Evaluation of the metabolomic profile through ^{1}H-NMR spectroscopy in ewes affected by hyperketonemia

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Abstract

Ketosis is one of the most important health problems in dairy sheep. The aim of this study was to evaluate the metabolic alterations in hyperketonemic ewes. Forty-six adult Sardinian ewes were enrolled between 7 ± 3 days after partum. Blood samples were collected in Venosafe tubes containing clot activator from jugular vein after clinical examination. The β-hydroxybutyrate (BHB) was evaluated on serum and used to divide ewes into two groups: Group H (serum BHB < 0.86 mmol/L) and Group K (serum BHB ≥ 0.86 mmol/L). Animal data and biochemical parameters of groups were examined with one-way ANOVA, whereas the metabolite significant differences were examined by t-test. A robust principal component analysis model and a heatmap were used to highlight possible common trends among metabolites. Over-representation analysis was performed to investigate metabolic pathways potentially altered in connection with the studied BHB alterations. The metabolomic analysis identified 54 metabolites and 14 were significant. These metabolites indicate altered ruminal microbial populations and fermentations; an interruption of the tricarboxylic acid cycle; initial lack of glucogenic substrates; mobilization of body reserves; the potential alteration of electron transport chain; influence on urea synthesis; alteration of nervous system, inflammatory response, and immune cell function.

Introduction

In dairy ruminants, high metabolic demand often occurs during late gestation and early lactation due to fetal growth and milk production [1]. Indeed, the greater fetus' growth (from 60 to 80% fetal body weight increase) occurs in the last month of pregnancy [2]. This demand tends to be concurrent with milk production, because pregnant animals are usually kept in lactation [1]. The mammary gland cells use around 80% of the blood metabolites such as amino acids, glucose and fatty acids to produce milk [3]. When energy demands are not met by dry matter intake (DMI), animals develop a negative energy balance (NEB) status with mobilization of body resources from adipose and muscle tissues [4,5]. The NEB increase the risk of metabolic diseases, such as ketosis [1,6].

Ketosis or pregnancy toxemia can occur in late gestation [7], while subclinical ketosis occurs mainly within the first two weeks postpartum and up to sixth week postpartum [8]. This metabolic disorder is characterized by a low plasma concentration of glucose and increase in plasma concentrations of nonesterified fatty acids (NEFA), derived from adipose tissue mobilization, and ketone bodies, derived from partially oxidation of NEFA [9,10]. In ruminants, the β-hydroxybutyrate (BHB) is the predominant ketone body in the blood [11]. It is commonly used to calculate energy balance during the last weeks of pregnancy and during the first weeks of lactation [2]. It represents the gold standard for diagnosing ketosis [12,13]. The blood BHB cut-off for subclinical ketosis in sheep is ≥ 0.86 mmol/L [14,15]. Furthermore, recent literature reveals that increased BHB concentration or hyperketonemia alone leads to a different BHB course compared to the control group in dairy cows. Therefore, preventing hyperketonemia is more important than managing or treating it [16].

Ketosis represents one of the most important health problems in high-producing dairy livestock (cows, sheep and goats) [17]. Major economic losses in ketotic sheep result from death of the affected animals, medication costs, production losses and triggering of secondary diseases [17]. Inadequate energy balance in pregnant sheep is dangerous not only to mothers, but also to pre- and perinatal viability and performance of lambs [1]. Indeed, limited metabolite availability during pregnancy may increase risk of low birth weight and perinatal mortality [1].

Metabolomics is a newest 'omics' science and a powerful tool to elucidating disease etiology, developing biomarkers to detect and characterize diseases as well as to monitor and predict complex diseases [18]. Nowadays, the prevalent technologies of metabolomics include nuclear magnetic resonance spectroscopy (NMR)[19]; gas chromatography-mass spectrometry (GC-MS)[20]; high performance liquid chromatography-mass spectrometry (HPLC-MS)[4] and thin layer chromatography - gas chromatographic techniques (TLC-GC)[21]. Among these techniques, the ^{1}H-NMR has important advantages, including its ability to provide more quickly results [22], requires easy sample preparation providing high sample efficacy and reproducibility [23].

In this study, we hypothesized that the development of hyperketonemia in ewes is associated with different groups of metabolites potentially related to ketosis development. These alterations can be detected using metabolomics approach and can better explain the etiopathogenesis of the ketosis process to safeguard animal health. In view of the above considerations, the aim of the current study was to evaluate the serum of hyperketonemic ewes using ^{1}H-NMR to assess the metabolic profile alteration.

Results

Main characteristics. The BHB, glucose and urea serum concentrations presented significant differences between the two groups (*p-value* < 0.001, *p-value* = 0.009, and *p-value* = 0.007, respectively). However, the two groups did not present a significant difference in NEFA, parity, BCS, DIM or daily milk yield (Table 1).

Table 1
Mean values ± standard of the mean (SEM) of clinical data and biochemical parameters of dairy ewes divided in group H and group K.

Parameters	Group H (n = 28)	Group K (n = 18)	*p-values*
Age (years)	4.60 ± 0.21	4.58 ± 0.20	NS[5]
Parity	3.19 ± 1.47	2.33 ± 1.50	NS[5]
BCS[1]	3.13 ± 0.69	2.61 ± 1.13	NS[5]
DIM[2]	4.88 ± 0.66	4.08 ± 1.22	NS[5]
Daily milk yield (Kg/day)	1.25 ± 0.06	1.22 ± 0.04	NS[5]
BHB[3] (mmol/L)	0.63 ± 0.12	1.35 ± 0.35	< 0.001
NEFA[4] (mEq/L)	0.17 ± 0.04	0.27 ± 0.05	NS[5]
Glucose (mmol/L)	4.07 ± 0.14	3.43 ± 0.19	0.009
Urea (mmol/L)	6.08 ± 0.33	7.66 ± 0.45	0.007
[1] Body condition score;			
[2] Days in milk;			
[3] β-Hydroxybutyrate;			
[4] Nonesterified fatty acids;			
[5] Not significant.			

Serum metabolome profile and robust principal component analysis (rPCA). In the serum samples were identified fifty-four different metabolites (Table 2). Among the identified metabolites, fourteen were significantly different between group H and group K (tyrosine, 3-methylhistidine, threonine, asparagine, glutamine, alanine, succinate, acetate, 3-hydroxyisobutyrate, methanol, ethanol, 2,3-butanediol, acetone and 3-hydroxybutyrate), and five tended to significance (valine, glutamate, histidine, arginine, and methionine). The first principal component (PC1) of its scoreplot, accounting for the 84.2% of all the samples' variance explained, nicely summarized the overall differences between samples of group K, appearing at low PC1 scores, and samples from group H, appearing at high PC1 scores (Fig. 1A). The loading plot (Fig. 1B) showed that the most representative metabolites of group K were 3-hydroxybutyrate, acetone, succinate, methanol, ethanol, 2,3-butanediol, acetate, 3-hydroxyisobutyrate, and 3-methylhistidine, while the most representative metabolites of group H were asparagine, alanine, glutamine, tyrosine, and threonine.

Table 2
Mean values and standard error of means (SEM) of identified metabolites (µmol/L) within the two groups (group H and group K).

Class	Metabolite	Group H	Group K	SEM	*p-values*
Amino acids and derivates	Asparagine	16.00	11.80	0.76	< 0.001
	Glutamine	59.20	47.50	2.00	< 0.001
	Alanine	57.40	48.90	1.78	0.001
	Tyrosine	9.94	7.70	0.44	0.001
	Threonine	36.70	29.80	2.02	0.013
	3-Methylhistidine	10.40	13.40	0.83	0.015
	Histidine	15.80	14.50	0.49	0.064
	Arginine	67.00	58.50	4.99	0.076
	Methionine	4.58	4.09	0.20	0.095
	Glutamate	61.10	55.50	2.47	0.096
	Valine	51.00	43.70	2.61	0.097
	Proline	20.30	18.90	0.76	NS[1]
	Serine	24.90	23.00	1.89	NS[1]
	Aspartate	1.63	1.41	0.10	NS[1]
	Lysine	14.40	14.10	0.84	NS[1]
	Isoleucine	25.90	25.10	1.29	NS[1]
	Leucine	49.80	47.70	2.12	NS[1]
	Dimethylglycine	3.63	4.05	0.24	NS[1]
	Glycine	125.00	139.00	7.79	NS[1]
	Betaine	20.90	20.20	1.87	NS[1]
	Phenylalanine	6.45	5.62	0.27	NS[1]
	Creatine	39.10	41.60	1.87	NS[1]
	Creatinine	1.16	1.14	0.10	NS[1]
	Taurine	24.70	21.20	2.41	NS[1]

Class	Metabolite	Group H	Group K	SEM	*p-values*
	Sarcosine	0.57	0.54	0.02	NS[1]
	N6-Acetyl-Lysine	6.76	6.58	0.37	NS[1]
	2-Aminobutyrate	6.47	6.56	0.28	NS[1]
Organic acids	Succinate	1.55	2.19	0.11	< 0.0001
	3-Hydroxyisobutyrate	3.60	4.52	0.25	0.009
	Acetate	134.00	170.00	11.55	0.025
	Formate	7.59	8.41	0.52	NS[1]
	Pyruvate	4.53	3.99	0.25	NS[1]
	Lactate	283.00	288.00	23.40	NS[1]
	Citrate	22.60	26.90	2.09	NS[1]
	Fumarate	0.68	0.76	0.04	NS[1]
Alcohols	2,3-Butanediol	0.86	2.35	0.31	0.002
	Ethanol	2.12	5.56	0.94	0.008
	Methanol	15.40	48.10	11.15	0.019
	Glycerol	18.40	19.00	2.09	NS[1]
	myo-Inositol	11.10	12.10	0.80	NS[1]
Carbohydrates	Glucose	1093.00	1017.00	46.70	NS[1]
	Mannose	7.03	8.12	0.72	NS[1]
	Lactose	10.90	11.40	1.86	NS[1]
Amine and derivates	TMAO	53.70	51.60	4.11	NS[1]
	Dimethylamine	0.40	0.47	0.03	NS[1]
Fatty acids	Isovalerate	3.63	4.05	0.24	NS[1]
	Methylsuccinate	0.57	0.78	0.12	NS[1]
Ketone bodies	3-Hydroxybutyrate	40.20	103.30	7.34	< 0.0001
	Acetone	6.05	19.52	1.88	< 0.0001

Class	Metabolite	Group H	Group K	SEM	*p-values*
Sulfone	Dimethyl sulfone	10.30	10.30	0.74	NS[1]
Vitamin	Choline	2.17	2.32	0.25	NS[1]
Imidazole	Allantoin	10.50	10.30	0.55	NS[1]
Nucleoside	Uridine	4.05	3.81	0.19	NS[1]
Guanidine	Methylguanidine	1.02	1.13	0.05	NS[1]
[1] Not significant.					

Over representation analysis (ORA). An over representation analysis of the significant metabolites was performed using “Enrichment Analysis” in the MetaboAnalyst 5.0 software to define which metabolic pathways were affected in group K. The identified metabolic pathways were presented graphically as a dot plot (Fig. 2). Although a total of 21 metabolic pathways were found to be associated with significant metabolites, only 6 metabolic pathways showed a significant alteration in group K (Table 3): i) Aminoacyl-tRNA biosynthesis; ii) Alanine, Aspartate and Glutamate metabolism; iii) D-Glutamine and D-glutamate metabolism; iv) Glycolysis / Gluconeogenesis; v) Glyoxylate and Dicarboxylate metabolism and vi) Phenylalanine, Tyrosine and Tryptophan biosynthesis.

Table 3
Metabolic pathways influenced by significative metabolites in hyperketonemic ewes with their *p-value*.

Metabolic pathways	Total[1]	Hits[2]	Metabolites[3]	*p-value*
Aminoacyl-tRNA biosynthesis	48	5	Asparagine, Glutamine, Alanine, Threonine and Tyrosine	< 0.0001
Alanine, Aspartate and Glutamate metabolism	28	4	Asparagine, Alanine, Glutamine and Succinate	< 0.0001
D-Glutamine and D-Glutamate metabolism	6	1	Glutamine	0.001
Glycolysis / Gluconeogenesis	26	2	Ethanol and Acetate	0.023
Glyoxylate and Dicarboxylate metabolism	32	2	Acetate and Glutamine	0.033
Phenylalanine, Tyrosine and Tryptophan biosynthesis	4	1	Tyrosine	0.036

[1] Total number of metabolites in the pathway based on the KEGG database;

[2] Number of metabolites influenced the pathways in this experiment;

[3] Metabolites identify by KEGG database

Discussion

Increased nutrient demand and decreased intake during early lactation may results in a NEB status [24,25]. Because of this, fatty acids and amino acids are mobilized from adipose and muscle tissues, respectively, predisposing animals to the risk of metabolic disease such as ketosis. Subclinical ketosis is less studied in dairy ewes but not less important because it can significantly affect farm economy [1,4]. Further studies about the metabolomics profile of animals would be of critical importance to improve the understanding about ketosis on small ruminants and to develop biomarkers for an early diagnosis [26,27]. Because of this, the aim of this study is the metabolomic analysis of serum in hyperketonemic ewes by ^{1}H-NMR.
The presence of hyperketonemia, hypoglycemia and uremia are consistent with an early subclinical ketosis status [28]. The group K showed a higher concentration of BHB and urea, and a lower concentration of glucose. The urea serum concentration was slightly over physiological range in sheep (2.86–7.14 mmol/L), whereas glucose concentration was within it (2.78–4.44 mmol/L), although reduced compared with group H [29]. The rPCA analysis is a multivariate statistical method used as an explanatory clustering technique to identify differences between groups of metabolome [30]. Our rPCA revealed a clear difference between the serum metabolome of groups. This might suggest that the metabolomic profile of ewes was firmly associated with different BHB concentration, even though group K was only hyperketonemic. From this point of view, hyperketonemic ewes may be considered separately from properly healthy animals.

The mobilization of adipose tissue is related to triacylglycerols break down into its components, NEFA and glycerol, with a release in blood stream [31,32]. In our study, the similarity both in NEFA and glycerol concentrations, may indicate that adipose tissue was not mobilized in group K. However, it is possible that an initial increase in NEFA and glycerol may be used by mammary gland to synthetize milk fat: an increase in fat percentage in milk is a common feature during ketosis [33]. The NEFA can be partially oxidized to ketone bodies such as acetoacetate, BHB and acetone in hepatic tissue [34]. BHB and acetone were identified by metabolomic approach in our study and they showed an increase in group K. Ketone bodies are responsible for alterations of inflammatory response [18]. They may also derive from ketogenic amino acids (lysine, leucine and isoleucine) [22] which did not changed in this study suggesting non-use to produce ketone bodies.

Leucine, isoleucine, and valine are branched-chain amino acids (BCAAs) used for protein synthesis in muscle. Low concentration of BCAAs are positively related to alanine concentration, which is a glucogenic amino acid highly concentrate in muscle [23,35,36]. Both alanine and valine showed a reduction in group K. The 3-methylhistidine come from muscle protein breakdown and it is considered as a biomarker of protein mobilization [4]. This metabolite showed an increment in group K. The lower concentrations of valine and alanine, and the higher concentration of 3-methylhistidine suggest an amino acids mobilization in group K. Tyrosine is also related to muscle metabolism. Indeed, its low concentration is an indicator of reduced muscle growth [37]. In this study, tyrosine was decreased in group K, in agreement with previously hypothesized. Creatine and its breakdown product, creatinine, are related to total muscle mass [19,38]. Creatine concentration is related to subclinical ketosis in dairy cows and weight loss in different ewe breeds [37,39]. The absence of changes in their concentrations may suggest that the total muscle mass was not still affected by protein break down.

There was an increase in methanol and ethanol concentrations in hyperketonemic ewes as well as in dairy cows[13]. Methanol is a potentially toxic compound which target is the retina in eyes and may explain the clinical sign of impaired vision during ketosis [14]. Methanol can be derived from methane, a gas produced during ruminal fermentation by microbial cells [40]. However, dimethylsulfone and formate showed similar concentrations between groups indicating that methane production was not affected [41]. Increase in methanol may also be related to an increase in ethanol concentration that inhibits the methanol's utilization by microorganisms [42]. Ethanol derives from anaerobic fermentation by yeasts. This alcohol is an agonist of GABA receptors, so it has a depressive effect. The major product of ethanol in hepatic tissue is acetate to provide energy. Acetate is a volatile fatty acid (VFA) produced by ruminal fermentation that increases during ketosis as reported in the present study. Acetate is an important energy substrate when bound to coenzyme A to produce acetyl-CoA and enter in tricarboxylic acid cycle (TCA). Moreover, it may be used in brain metabolism, specifically in glial cells, potentially causing mitochondrial permeability and excitotoxic neuronal death [12,23,43]. Acetyl-CoA can also be derived from 2,3-butanediol [44], a ruminal and intestinal microbial product that was increased in group K. Another VFA is propionate [12,45] which was not identified in our study, although the 3-hydroxyisobutyrate may result

from propionate and it increased in group K. The above changes may suggest an increment and alteration in ruminal fermentations in hyperketonemic ewes with potential relationships with the pathogenesis and symptoms of ketosis.

Myo-inositol is a stereoisomeric form of inositol and represents an insulin mimetic metabolite because it promotes adipose tissue lipid storage and limits lipolysis rate [46]. In our study, myo-inositol was similar between groups suggesting that the lipolysis process was not limited. Choline supports the transport of fatty acids, increases their oxidation and reduces the risk of hepatic lipidosis [22]. Choline can be converted in TMAO, a marker of oxidative stress because it reduces glycolysis and enhances β-oxidation of fatty acids [13]. Another metabolite related to β-oxidation is allantoin, a product of uric acid. Uric acid is related to triglycerides metabolism and its increase may limit enzymatic activity for their catabolism [39]. The analogous concentration of choline, TMAO, and allantoin may suggest that the β-oxidation of fatty acids was not influenced in our hyperketonemic ewes. Although subclinical ketosis is associated with an increment and alteration in lipid metabolism, group K did not show these characteristics. As previously mentioned, a possible increment of NEFA may be hidden by mammary gland, leading to a lack of fatty acids oxidation's alterations.

TCA begins with the combination between acetyl-CoA and oxaloacetate. Acetyl-CoA may derive from fatty acid catabolism or pyruvate oxidation. Pyruvate may derive from amino acids (glycine, serine and alanine) [47] among them only the alanine reduction in group K was significant. Glycine and serine are biosynthetically linked and they represent important regulators of glutathione synthesis to manage the oxidative stress [48]. However, they did not change suggesting the absence of an oxidative stress state, in accordance with the lack of influence of fatty acids oxidation. Alanine represents one of the major resources for gluconeogenesis, therefore it affects carbohydrate metabolism. Its lower concentration is related to ketosis and fatty liver in dairy cows [49,50]. Pyruvate did not change in group K as well as glucose identified by metabolomic analysis. Indeed, pyruvate can be used for gluconeogenesis to produce glucose [51]. These findings confirm that glucose concentration depends on pyruvate and a reduction of pyruvate due to lack of its precursors (glycine, serine and alanine) can significantly affect glycemia and the development of ketosis. Asparagine is one of oxaloacetate precursors [47] and showed a reduction in our hyperketonemic ewes in agreement with other studies [52]. This metabolite is involved in cell functions of nerve and brain tissue, and it is a nontoxic carrier of residual ammonia. In this study, oxaloacetate was not identified. However, the reduction of its precursor may indicate an oxaloacetate reduction and an alteration of TCA.

The following intermediates of TCA are citrate and isocitrate, which are maintained in equilibrium in the cell [53]. Isocitrate is subsequently converted to α-ketoglutarate that may derive from glutamate. Histidine, proline, glutamine and arginine are all metabolites related to glutamate production [54,55]. In accordance with the study of Zhang et al. (2017a), glutamate is an immune-regulator amino acid because it is involved in the activation and proliferation of immune system cells; genetic expression and production of cytokines and antibodies; and cellular oxide-reduction. Histidine presents antioxidant and anti-

inflammatory qualities for scavenging reactive oxygen species (ROS) generated by cells during acute inflammation [56] and for suppressing pro-inflammatory cytokine expression [57]. In this study, glutamate and histidine described a reduction in hyperketonemic ewes suggesting a potential oxidative stress state and immune suppression if the metabolic state progresses to ketosis. Glutamate may act as a neuroactive ligand for glutamate receptor 1, with a consequent excitatory effect. The reduction of glutamate may play an important role in nervous depression if ketosis develops. Glutamine can be converted in glutamate and after pyrroline-5-carboxylate, which links TCA and urea cycle. Arginine is converted into ornithine and urea in the final phase of the urea cycle [55]. The reduction of glutamine, glutamate, and arginine may suggest an alteration of this cycle, with a possible lack of urea synthesis in hyperketonemic ewes.

Succinate is the subsequent intermediate of TCA whose precursors are threonine and methionine [32]. Threonine is related to collagen production, regulation of the immune system, and secondary ketosis due to fatty liver syndrome [58,59]. Methionine is involved in protein synthesis, antioxidant production and methyl group donation. It can also be synthetized from choline oxidation. The choline production from methionine is relatively high in ruminants due to its degradation in the rumen [60]. In this study, threonine and methionine described a reduction in group K. These findings suggest a potential alteration of the inflammatory response, immune system functions and management of oxidative stress status other than their use to produce succinate, which was increased in group K, to obtain oxaloacetate for gluconeogenesis at the end of the cycle. However, fumarate is the next intermediate of TCA and not showed difference between groups. This metabolite may be synthetized by phenylalanine and tyrosine [47]. As previously mentioned, tyrosine showed a reduction in group K as reported in other studies [61]. These results may indicate that there was a disturbance of succinate dehydrogenase function and fumarate was maintained by its precursor. Succinate dehydrogenase is the only enzyme involved in TCA and in the electron transport chain (ETC) [62]. An alteration in succinate dehydrogenase might suggest an influence on ETC in hyperketonemic ewes as well as in hyperketonemic dairy cows [13].

The main function of aminoacyl-tRNA biosynthesis is to catalyze the aminoacylation of transfer RNAs (tRNAs) involved in protein synthesis, angiogenesis and immune regulation [63]. During Alanine, Aspartate, and Glutamate metabolism there is the biosynthesis of some amino acids (alanine, aspartate, asparagine, glutamate, and glutamine) and intermediates of TCA (oxaloacetate, citrate, succinate, fumarate). Therefore, this metabolic pathway is related with lipid, carbohydrates, and amino acid metabolisms. Furthermore, in human patient this pathway can be involved in the pathogenesis of metabolic syndrome [64]. The D-Glutamine and D-Glutamate metabolism was linked to Alanine, Aspartate, and Glutamate metabolism and concerns the glutamine/ glutamate cycling. The influence of Glycolysis/Gluconeogenesis further emphasizes how much energy production and glucose synthesis are required in hyperketonemic ewes. Glyoxylate and Dicarboxylate metabolism is used to produce carbohydrates from fatty acids in bacteria, protozoa, protists, and fungi. The influence of this metabolic pathway could result from an alteration of animal's microbiomes. Phenylalanine, Tyrosine, and

Tryptophan biosynthesis might be important because it was related to ubiquinone synthesis, which was involved in oxidative phosphorylation during TCA.

Conclusions

Our study demonstrates that the ^{1}H-NMR metabolomic approach allows detection of metabolic changes that occur in hyperketonemia and that may be related to the pathogenesis of ketosis. Indeed, the metabolic state of our hyperketonemic ewes suggests an alteration of ruminal fermentation; mobilization of body reserves; alteration in carbohydrates and amino acids metabolism; a potential alteration in ETC; influence on urea synthesis; alteration in nervous system, inflammatory response, and immune cell function.

Methods

All the procedures related to animals were conducted according to Directive 2010/63/EU of the European Parliament and of the Council of 22nd September 2010 on the protection of animals used for scientific purposes (Article 1, Paragraph 1, Letter b) and the Italian legislation (D. Lgs. n. 26/2014, Article 2, Paragraph 1, Letter b). The study has received the approval of the Ethics Committee of Sassari University, Protocol number 128469/2019. This study was carried out in compliance with the ARRIVE guidelines. Informed consent was obtained from the owners for handling the animals and for the clinical activity of the Veterinary Teaching Hospital, University of Padua.

Animals, Experimental design, and Blood analysis. A cross-sectional experimental design was used. The experiment was conducted on forty-six adult ewes, selected in the immediate post lambing within 10 days in milk (DIM) from a flock located in a commercial farm in North Sardinia (Italy). The animals enrolled for the present study belong to the same subjects evaluated in Fiore et al. (2021) previous study. In the study, all animals lambed twins and there were 11 primiparous, 11 animals with two parities, 10 with three parities, and 14 with at least four parities. All ewes were fed a total mixed ration (TMR) composed of 700 g of haylage banded, 400 g of hay, 200 g of silage maize, 150 g of soya, 150 g of flaked corn and 150 g of beet pulp according to animals' physiological and productive status. Milking sheep were fed a TMR formulated for lactating sheep (40–50 kg of body weight—BW) with 15% of protein content and a total energy value of the ration of 9.5 ME (Mj/kg DM). The ewes grazed 1 h/day natural pasture. Body condition score (BCS) was rated on a scale of 1 to 5 points, with 1 being emaciated and 5 being extremely fat [66], on the same day as clinical examination and blood sampling in each subject. Age and parity were also considered among characteristics of the ewes. Biological samples were collected from clinical healthy ewes that were examined by the Veterinarian of the University of Sassari (Italy).

Blood samples were collected at 7 ± 3 DIM from jugular vein through vacutainer system and stored in tubes containing a clot activator (9mL; Terumo Venosafe, Leuvel, Belgium) as described in the study of Fiore et al. (2021). The biological material was refrigerated at 4°C and transported at the same

temperature to the laboratory of the University of Sassari (Italy) within 1 h from the sampling. The blood samples containing clot activator were immediately centrifuged at 3000 rpm × 10 min in the laboratory (Hettich® EBA 20 centrifuge, Stuttgart, DE, Germany). Two aliquots of serum were extracted and sent to the University of Padua (Italy), at the department of Animal Medicine, Production and Health (MAPS) in dry ice within 24 hours. One aliquot of the serum was stored at − 20°C for the biochemical analysis, and one aliquot was stored at − 80°C for the metabolomic analysis by ^{1}H-NMR.

Serum biochemistry was performed in the laboratory of the Experimental Zooprophylactic Institute of Umbria and Marche (IZSUM, Perugia, Italy) as described in the study of Fiore et al. (2021).
All animals were divided in two groups based on their BHB blood concentration. Group H or healthy group enrolled 28 ewes with a blood BHB < 0.86 mmol/L and Group K or hyperketonemic group enrolled 18 ewes with a blood BHB ≥ 0.86 mmol/L [15]. Clinical data and parameters of the two groups were shown in Table 1.

Metabolomic analysis. An NMR analysis solution was created with 10 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) in D2O, set at pH 7.00 ± 0.02 by means of 1 M phosphate buffer containing 2 mM NaN3. TSP was used as an NMR chemical-shift reference, while NaN3 avoided microbial proliferation as suggested by Zhu et al. (2020). Serum samples were set up for ^{1}H-NMR by thawing and centrifuging 1 mL of each sample for 15 min at 18630 g and 4°C. 700 µL of supernatant were added to 100 µL of NMR analysis solution. Finally, each samples was centrifuged as previously mentioned.

¹H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with the software Topspin 3.5. Following Zhu et al. (2018), the signals from broad resonances originating from large molecules were suppressed by a CPMG filter comprised of 400 echoes with a τ of 400 µs and a 180° pulse of 24 µs, for a total filter of 330 ms. The HOD 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 256 transients using 32 K data points over a 7184 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 [68], more reliable than the once more common normalization on creatinine. The spectral phase was manually adjusted in Topspin, while the subsequent adjustments were performed in R computational language by means of a script developed in-house [69]. After the removal of the residual water signal, the ¹H-NMR spectra were baseline-corrected by means of peak detection, according to the “rolling ball” principle [70], implemented in the baseline R package [71]. The signals were assigned by comparing their chemical shift and multiplicity with the Chenomx software library (Chenomx Inc., Canada, ver 8.3).

In order to apply NMR as a quantitative technique [72], the recycle delay was set to 5s, 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 [73]. Molecule quantification was performed by means of rectangular integration, considering one of the corresponding signals, free from interferences [74].

Statistical analysis. Animal data and biochemical parameters were analyzed using the S.A.S. system software (version 9.4; SAS Institute Inc., Cary, North Carolina, USA) with a one-way ANOVA to evaluate the differences within the two groups (Group H vs. Group K). Statistical analysis of metabolites was conducted in R ver. 4.0.3 (R core team, Vienna, Austria) [69] computational language. The distribution of metabolites was assessed using Shapiro normality test. Data not-normally distributed were transformed according to Box and Cox [75]. The metabolites' differences between the two groups were evaluated by t-test for unpaired samples. A significance limit (p-$value$) ≤ 0.05 was accepted. Metabolites with p-$value$ between 0.05 and 0.1 were considered as trend to significance.
A robust principal component analysis (rPCA) model [76] was built up through the PcaHubert algorithm, implemented in the "rrcov" package of R the software in order to summarize the structure of the data. Firstly, the algorithm detects outlying samples, by computing their distance from the others along and orthogonally to the PCA plane. The optimal number of principal components (PCs) is finally determined. The rPCA model is summarized by a score-plot and a correlation plot. The score-plot highlights the overall structure of the data, by showing the samples in the PC space. The second plot highlights the molecules that mostly determine the structure of the data, showing the correlations between the concentration of each molecule and the PCs.

MetaboAnalyst 5.0 software (www.metaboanalyst.ca/MetaboAnalysts) is a comprehensive web-based tool designed to help users easily perform metabolomic data analysis, visualization, and functional interpretation [77]. The software function "Enrichment analysis" was used to perform an over representation analysis (ORA) on significant metabolites to understand which metabolic pathways were associated with greater BHB concentration. Through MetaboAnalyst software, the website of PubChem (https://pubchem.ncbi.nlm.nih.gov/), Human Metabolome Database (HMDB; https://hmdb.ca/metabolites/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) were consulted to understand metabolites functions.

Declarations

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Author contributions

E.F., and F.F. designed the study. F.F., and N.S. collected the samples. L.L., C.Z., and L.M. executed laboratory analyses. A.L., L.L., and V.F. performed the data analysis. A.L., L.L., and V.F. writing-original draft. A.L., L.L., F.F., K.H., E.M., and E.F. reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Data Availability Statement

The data are available by sending an email to the corresponding author.

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Figures

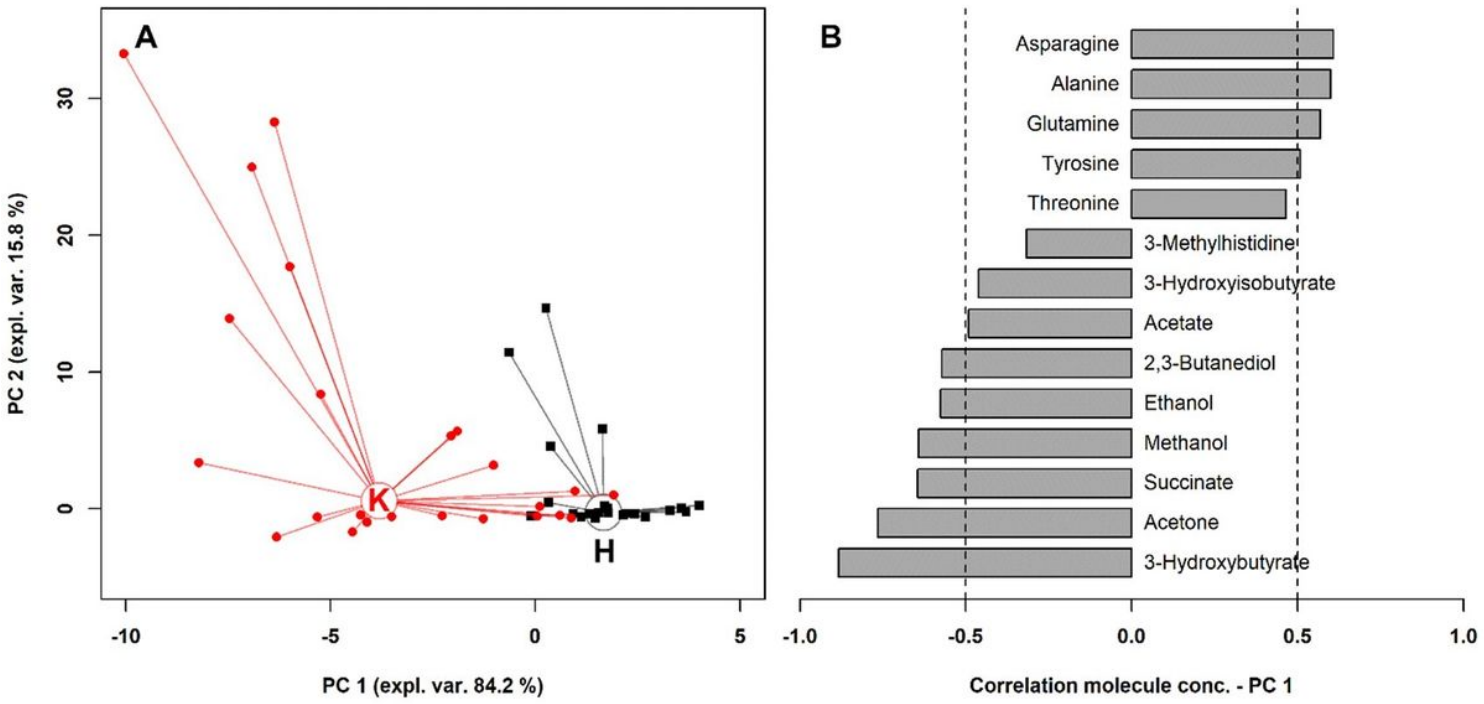

Figure 1

rPCA model built on the space constituted by the concentration of the molecules which showed a statistically significant difference between different groups. In the scoreplot (A), samples from the two groups are represented with black squares (Group H) and red circles (Group K). The wide, empty circles represent the median of each samples' group. The loading plot (B) reports the significant correlation between the concentration of each substance and its importance over principal component 1 (PC 1; $p < 0.05$).

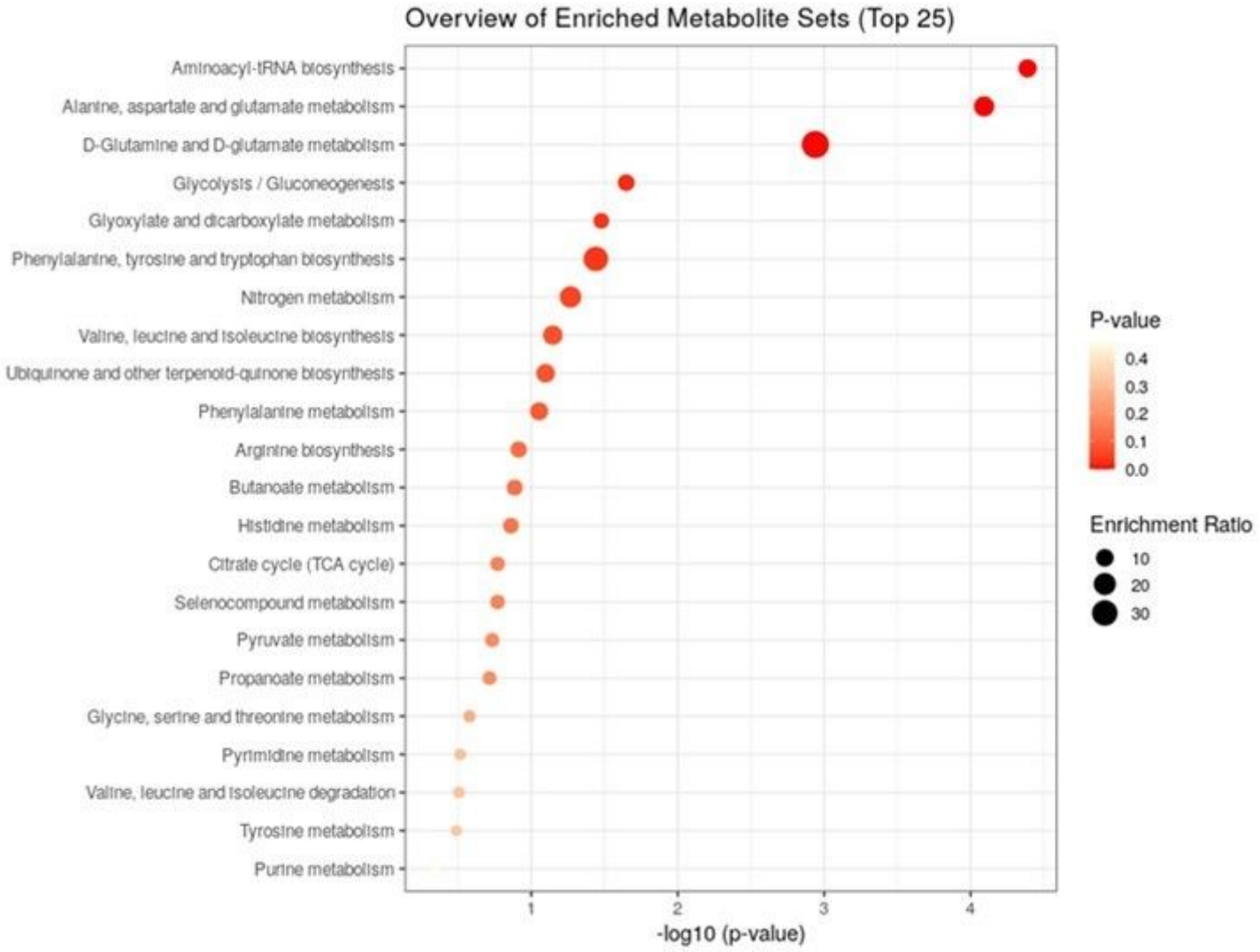

Figure 2

Dot plot of metabolic pathway influenced by statistically significant metabolites in hyperketonemic ewes or group K. Color gradient and symbol size represent significant metabolite changes in the corresponding pathway.