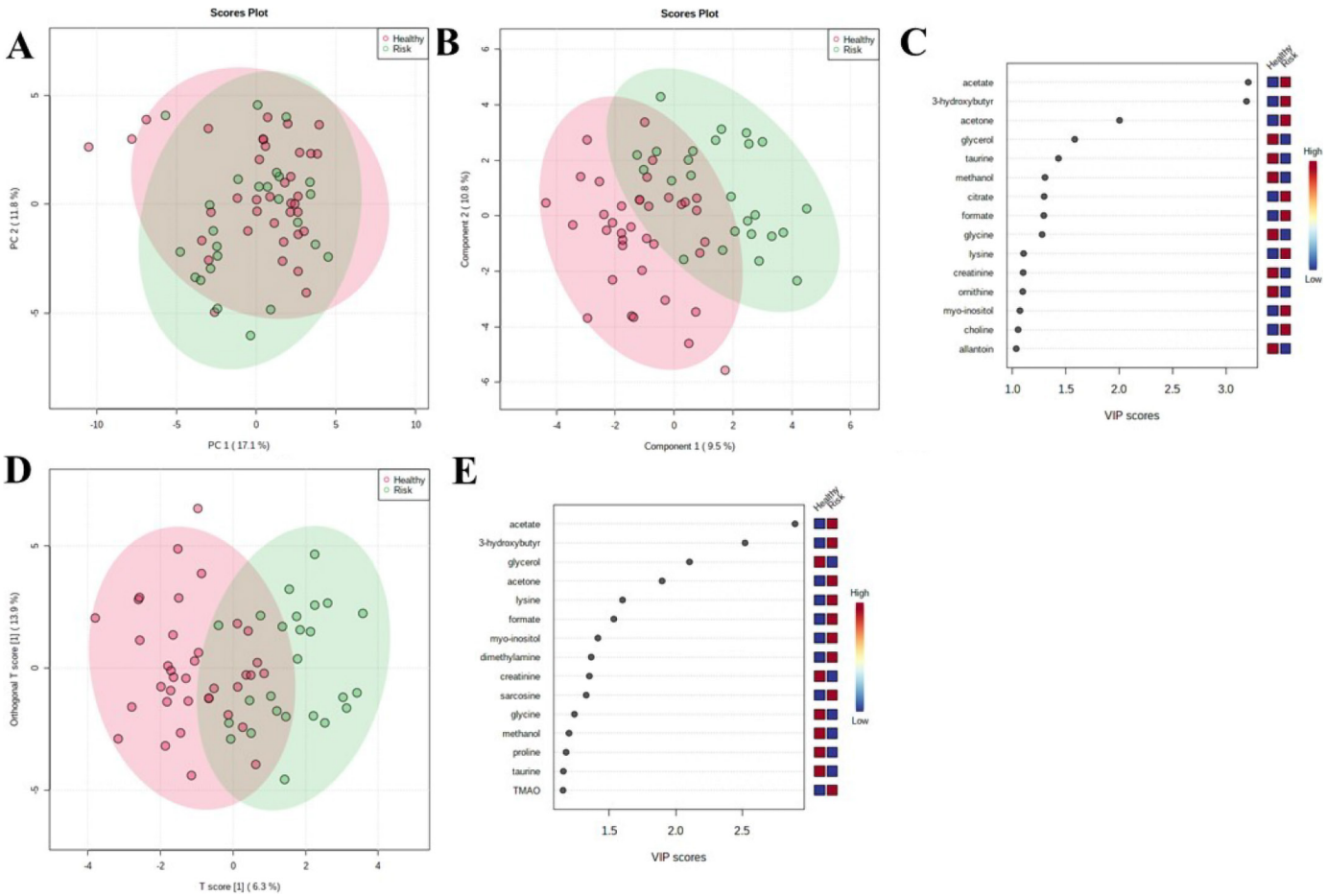


**Figure 1.** Results obtained by the Chemometrics Analysis performed with MetaboAnalyst 5.0. (A) Scores plot of principal component analysis between principal component (PC) 1 and 2 applied to group H (Healthy) and group K (Risk). (B and D) Scores plot of partial least squares of discriminant analysis (B; PLS-DA) and of orthogonal partial least squares of discriminant analysis (D; OPLS-DA) applied to the groups. The variance displayed in the plot is the explained variance for X. (C and E) Variable importance in projection (VIP) scores of the most important metabolites that differ between groups for the PLS-DA (C) and for OPLS-DA (E). The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in the 2 groups.

higher energy demands, which leads to an increase in NEFA concentrations. Nonesterified fatty acids are metabolized in hepatic tissue via complete oxidation to energetic production or partial oxidation to generate ketone bodies. Moreover, NEFA increase is often associate with BHB increases (Fiore et al., 2017; Lisuzzo et al., 2022a). In this study, NEFA concentration was not different between the groups which may be due to the NEFA increase occurring before study measurements began (McCarthy et al., 2015). Liver injury may lead to an increase of hepatic enzymes such as AST. According to the literature, increased AST of 122 units/L has been previously reported in ketotic buffaloes due to fat infiltration in the liver (Russell and Roussel, 2007; Youssef et al., 2010). In this study, AST concentrations were different between groups with a concentration of 142 and 164 units/L in group H and K, respectively. The higher values of AST may suggest a potential state of hepatic lipidosis in both groups, with a possibly worse state in group K. However, the normal range of cholesterol concentration suggested the absence of fatty liver suggesting that the increment of AST is due to another type of liver or muscle injury (Van Saun, 2000; Russell and Roussel, 2007).

### *Assessment of Metabolome Trends*

The PCA, PLS-DA, and OPLS-DA analyses are multivariate statistical methods that summarize and transform hundreds of metabolite features into few key components (Xia et al., 2013). Among them, the PCA analysis is generally used as an exploratory clustering technique, whereas the PLS-DA is a supervised method which maximize group separation. Furthermore, the

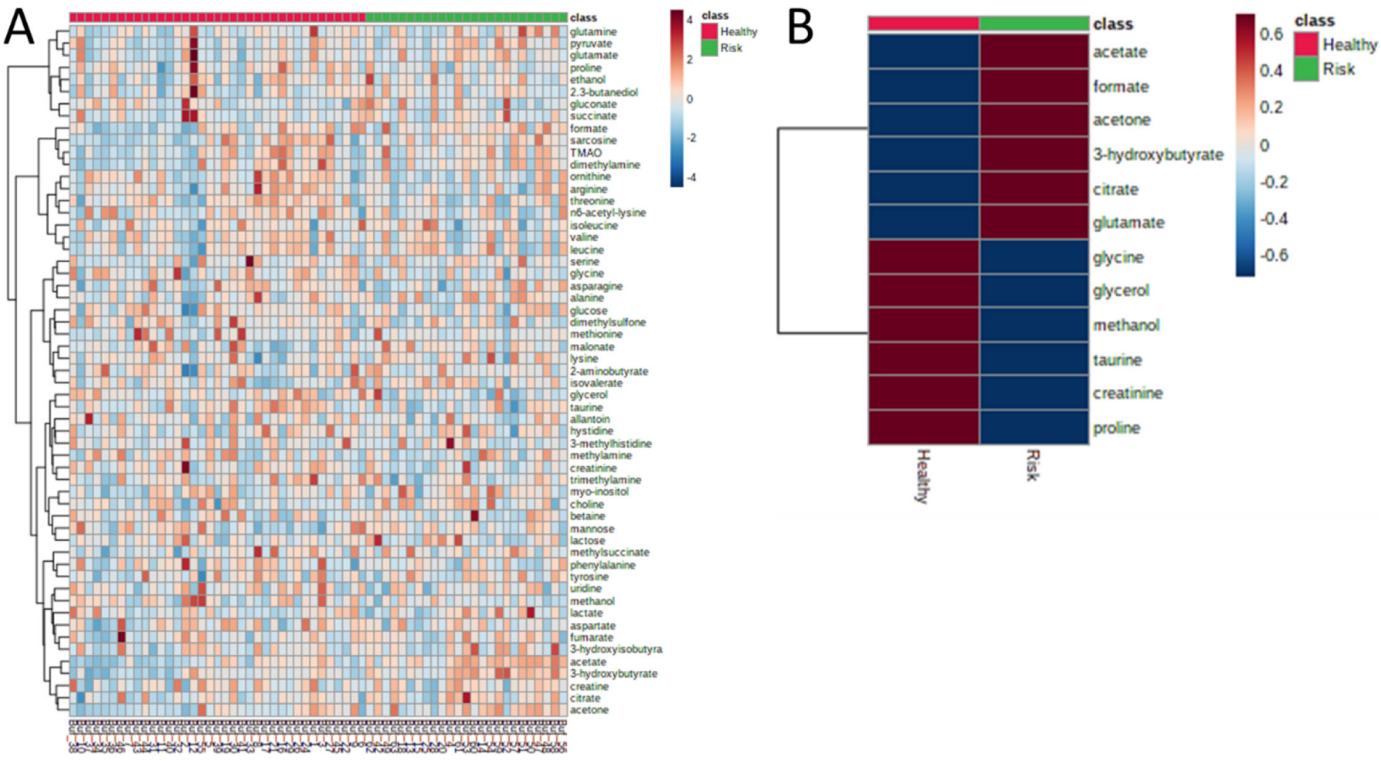


**Figure 2.** Results obtained by the Cluster Analysis performed with MetaboAnalyst 5.0. (A) Heatmap of all metabolite concentrations on 62 Mediterranean buffaloes (MB). (B) Heatmap of significantly different metabolite concentrations in group H (Healthy) and group K (Risk). The top right shows the colorimetric scale: if the color tends to dark red, the metabolite concentration was increased; if the color tends to dark blue, the metabolite concentration was decreased. On top right is also present the class of group H (Healthy; red) and group K (Risk; green), which is represented in the first line of each heatmap.

OPLS-DA removes the systematic variation that is not correlated with classes improving the interpretation (Sundekilde et al., 2013). Our PCA analysis failed to generate a net division between groups. On the contrary, the PLS-DA and OPLS-DA analysis and its VIP plots showed a separation between groups principally due to acetate, 3-hydroxybutyrate, acetone, and glycerol concentrations. The first 3 metabolites showed greater concentrations in group K, while glycerol concentration was greater in group H.

***Metabolomic Analysis: The Lipid Mobilization.*** The mobilization of fat stores due to high energy requirement led to an increase of lipolysis in ruminants with a NEB status (de Vries and Veerkamp, 2000; Li et al., 2014). During lipolysis, the triacylglycerols were catabolize in their component, glycerol and NEFA, and released into blood stream (Contreras and Sordillo, 2011). Glycerol represent one of the glucose precursors that animals can use for gluconeogenesis (Drackley et al., 2005; Zhang and Ametaj, 2020). The glycerol concentration of our MB showed a reduction in group K. This reduction may be associated with its utilization for gluconeogenesis, possibly indicating a higher energy requirement supplied by glucose precursors. This context may suggest that group K presented a change of metabolism with early ketosis state. Furthermore, ketone bodies are products of fat metabolism that commonly increase during the higher energy requirements and the NEB status (Ceciliani et al., 2018; Puppel et al., 2019). Two ketone bodies (acetone and BHB) were also identified by metabolomic analysis. These metabolites increased in the K group although with a different absolute value than the biochemical analysis probably related to the different sensitivity of analysis.

***Metabolomic Analysis: Muscle Mobilization.*** Muscular metabolism is used to supply the energy requirement (Drackley et al., 2005). The mobilization of this tissue is related to a reduction of creatinine (Grasso et al., 2004). Creatinine is a spontaneous product of creatine or creatine phosphate breakdown and they are related to total muscular mass and cell energy metabolism (Kohlmeier, 2015; Megahed et al., 2019; Yanibada et al., 2020). In this study, creatinine concentration was reduced in group K, likely due to increased muscle metabolism, in agreement with previous reports (Nozad et al., 2012; Zhang and Ametaj, 2020).

***Metabolomic Analysis: Ruminal Fermentations.*** Acetate is a short-chain fatty acid produced

**Table 4.** Metabolic pathways influenced by the increase of BHB concentration (group K; BHB ≥0.70 mmol/L)[1]

| Metabolic pathway | Total[2] | Hits[3] | Metabolites[4] | *P*-value |
|---|---|---|---|---|
| Glyoxylate and dicarboxylate metabolism | 32 | 8 | Acetate, citrate, formate, glycine, glutamate, glutamine, pyruvate, and serine | <0.0001 |
| Pyruvate metabolism | 22 | 4 | Pyruvate, lactate, acetate, and fumarate | <0.0001 |
| Glycolysis/gluconeogenesis | 26 | 4 | Ethanol, pyruvate, lactate, and acetate | <0.0001 |
| Glycerolipid metabolism | 16 | 1 | Glycerol | 0.022 |
| Taurine and hypotaurine metabolism | 8 | 1 | Taurine | 0.040 |

[1]Results obtained by the enrichment analysis performed with MetaboAnalyst 5.0.
[2]Total number of metabolites related to metabolic pathway.
[3]Number of identified metabolites related to metabolic pathway.
[4]Name of identified metabolites related to metabolic pathway.

during ruminal fermentation, particularly by fibrous components of the diet (Drackley et al., 2005; Zhu et al., 2019). In this study, acetate concentration increased in group K in agreement with previous studies (Zhang and Ametaj, 2020; Lisuzzo et al., 2022b). Formate is another metabolite produced during ruminal fermentation from methanethiol (Saleem et al., 2013; Yanibada et al., 2020). The conversion of methanethiol to formate produces hydrogen sulfides and consumes hydrogen. This conversion is performed by anaerobic microbes that compete with methanogens, and for this reason formate level is negatively related to methane (Yanibada et al., 2020). Methanogens microbes can synthetize methane from methanol (Hook et al., 2010). In this study, formate increased in group K while methanol was reduced in the same group. These findings associated with the greater concentration of acetate may suggest an alteration of ruminal microbial population and fermentation according to BHB concentration in this study.

***Metabolomic Analysis: AA Metabolism.*** In this study, 4 AA (Gly, taurine, Glu, and Pro) were different between groups. Glycine, taurine, and Pro concentrations were reduced in group K, while Glu increased in the same group. Changes in these AA may suggest links with the urea cycle, tricarboxylic acid (**TCA**) cycle, oxidative stress, and thyroid hormone synthesis. However, further more specific studies are needed to assess their possible influence.

***Effect on Urea Cycle.*** Glutamic acid and Pro are linked to each other, because Pro is a precursor of Glu trough pyrroline-5-carboxylate (Kohlmeier, 2015; Albaugh et al., 2017). Furthermore, pyrroline-5-carboxylate is a precursor of Orn, an intermediate of the urea cycle (Zhang et al., 2013; Albaugh et al., 2017). Orn concentration was slightly reduced in group K. However, it was not significant in our study with a *P*-value of 0.12. The reduced concentration of Orn and consequent influence of the urea cycle were found in other studies related to ketosis (Zhang et al., 2013; Guo et al., 2019). The reduction of only Pro may suggest an initial influence of the urea cycle before reaching subclinical ketosis state.

***Effect on the Oxidative Stress Status.*** The oxidation of Pro to pyrroline-5-carboxylate generates a reactive oxygen species and creates a state of cellular oxidative stress (Krishnan et al., 2008; Kohlmeier, 2015). The presence of free radicals can be controlled by antioxidants such as glutathione (McPherson and McEneny, 2012). Glutamic acid and Gly are 2 of the 3 AA involved in glutathione synthesis. Moreover, hypotaurine, the reduced form of taurine, is reported to be protective for the cell's oxygen free radicals damages (Kohlmeier, 2015). The reduction concentrations of Pro, Gly, and taurine may suggest an increase of reactive oxygen species production and the use of antioxidant systems represented by glutathione and hypotaurine. This state may indicate an initial oxidative stress status according to the increase of BHB concentration.

## Relationships with Thyroid Hormone Synthesis

Glutamic acid and taurine can form the glutaurine, a metabolite involved into the regulation of thyroid hormone synthesis (Bittner et al., 2005; Kohlmeier, 2015). The glutaurine concentrations were reported as inversely linked to triiodothyronine (T3) levels (Bittner et al., 2005). According to the study of Fiore et al. (2018) on dairy buffaloes, the thyroid hormone levels tended to decrease during the postpartum period. The reduction of taurine may indicate an influence on glutaurine concentrations and a consequent effect on thyroid hormone levels.

## Relationships with the TCA Cycle

Amino acids are glucogenic substrates that can influence the TCA, with a consequent risk of developing ketosis. Glycine and Glu are precursors of pyruvate and α-ketoglutarate, respectively (Xue et al., 2018; Lisuzzo et al., 2022b). Pyruvate represents the starting

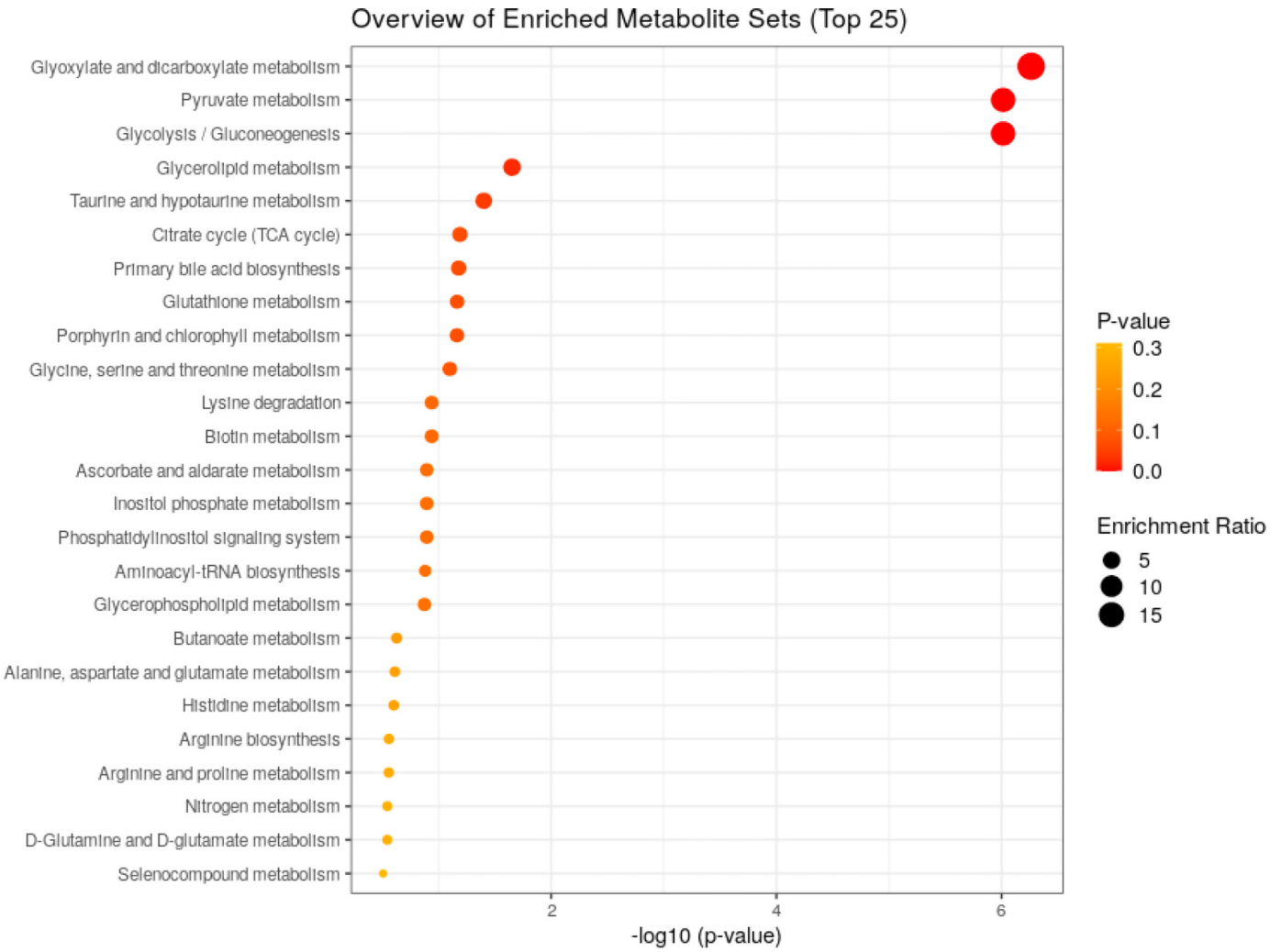


**Figure 3.** Dot plot of metabolic pathways influenced by the increase of BHB concentration (group K). Color gradient and symbol size represent significant metabolite changes in the corresponding pathway. Results obtained by the enrichment analysis performed with MetaboAnalyst 5.0.

point for TCA, by its conversion in acetyl-CoA, and gluconeogenesis. The Gly reduction may also suggest a potential reduction of pyruvate. However, it is the only precursor that presented a difference between the 2 groups. Furthermore, the joining of acetyl-CoA with oxaloacetate generates citrate, an intermediate of TCA (Duffield, 2000; Xu et al., 2020). In this study, citrate concentrations showed a tendency toward significative increment in group K. This finding associated with the higher concentration of Glu may suggest that the TCA cycle was not influenced in the group at risk of hyperketonemia.

## CONCLUSIONS

The higher levels of BHB in our group identified as being at risk of hyperketonemia may suggest metabolic changes related to ketosis such as (1) initial mobilization of body resources, (2) a potential state of oxidative stress, (3) possible changes in rumen microbial populations and fermentations, (4) an initial influence on the urea cycle, and (5) thyroid hormone synthesis. These results thus suggest the need to further refine BHB levels in buffaloes by identifying a specific threshold value for this species to clearly define animals as healthy or ketotic.

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J.C., M.C.A, and P.C.; analysis of data: E.F., A.L., L.L., V.F., B.C., and C.Z.; interpretation of data: E.F., J.G., M.C.A, and A.L.; drafting the work: E.F., J.G., M.C.A., A.L., V.F., and P.C.; revising the original draft: E.F., A.L., L.L., K.J.H., E.M., M.C.A., C.Z., B.C., V.F., and J.G.; and final approval of original draft: E.F., A.L., L.L., K.J.H., E.M., M.C.A., C.Z., B.C., V.F., J.G., and P.C. The authors have not stated any conflicts of interest.

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