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Agricultural waste byproduct utilization: Date pit powder ^{1}H NMR metabolite identification for goat (*Capra hircus*) milk pathway analysis

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ABSTRACT

Agricultural waste byproducts (Date pits), are discarded in tons yearly and possess several nutritional advantages. Researches indicated that goat milk holds equivalent or superior potential than cow milk. Pathway analyses aid in uncovering biological mechanisms embedded within the dataset. This study concentrated on date pit powder (DPP) as a potential supplement for dairy goats to enhance milk quality, emphasizing on the route analyses. ^{1}H NMR analyses on DPP extracts was executed followed by chemometrics. Dairy goats were categorized into six groups ($n = 4$ / group): control, 10 g and 20 g for both Ajwa DPP (high quality) and Mariami DPP, and an additional 30 g for Mariami DPP exclusively. DPP supplementation was administered daily throughout a three-month duration. The goat milk was subsequently subjected to ^{1}H NMR studies, followed by chemometrics (PCA and PLS). Milk metabolites were subsequently analyzed using MetaboAnalyst software, with visualizations shown as heatmaps and pathway analyses. The ^{1}H NMR data of DPP samples from TCA and PB extracts (non-polar) successfully eliminated most metabolites in comparison to the aqueous ACN and MeOH extracts (polar). Following which, Ajwa and Mariami DPP exhibited nearly identical concentrations of the chemicals α-Glucose and flavanone, resulting in PCA clustering. The heatmap revealed varying milk metabolite concentrations, reflecting the animals' condition at months 1 and 3 due to DPP treatment. Six significant metabolic pathways ($p < 0.05$), with alanine, aspartate, and glutamate metabolism exhibiting the lowest p-value. DPP, an agricultural waste byproduct, is a viable alternative nutritional supplement at enhancing milk quality, as evaluated by metabolic pathways, in addition to reports of increased milk yield in other studies. This indirectly is in line with the sustainable development goals (SDG) in particular targeting on zero hunger; and good health and well-being.

1. Introduction

Approximately with date fruit yields of 9612,884 tons per year (Faostat, 2020; Faostat, 2023), dates produce abundant amounts of non-edible parts including, date seeds (Mateus et al., 2024) as agricultural wastes and that one of the biggest global concerns is food waste, due to its negative impact on the economy and environment (Osorio et al., 2021). However, some efforts have been made to minimize wastage. Milad et al. (2014) applied the ground date pits on goats, but to view the performance of growing male goats. Date pits are now used mainly for animal feeds in the cattle, sheep, camel and poultry industries, among others.

AL-Suwaiegh (2016) stated that goat milk is easier to digest and has longer shelf life compared to cow milk. In the developing world,

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majority of the people have low economic status, this situation make goats suitable to be readily be adopted as dairy animals. Moreover, Abd Rahman et al. (2022) supplemented date pit powder to dairy goats and found that with agricultural waste byproduct as a supplement, milk yield enhancement was possible. This was further emphasized by Zubairova et al. (2022) whereby nutritional or biotechnological technologies for dairy animals, it exhibited positive effects on milk yield and the general quality of milk and meat of cows.

Metabolites are intermediates in metabolic pathways in living organisms and their analyses due to nutritional effects would be of importance (Kanehisa et al., 2014). Cubero-Leon et al. (2018) specified that the objective of any metabolomics study is to screen and monitor many molecules over a diverse chemical spectrum and concentration range. The aim of such analyses is generally to identify and quantify small metabolites (compounds of low molecular-weight, Mw < 1.5 kDa) that result from the intervention. Metabolic pathways and chemical classes of compounds from databases offer apriori information that can be applied to assess altered trends with respect to groups of specific-related metabolites (Boccard & Rudaz, 2014). MetaboAnalyst is a software tool and has been used widely among others to elucidate key metabolic differences in breast cancer of African-American and Caucasian women, identify highly predictive biomarkers of ketosis in dairy cows, understand alterations in the intestinal metabolome during enteric infections and comprehend many other biological processes and complex diseases (Chong et al., 2018).

NMR has been an analytical platform for studying metabolites in plants (Hendrawati et al., 2006; Wu et al., 2014) and animals (Klein et al., 2010; Sun et al., 2014), among others. Hence, this study aims to highlight the profound metabolites in Ajwa and Mariami DPP extracts and the metabolic pathways affected by the DPP supplementation by analyzing the milk of the researched dairy goats.

2. Materials and methods

2.1. Animal ethics approval

Animal studies were approved by the Animal Ethics Committee (AEC) of Universiti Sains Islam Malaysia [USIM / AEC / AUP / 2016 (3)].

2.2. Chemicals

Trichloroacetic acid (TCA), phosphate buffer (PB) (pH 7.4), deuterium oxide (D_2O) and 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP) was acquired from Sigma-Aldrich, Germany.

2.3. Sample analysis

Prior DPP analysis, dried date pits were readily grounded to attain the powder form suitable for goat consumption and purchased from Syarikat Abdul Gaffar (SAG) in Penang, north of the Malaysian Peninsular. The method of (Wu et al., 2014) was used for the extraction of analytes from DPP. Four different solvents of 1.5 mL each were added to the DPP cultivars (100 mg) in parallel, which comprised of PB, aqueous acetonitrile (ACN) (50:50), aqueous methanol (MeOH) (50:50) and 1 M TCA. Two biological replicates for both Ajwa and Mariami DPP were used for each solvent group. All the mixtures were vortexed for 10 s and then kept in the chiller for 10 min. Extraction was further repeated twice and the three resultant supernatants were pooled together for assessment. The bulk of the organic solvents were removed by drying process, *via* evaporation using a vacuum concentrator (Concentrator Plus, Eppendorf, Germany).

Dried extracts from MeOH and ACN solvents were reconstituted in 1200 μL PB (0.1 M, pH 7.4) containing 10 % D_2O and 0.15 mM TSP. The extract from PB was added to 1080 μL water and 120 μL D_2O containing 1.5 mM TSP. Meanwhile, the extract from TCA was added to 920 μL water, 40 μL K_2CO_3 solution (1 M), 120 μL PB (1.5 M, pH 7.4), and 120 μL D_2O containing 1.5 mM TSP. For all the extracts, 540 μL of the final solution was added to the NMR tubes (i.d., 5 mm) for NMR measurements.

The ^1H NMR tests were conducted on DPP samples using the methods recommended by Klein et al. (2010). For the test on the samples, 500 MHz NMR spectroscopy (Varian Inc., Palo Alto, California, USA) was used with a ONE NMR probe with z-gradients. For each sample, the probe was automatically secured, calibrated, adjusted, and aligned. An optimized standard-shim file for the samples served as the first reference for the automated shimming process. The solvent signal was attenuated using the pre-saturation technique. Each sample underwent 64 scans. All spectra were recorded at 298 K (25 °C).

2.4. Data analysis

The ^1H NMR spectra were pre-processed utilizing Chenomx Processor, version 6.1 (Chenomx, Edmonton, Canada). The spectral areas with residual solvent signals for water (δ 4.50–5.24 ppm) were excluded to remove solvent signals. Furthermore, a binning approach was employed to extract the features of the metabolites from the spectra of the DPP extracts.

The binned integrated ^1H NMR spectra were transferred to Unscrambler X software (Camo Software AS, Oslo, Norway) for multivariate analysis. The data underwent maximum normalization before chemometric analysis, and all measured metabolites were analyzed using principal components analysis (PCA), an unsupervised multivariate pattern recognition technique designed to discern variations in metabolite profiles among the examined DPP extract samples. A supervised regression method, partial least squares (PLS), was subsequently employed to assess the individual effects. All PCA and PLS models underwent validation by segmented cross-validation (CV), with each segment corresponding to the replicates of each sample. Q2 quantifies this coefficient of variation.

2.5. Goat and goat milk handling

The goats were identified, managed and supplemented with DPP following the protocols in Abd Rahman et al. (2022). In brief, the study was conducted at a private farm in Sg. Buloh, Selangor, Malaysia (GPS: 3.197592; 101.524848). Six groups of one year old Saanen-Boer cross female goats ($n = 4$ per group) with a mean body weight of 24.89 ± 3.08 kg were used in the experiment. The off-spring (kids) were left to breast-feed for 1 week aft birth until which they were separated in the morning before feeding trial commenced starting on day 8th and lasted for 12 weeks for milk sampling. Individual milk samples on the specific monthly interval day (based on the onset of DPP supplementation) were then collected in 50 mL Falcon tubes that were separately labelled before being transferred using an icebox to a −20 °C freezer on the same day. Three biological replicates were applied from each goat.

2.5.1. Milk extracts

The milk extraction was performed according to the guidelines established by Klein et al. (2010). Before analyzing the milk sample, 100 mL of phosphate buffer (pH 7.4) was produced by combining 8.02 mL of 1 mol/L aqueous K_2HPO_4 solution with 1.98 mL of 1 mol/L aqueous KH_2PO_4 solution, and then diluting to 100 mL with deionized water. The buffer was stored in the chiller for future utilization. Subsequently, 400 μL of the ultra-filtrate is combined with 200 μL of 0.1 mol/L phosphate buffer (pH 7.4) and 50 μL of 29.02 mmol/L TSP in D2O as the internal standard.

Milk samples from the end of months 1, 2, and 3 of DPP supplementation in individual goats were thawed overnight at 4 °C, properly mixed, and centrifuged in an Eppendorf centrifuge at 4 °C for 20 min at 3000 rpm. The resultant upper layer (lipids) was extracted. A 3 % TCA was subsequently added to the solution and centrifuged at 4 °C for 15 min at 10,000 rpm. The supernatant was subsequently filtered using a 0.2 μm PTFE membrane filter and stored at −20 °C until further metabolomic and pathway analysis. An extra centrifugation stage at 5000 x G for 10 min was necessary before the analyses (Gaze et al., 2015).

2.6. Pathway and heatmap analysis

MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca) was utilized to produce the milk pathway analysis (depicted using the 'metabolome view') and heatmap analysis. Prior to the analyses, data were normalized by a reference sample, log-transformed and either mean-scaled, auto-scaled or Pareto-scaled for better data homogenization. The best-normalized data resulting from the data pre-processing procedure should have both kurtosis and skewness values near zero (Chong et al., 2018).

3. Results and discussion

3.1. Putative compounds in DPP extracts

Fig. 1 shows the ^{1}H NMR spectra of Mariami and Ajwa DPP extracts, respectively and pinpoints some of the different putative compounds extracted by the various solvents with references from Wu et al. (2014) and the library database in the Chenomx Profiler software. Satrio et al. (2024) emphasized that in an omics-based study, the selection of solvents to extract both targeted and untargeted compounds (metabolites) is crucial. Prior to seed metabolomics analysis, Kim et al. (2010) stated that a good solvent is characterized by its optimal extraction and its capability in conserving the stability of the chemical structure of desired compounds. Therefore, the type of extraction solvent and its polarity may have a significant influence on the level of extracted polyphenols, among others. One single solvent is not likely to extract all groups of metabolites as desired for meeting the objective of metabolomics.

It was observed that by using aqueous MeOH and aqueous ACN as solvents, relatively small numbers of peaks and low concentrations of compounds, as indicated by the peak height both for Ajwa and Mariami DPP extracts were produced in comparison to the phosphate buffer (PB) and trichloroacetic acid (TCA) solvents. PB and TCA buffers were able to extract much more and higher compound concentrations from the DPP extracts giving an overview of the date pit metabolite dynamics. Even though both aqueous MeOH and aqueous ACN solvents have higher polarity capacity compared to TCA and PB solvents, the latter extracts presented a higher concentration of non-polar compounds than those being polar when applying the extraction method by Wu et al. (2014).

The abundance of metabolites extracted by the four solvents was in the sugar region (5.5 – 3 ppm) of the ^{1}H NMR spectra for both DPP cultivars. For NMR, there is less limitation in solvent choice, though in view of the array of metabolites being extracted, a mid-polar solvent arrangement such as aqueous MeOH is often applied (Kim & Verpoorte, 2010). Aqueous MeOH is commonly used as an extraction solvent alone (Hendrawati et al., 2006; Alfaleh & Sindi, 2024) and / or in combination with CH_3Cl in a two-phase system yielding non- and polar extracts (Choi et al., 2004). However, a two-phase solvent system imposes an added separating step, which is the vaporization of solvents and redissolving of the residue in proper NMR solvent. Kim and Verpoorte (2010) showed that of all possible mixtures of MeOH–water that was experimented on a Brassica model, the MeOH and water (1: 1) combination appeared to be the most appropriate solvent as a diverse group of metabolites, including flavonoids, glucosinolates and phenylpropanoids that were all extracted. Moreover, Mallhi et al. (2014) reported that MeOH and acetone solvents were applied to Ajwa date seed, and the resulting extracts

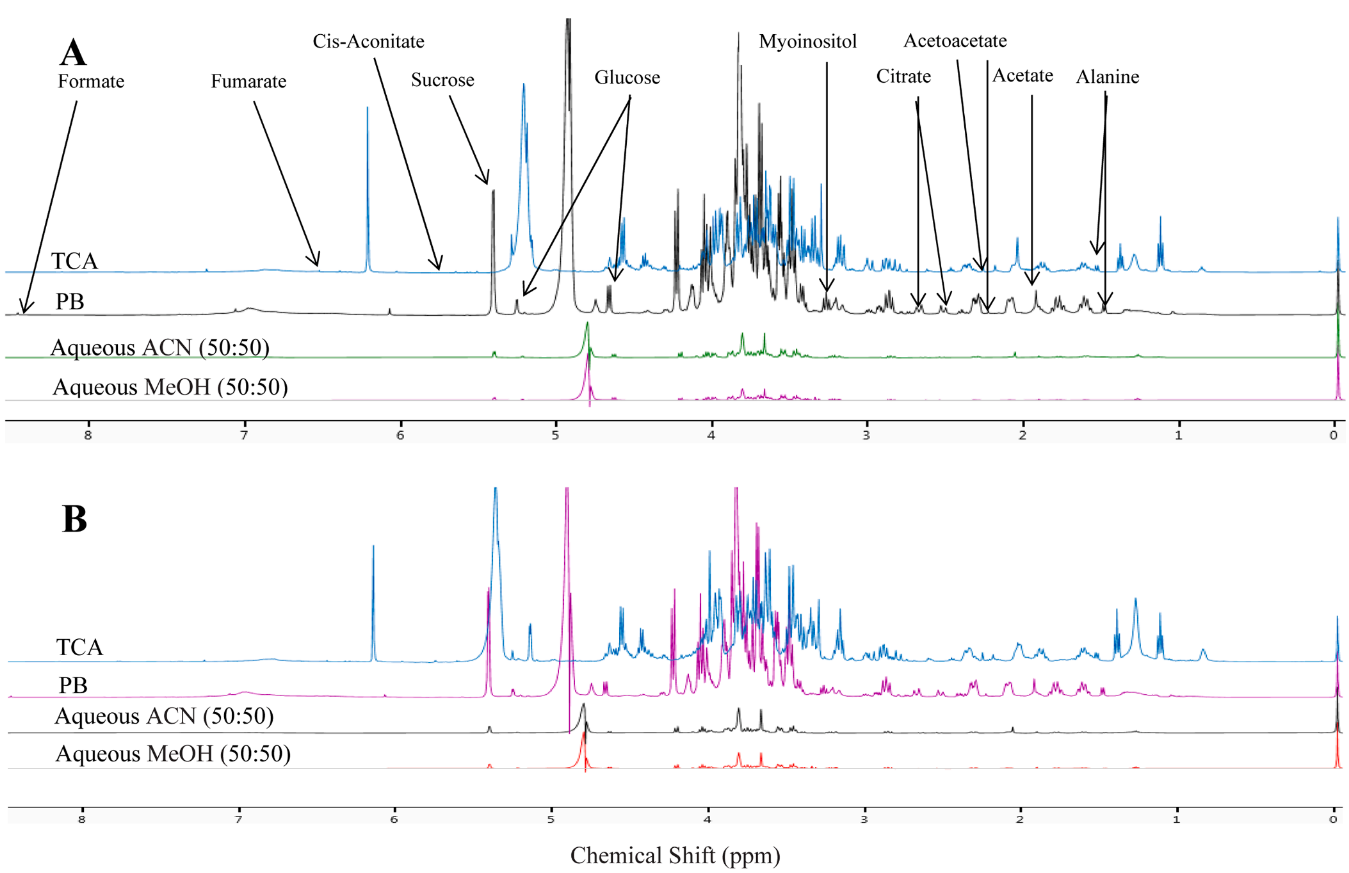

Fig. 1. ^{1}H NMR spectra of Mariami (**A**) and Ajwa (**B**) DPP extracts. Individual spectrums from the bottom in ascending order are as follows:- MeOH=Methanol; ACN=Acetonitrile; PB=Phosphate buffer; TCA=Trichloroacetic acid.

revealed a 1.15 and 1.40 (mg/ mL) of microbial inhibition concentration (MIC), respectively. In spite of the solvents mentioned above, Wu et al. (2014) also used perchloric acid, icy and hot buffer solvents but to mungbeans for metabolite extraction in a NMR-based plant seed metabolomic analyses.

The DPP was extracted twice for better metabolite coverage (Wu et al., 2014; Demiwal et al., 2024), who reported that a single extraction was not enough to attain sufficient metabolite extraction to exclude the tissue-to-solvent ratio (TSR) effects on metabolite composition. Results from their research indicated that for tissue extraction, the optimal TSR is also critically important especially when considering the saturation effects with high TSR.

The polarities of some compounds (*e.g.* polyphenols) extended from polar to non-polar. Nevertheless, an optimum extraction of polyphenols is usually attained in the polar solvent which have a better efficacy of solvation as a result of interactions (hydrogen bonds) between the polar sites of the antioxidant compounds and the solvent than the nonpolar compound (Farooq et al., 2021). Hence, water and an aqueous mixture of MeOH and EtOH are frequently applied for recovering polyphenols. Likewise in this current study, aqueous MeOH and aqueous ACN were used for the metabolite extraction purposes which then resulted in almost similar spectrum being produced from both Ajwa and Mariami DPP extracts. For acetone, however, it would give a lesser number of antioxidant compounds because of their lower efficiency of solvation. The acetone molecules are known as proton acceptors while MeOH and water are recognised as proton donors (Thouri et al., 2017).

3.2. PCA of DPP extracts

The PCA model in Fig. 2 displayed almost similar metabolites being extracted by all the solvents even though the DPP was of different cultivars. The 3.82 ppm signal was found to be pronouncedly increased in the PB solvent while TCA solvents extracted α-Glucose (5.18 ppm). The assignment at 3.82 ppm is a typical methoxyl signal and is associated with flavanone, as suggested by Nakashima et al. (2024). Furthermore, both aqueous MeOH and aqueous ACN solvents were capable of extracting compounds represented by the 4.78 and 4.82 ppm signals. These signals came from the solvent that was not completely removed, which was the water solvent of the aqueous MeOH and aqueous ACN. All these metabolites were unique to each of the respective solvents.

Metabolites (Fig. 2) in the yellow ellipse were increased in the TCA group but decreased in the aqueous MeOH and aqueous ACN groups. This is *vice versa* for metabolites in the green ellipse. This same concept is applied to the metabolites that are grouped in the red ellipse. They are increased in the PB group but decreased in the TCA-based extracts. These findings propose that there are not many differences in the DPP metabolite composition between Ajwa and Mariami DPP when comparing the different solvents.

Hamad et al. (2015) indicated that PCA is mostly applied to assess the variances among plant varieties at the metabolic level. Their resemblances and differences in the chemical composition among researched date cultivars can be clarified on the basis of the numerous metabolic reactions and environmental settings. Accounting for the number of replicates for the plant-based ^{1}H NMR experiment, Kim and Verpoorte (2010) used three biological replicates for each situation as compared to this study which only utilized two biological replicates. The results, however, depicted that it was still in a clustered pattern.

3.3. Metabolites and milk pathway analysis of DPP-treated goats

Diet is an essential factor that is vital to deliberate into in a metabolomics study (Chowdhury et al., 2023). Although DPP supplementations varied among the groups, experiments applying a biological setting would involve some variability, including genetic, that cannot be eliminated. The distortion triggered by these factors can be substantial and make statistical analysis less certain. Stressing on a specific pathway may limit the number of compounds needed for statistical analyses and possibly reducing only the effect of diet and, optimistically, some other potential factors. From a more thorough analysis, once a set of compounds of concern have been recognized, two kinds of tools can be useful to expand biological comprehension into the investigational results: **(i)** statistical enrichment analysis of metabolite annotations and **(ii)** mapping and visualization of pathways (Chagoyen & Pazos, 2012).

To further extend knowledge about the goat milk metabolome and the related metabolic pathways due to DPP supplementation, all significant matched pathways according to only p values of < 0.05 from pathway enrichment analysis and pathway impact values from pathway topology analysis (impact values > 0) were extracted as listed in Table 1. It was noted that inclusion of DPP in the goat's diet increased the levels of some metabolites associated with energy metabolism, suggesting a

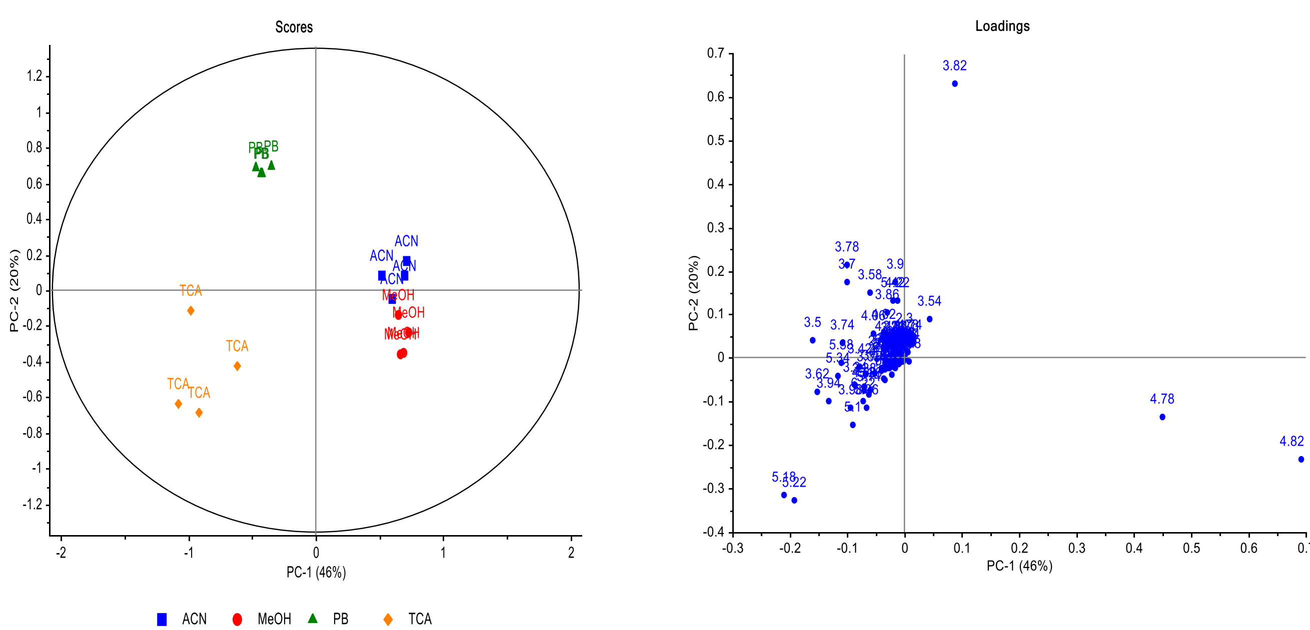

Fig. 2. PCA model of different DPP extracts from different solvents. Similar corresponding coordinates are correlated in the scores and loadings plots. ACN=aqueous acetonitrile (50:50); MeOH=aqueous methanol (50:50); PB=phosphate buffer; TCA=Trichloroacetic acid.

Table 1
Metabolic pathway analysis of pathways ($p < 0.05$) among all the experimental groups.

No.	Treatment	Metabolic Pathways	p-value	FDR	Impact
1	C *vs* A M1	Alanine, aspartate & glutamate metabolism (AAGM)	0.003	0.067	0.277
2	C *vs* M M1	Pyruvate metabolism (PM)	0.028	0.427	0.243
3		Glycolysis or gluconeogenesis	0.028	0.427	0.128
4	C *vs* A M2	–	> 0.05	–	–
5	C *vs* M M2	Glyoxylate & dicarboxylate metabolism (GDM)	0.014	0.460	0.407
6	C *vs* A M3	–	> 0.05	–	–
7	C *vs* M M3	Valine, leucine & isoleucine de gradation (VLID)	0.004	0.116	0.021
8		Synthesis & degradation of ketone bodies (KBM)	0.049	0.408	0.6

Note: FDR=False discovery rate; *C*=control; *A*=Ajwa DPP; *M*=Mariami DPP; M1=Month 1; M2=Month 2; M3=Month 3; Maximum limit of pathway impact=1.0.

metabolic disturbance in the goats and is supported by Abdelkrim et al. (2023), among others.

In interpreting metabolomic profiles using unbiased pathway models, myoinositol was among the content of the overlapped signals that comprised simple (glucose, lactose and myoinositol) and complex sugars, which is in line with an NMR-based study of human milk (Pratico et al., 2014) which was also detected in the DPP extract in Fig. 1. Caboni et al. (2017) acknowledged greater myoinositol levels in sheep compared to goat's milk in a metabolomics-based research. From another perspective, an altered glucid metabolism during fetal growth in neonates with intrauterine growth retardation (IUGR) has been estimated to be reflected by the escalation in extracellular myoinositol that may be revealed as a valid predictive marker of the development of obesity and type 2 diabetes in adulthood (Dessi et al., 2011).

Milk metabolites that are regarded as highly conserved either in month 1 or month 3 of the study are depicted in Fig. 3 (Heatmap). This is possible because the metabolism of individual goats differs at various times depending on the physiological state of the goat. Among the examples of the detected metabolites, glycerol was found to be considerably high in fluctuation (range). Glycerol is a key precursor for the synthesis of glucose *via* the gluconeogenesis pathway. When energy consumption escalates, the breakdown of body fat *via* the lipolysis pathway is also intensified, discharging glycerol and fatty acid into the blood. Then, in the liver, glycerol is altered to glucose to supply energy for subsequent cell metabolism (Sun et al., 2014). Citrate, on the other hand, was found to increase tremendously at the end of the feeding trial in the control group. However, the results were in contrast to (Wu et al., 2016), who detected a decline in milk citrate levels. Such a decrease is likely a response to the increased lactose secretion, hence balancing the osmotic pressure. Citrate is greatly an abundant component of milk and affects milk processing, fluidity and flavour features. It also plays a central role as an intermediary in cell energy metabolism and the TCA cycle (Wu et al., 2016).

Pyruvate secures energy *via* the TCA cycle. However, diminished levels of glucose *via* glycolysis prior to TCA impede the alteration of pyruvate to acetyl-CoA as in a negative feedback mechanism prior to entering the TCA cycle. Thus, pyruvate will yield more ketones to supply energy to extrahepatic tissues. The reduced consumption of pyruvate *via* the TCA cycle can affect the buildup of ketone bodies in the blood, leading to ketosis (Sun et al., 2014). This scenario was however unparalleled with this study's results whereby d-glucose levels were the least at month 2, yet at the same time having low levels of ketone bodies.

The metabolome view maps were depicted in Fig. 4, indicating significant ($p < 0.05$) metabolic pathways involved at months 1, 2 and 3, respectively, that clearly shows the relevancy and impact to the overall metabolism process which involved the detected metabolites. The *p*-value *vs*. pathway impact graph for the computed metabolic pathways permits the researcher to focus on the main significantly impacted pathways for further investigation (Farag et al., 2020). Based on the above metabolic pathway analysis, **(i)** alanine, aspartate and glutamate metabolism; **(ii)** pyruvate metabolism; **(iii)** glycolysis or gluconeogenesis; **(iv)** glyoxylate and dicarboxylate metabolism; **(v)** valine, leucine and isoleucine degradation and **(vi)** synthesis and degradation of ketone bodies were the key different metabolic pathways identified from the milk which may also be the most essential pathways linked with metabolic changes in this biofluid (milk) from goats supplemented with the DPP diet. Although tyrosine metabolism was significant in this study in month 3 (Figure not shown), it did not give an impact in the overall study compared to Sun et al. (2015), who identified that tyrosine metabolism from the significantly different metabolites from the four biofluids (rumen fluid, milk, serum and urine), due to alfalfa hay and corn stover-based diets given to dairy cows gave an impact to the animal's metabolism. The same researchers also revealed that the integrated key metabolic pathway study presented the dietary effects on dairy cows that may have more widespread amino acid metabolisms, proposing the metabolite-associated pathways may serve as biomarkers for higher milk production and improved milk protein quality.

The valine, leucine and isoleucine biosynthesis pathway attained the highest impact over the others, as shown in the metabolome view map of the common metabolites recognized, and interestingly, the opposite pathway (valine, leucine and isoleucine degradation pathway) was recognized at month 3 when Mariami DPP was given to the goats. In addition, metabolite comparative analyses among various animal species revealed that increased milk metabolites related to metabolic pathways (valine, leucine and isoleucine biosynthesis) might have also partly contributed to the higher milk protein contents in minor dairy animals (Jersey, buffalo, yak and goat) paralleled to Holstein cows. These results could provide new deliberations into milk protein synthesis in mammary glands among the animals (Yang et al., 2016). Lee et al. (2023) moreover analysed volatile metabolites in meat from two cow species that led to the flavor differentiation in them, hence empowering assessment for meat quality attainment.

In another view, Xi et al. (2017) discovered that the key metabolic pathways affected in subclinical and clinical mastitis groups of dairy cows consist of those directly associated with the carbohydrate and energy, lipid and protein metabolisms, which then gives researchers and farmers the opportunity to reduce the adverse effects of the conditions that leads to negative economic impact by doing necessary interventions.

3.4. Conclusions

The PCA presentation of ^{1}H NMR data of four DPP extracts revealed that Ajwa and Mariami DPP had almost similar highest compound concentrations giving a clustering effect depicted in the Scores plot. It is apparent that MetaboAnalyst software supports real-time interactive data analysis with six significant ($p < 0.05$) pathways that were recognized throughout the feeding trial due to DPP supplementation to the lactating goats as indicated in the metabolome view maps. However, there were no similar pathways identified being unique to the DPP supplementation. By identifying the metabolic pathways affected by the DPP nutritional intervention, this would guide researchers in manipulating potential milk quality improvement, for a positive economic impact in an animal-based setting as suggested by the United Nation (UN) in the sustainable development goals (SDG) in particular targeting on zero hunger; and good health and well-being.

CRediT authorship contribution statement

M.R.Abd Rahman: Writing – original draft, Methodology,

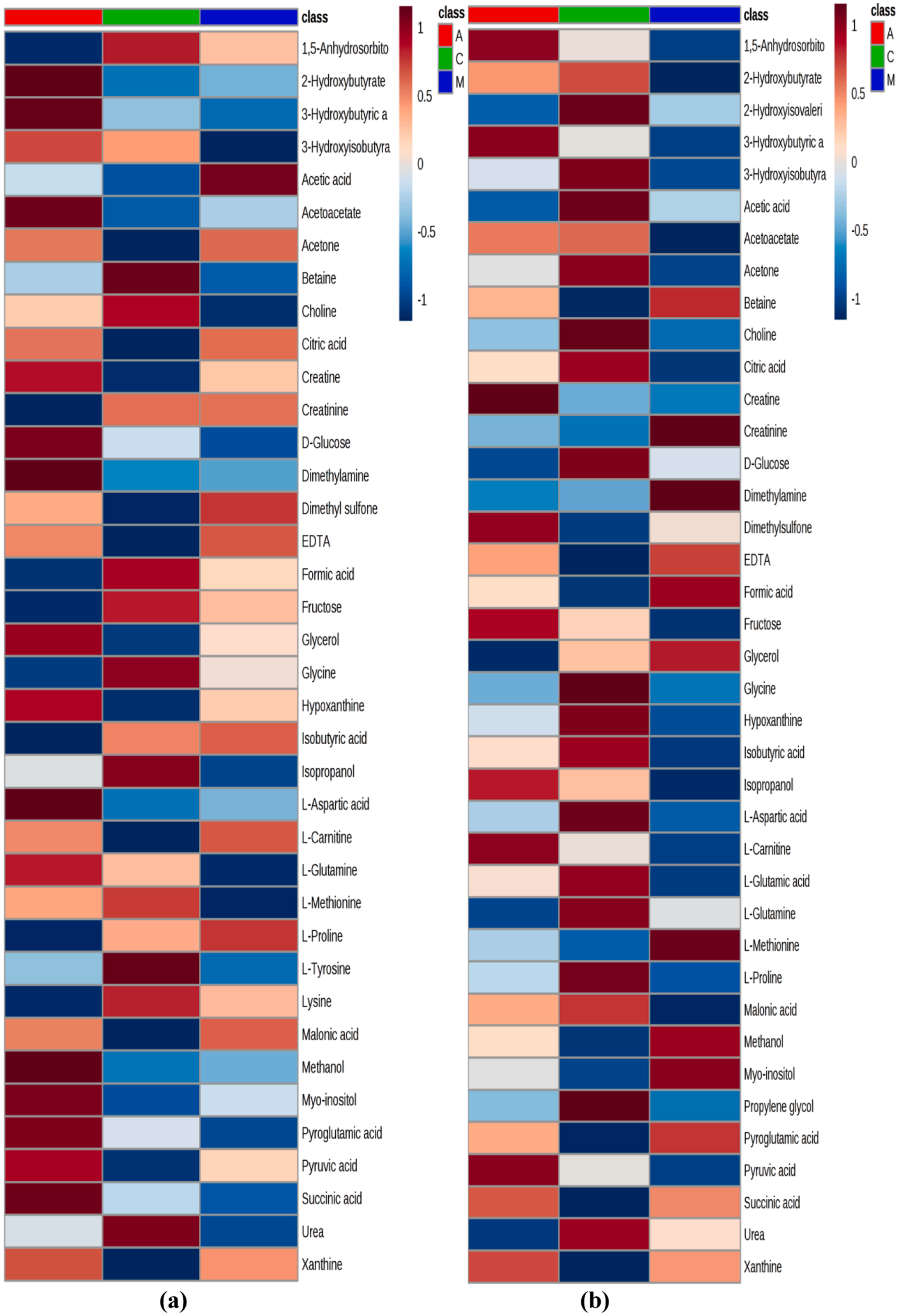

Fig. 3. The red-blue color system (Heatmap) of metabolite concentrations from the three groups at (**a**) month 1 and (**b**) month 3 of the trial. Green=mean of control groups; Red=mean of Ajwa DPP groups; Blue=mean of Mariami DPP groups.

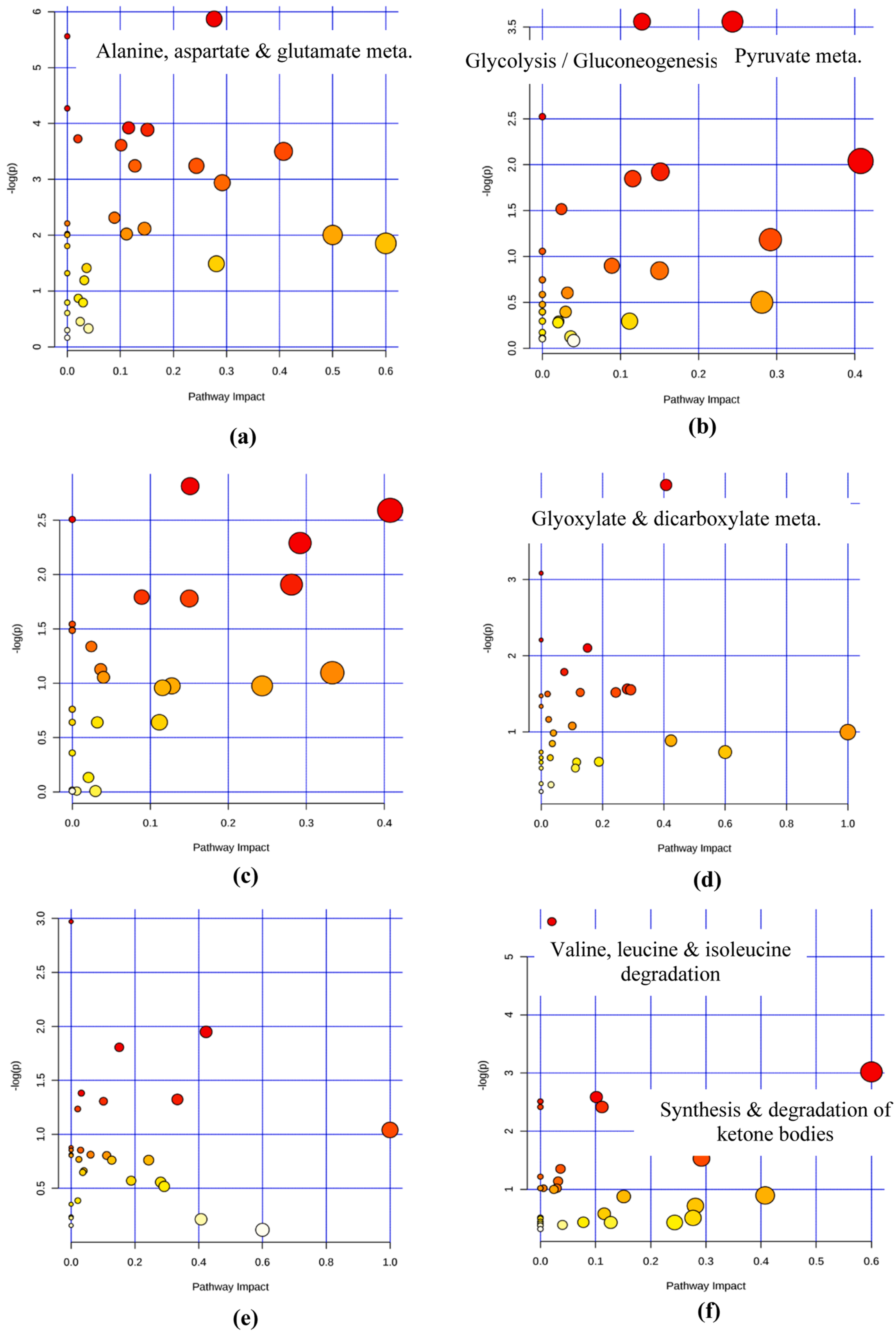

Fig. 4. Metabolome view map depicting metabolic pathway analysis ($p < 0.05$) among all the experimental groups at **months 1 (a & b), 2 (c & d) and 3 (e & f)** of the study. y-axis=pathway enrichment, x-axis=pathway impact; larger globule size=higher pathway impact; darker colours=higher pathway enrichment values; *Refer to Table 1 for respective analysis between group.

Investigation, Formal analysis, Conceptualization. **M.M. Aween:** Writing – review & editing. **Z. Hassan:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **M.S. Hassan:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **R. Hashim:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **I.S. Ismail:** Visualization, Software, Formal analysis. **M.S.M. Misenan:** Writing – review & editing. **R. Ranjithkumar:** Writing – review & editing. **K.F. Li:** Writing – review & editing. **L.S. Wong:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2025.101024.

Data availability

Data will be made available on request.

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