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Gas chromatography-mass spectrometry based metabolomic approach to investigate the changes in goat milk yoghurt during storage

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ABSTRACT

The overall goal was to utilize a gas chromatography spectrometry based metabolomics approach to investigate the metabolite changes in goat milk yoghurt during storage. A total of 129 metabolites were identified in goat milk yoghurt during 28 days refrigerated storage. Among 129, 39 metabolites were differentially regulated ($p < 0.05$) wherein 22 were upregulated (UR) and 17 were downregulated (DR). 17 (9 UR, 8 DR), 20 (11 UR, 9 DR) and 2 (both UR) differential metabolites were identified during storage period of 0–14, 14–28, and 0–28 days, respectively. Metabolic pathway analysis revealed that aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis and phenylalanine metabolism altered during 0–14 days storage; while fatty acid biosynthesis, and propanoate metabolism altered during 14–28 days of storage. Metabolite-gene interaction analysis identified genes regulated by differentially expressed metabolites. Functional annotation of interacted genes in corroboration with that of KEGG pathway analysis provided the probable mechanisms that altered the metabolites during storage. These findings reveal comprehensive insights into the metabolite alterations during storage. This research provides practical information for developing goat milk yoghurt with enhanced bioactivities and would aid in future investigations into the nutritional research and isolation of functional compounds.

1. Introduction

Yoghurt is the oldest fermented dairy product worldwide and one of the most prominent products from goat milk (Sepe & Argüello, 2019). It is produced by the action of a mixture of bacterial cultures namely, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus,* on milk components (Aryana & Olson, 2017). Yoghurt finds significant position in the human diet as it has been used to treat various ailments and diseased conditions (Weerathilake et al., 2014). Yoghurt is recommended for people afflicted with cow milk allergy (especially goat yoghurt), lactose intolerance, inflammatory bowel syndrome, gastrointestinal tract disorders, and immune related functions (Serafeimidou et al., 2012; Wasilewska et al., 2019). However, there are certain technological challenges associated with fermented goat milk products that render them to be more soft, friable (due to low αs_1 content) and have pronounced goaty flavour (Lucatto et al., 2020).

With the advent of metabolomics, fermented dairy products such as yoghurt could be viewed as a complex chemical mixture produced by a variety of cultures/processing treatments, which contain different metabolites and chemical entities in semi-solid/liquid matrix (Wishart, 2008). The type of metabolites formed depends on the raw materials, processing procedures and the type of cultures employed. The metabolites formed can directly or indirectly influence the technological and nutritional quality of food products (Pisano et al., 2016). . Metabolite profiling would help in building metabolic networks, their interaction with different genes regulating the specific pathways, and also can provide insights about their specific role in biological systems (Caboni et al., 2019; Li et al., 2020; Scano et al., 2014; Xia et al., 2020). Metabolites play a significant role in post-acidification changes of yoghurt during storage; thus it might reflect the metabolic behavior of starter cultures (Cavalcante et al., 2016). Thus, metabolomics is one of the most promising approaches for the investigation of metabolite changes in fermented dairy products and allows the determination of metabolite (<1500 Da) at very low concentrations (Caboni et al., 2019).

The nutritional profiling, suitability of microbial culture, and health benefits of goat milk yoghurt have already been studied (Adhikari et al., 2002; Bao et al., 2016; Lucatto et al., 2020; Miocinovic et al., 2016; Tamime & Deeth, 1980). Though sheep and goat milk and theior

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conversion to yoghurt have been studied using GC–MS metabolomics (Murgia et al., 2019), limited research has been focused on differential metabolites and key metabolic pathways occurring during storage of goat milk yoghurt. The level of monosaccharides, carboxylic acids, and amino-acids are reported to be altered during processing and storage of milk, while fatty acids are formed from the metabolic activity of various metabolites such as amino-acids, peptides, nucleosides etc. (Guo, 2003; Strzałkowska et al., 2010).

The regulation of metabolites formed in yoghurt during storage might influence its physical and functional properties. However, limited knowledge is currently available on the effects of storage on metabolite profile changes in goat milk yoghurt. Thus, we aimed to analyze and characterize the metabolite changes in goat milk yoghurt during storage using gas chromatography-mass spectrometry based non-targeted metabolomics approach. Identification of differential metabolites, metabolic pathways, and investigation of the metabolite-gene interaction along with associated biological processes were conducted to have better understanding and strengthening of scientific information about the alterations in the mechanisms and abundance of differential metabolites in goat milk yoghurt during storage.

2. Materials and methods

2.1. Chemicals and reagents

Solvents namely, methanol, chloroform were all GC–MS grade (Sigma Aldrich, Missouri, USA). Potassium chloride, MOX reagent (2% methoxamine hydrochloride in pyridine), BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) and other analytical standards such as succinic acid and ribitol presented > 98% purity (Sigma Aldrich, Missouri, USA).

2.2. Yoghurt culture

Lyophilized yoghurt culture, consisted of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (YO-MIX® 863 LYO 500 DCU), was procured from Danisco-DuPont, Kansas, USA.

2.3. Yoghurt preparation

Pasteurized goat milk (3.5 g/L fat, 8.3 g/L solids-not fat (SNF)) was procured from local stores, Stillwater, Oklahoma, USA. SNF of the milk was standardized using goat milk powder to 14 g/L SNF as per standards (Aryana & Olson, 2017). Further, milk was thermally treated at 85 °C for 15 min followed by cooling to 42–45 °C (desirable temperature for culture addition). Yoghurt culture (Danisco-DuPont, Kansas, USA) was added to the milk @ 20 DCU/100 L. Culture was mixed very slowly in the milk with ladle followed by filling in polystyrene cups and covering with aluminum lid. These filled cups were then incubated at 42 °C till pH reached around 4.5–4.6 (Nuaire Incubator, Plymouth, Minnesota, USA). Goat milk yoghurt samples were stored at refrigeration temperature (4 ± 1 °C) for 28 days (VWR Refrigerator, Suwanee, GA) and analyzed for metabolite profiling on day 0, 14 and 28. The whole experiment was performed thrice. Total yoghurt samples were 15 (five trails for three storage intervals, 5*3 = 15) and each sample was run in duplicate.

2.4. Non-targeted metabolite profile analysis

2.4.1. Metabolite extraction

Metabolites in yoghurt were extracted as per the methodology described by Mitacek et al. (2019) with modifications. Briefly, 100 μL of yoghurt sample was added with 1.5 mL mixture of methanol and chloroform (3:1, v/v) and incubated for 24 h at 4 °C in glass vials with PTFE lined caps where 2,2,3,3-d-4-succinic acid (1 mg/mL) was used as an internal standard. 1.5 mL of chloroform and 360 μL of 0.2 M potassium chloride were added to glass vials followed by centrifugation at 7000 g for 10 min. 200 μL of supernatant was added with ribitol solution (1 mg/mL; second internal standard) followed by drying under a steady stream of high-purity nitrogen gas. Further, 100 μL of MOX reagent was added to each vial and incubated for another 17 h at room temperature for derivatization. 50 μL of BSTFA was added and incubated at 50 °C for 30 min. Samples were then transferred to 200 μL spring inserts inside autosampler amber glass vials and capped with pre-assembled crimp caps. Quality control samples were also prepared by mixing the metabolite extracts of each sample from three groups (days 0, 14 and 28). These were also run with other samples in order to ensure the reliability of the experimental results.

2.4.2. Metabolite profiling

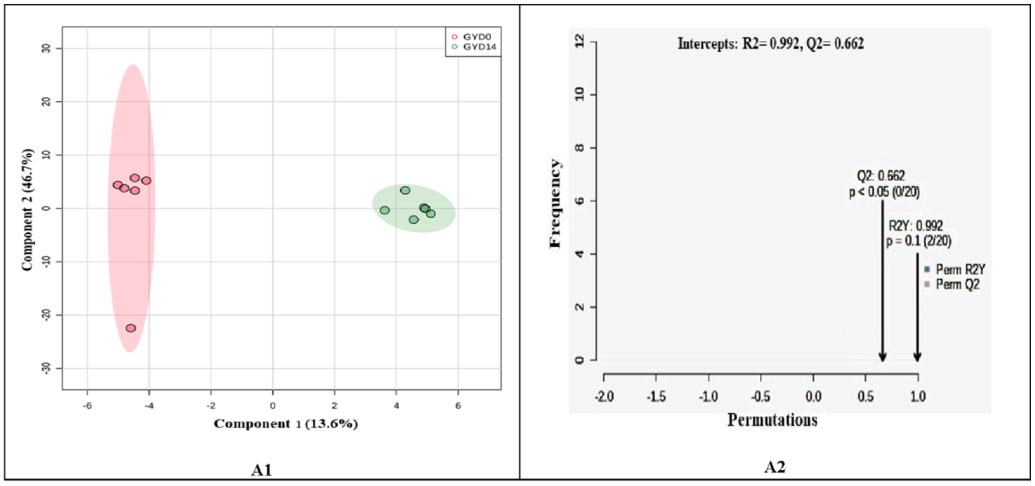
1 μL of the sample was injected with the Agilent 7683 autosampler in split-less mode onto a DB-5MS GC capillary column (Agilent Technologies, 60 m × 0.250 mm × 0.25 μ; Agilent 7890 GC) coupled to a 5975 mass selective detector (Agilent Technologies, Palo Alto, C.A, USA) with an electron ionization ion source which was set at 230 °C. The inlet temperature was set at 250 °C while the oven temperature was set at 50 °C for 2 min, with a subsequent temperature gradient of 5 °C per minute until a final temperature of 315 °C was obtained and held for 3 min. Each sample was run in duplicates beginning with a blank sample consisting of 200 μL of methanol. Ultra-pure helium gas (Stillwater Steel, Stillwater, OK) was used as a mobile phase. Mass spectra ranging from 50 to 650 m/z was recorded.

2.5. Data processing

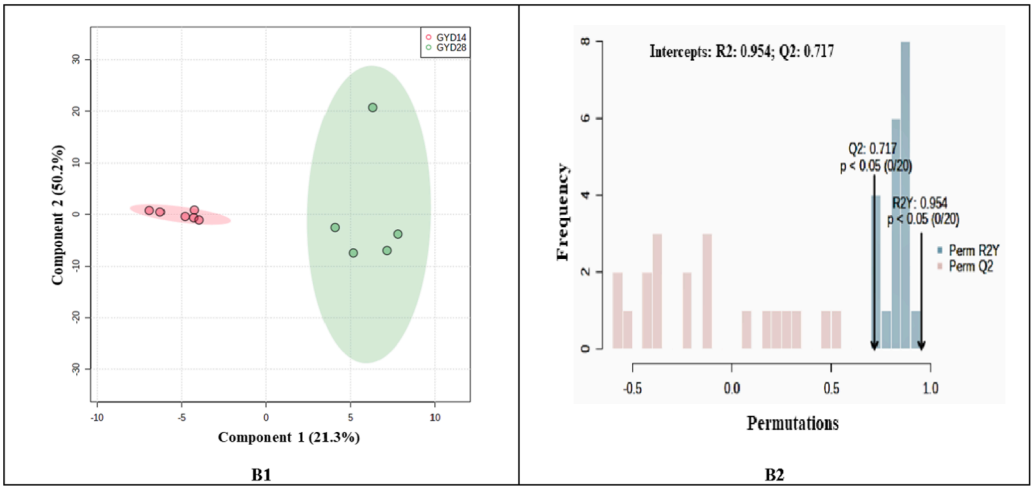
The raw data files from the Agilent Chemstation software were converted to .cdf format. A matrix of molecular features as defined by retention time and mass (m/z) was generated utilizing the XCMS (Smith, 2010) software in R for detection and alignment. The raw peak areas were normalized based on the total ion signal and outliers were detected based on the total signal and PC1 of the principal component analysis (PCA). A novel clustering tool, RAMClustR (Broeckling et al., 2014) was utilized to group features into spectra based on coelution and covariance across the full dataset. The spectra were then used to determine the identity of observed compounds in the experiment. Compounds were identified based on spectral matching to both NISTv17 (Natl. Inst. of Standards and Technology, Gaithersburg, MD, USA) and Golm metabolite library databases (Hummel et al., 2013). The confidence of each metabolite annotation was scored on a 1 to 4 scale based on guidelines provided by the Metabolomics Standards Initiative (Sumner et al., 2007). Peak areas for each feature in a spectrum were condensed using the weighted mean of all features in a spectrum into a single value for each compound.

2.6. Data analysis

The data obtained after annotating the metabolites were exported to MetaboAnalyst 4.0 for orthogonal partial least squares discriminant analysis (OPLS-DA) and permutation tests of OPLS-DA model, hierarchal cluster analysis, box and whisker plots, enrichment analysis and metabolite-gene interaction (Xia et al., 2020). The Venn diagram was constructed using the program web-based smart diagram® (https://cloud.smartdraw.com/). Pearson correlation coefficient of significant metabolites was calculated based on their respective intensity values using correlation calculator v 1.0.1, where the data was normalized to log 2 transformation for coefficient calculation. The data obtained was further used to generate a correlation matrix (Basu et al., 2017). The output file was exported to Cytoscape v 3.7 to build a correlation network (Shannon et al., 2003). Online databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.kegg.jp/kegg/), Human Metabolome Database (HMDB) (https://hmdb.ca/), PubChem (https://pubchem.ncbi.nlm.nih.gov/), the Small Molecule Pathway Database (SMPDB) (https://smpdb.ca/)



(A)



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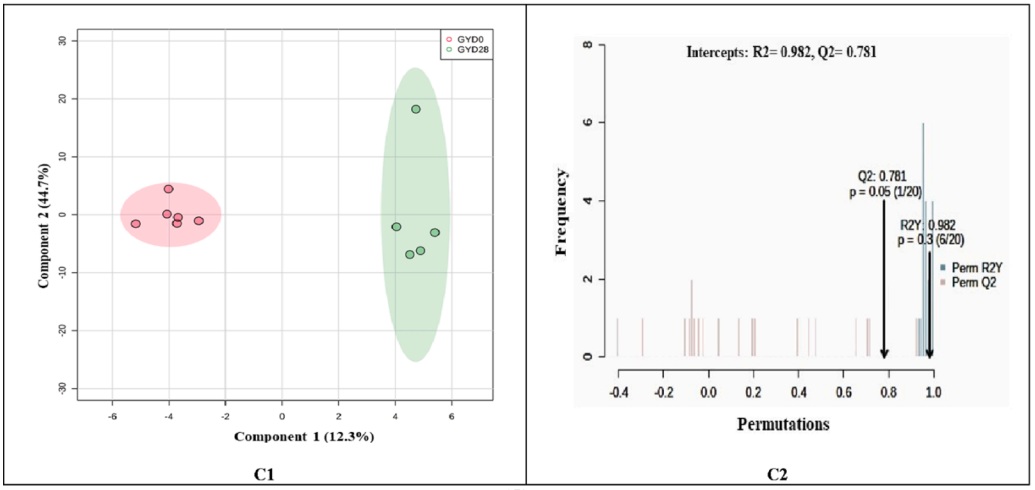


(C)

Fig. 1. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) depicting the score scatter plot (A1, B1, C1) and Permutation test for OPLS-DA model (A2, B2, C2) for different samples of goat milk yoghurt during storage: (A) day 0 versus day 14; (B) day 14 versus day 28; (C) day 0 versus day 28. (OPLS-DA exhibits clear separation between two groups of goat milk yoghurt during storage; R^2 indicates the measure of model fit to original data and Q^2 measures the consistency between original and cross-validation predicted data). A1: Component 1 accounts for 13.6% of variation in goat milk yoghurt between storage day 0 and day 14, Component 2 accounts for 46.7% of variation in goat milk yoghurt between storage day 0 and day 14. Fig. 1 (B1): Component 1 accounts for 21.3% of variation in goat milk yoghurt between storage day 14 and day 28, Component 2 accounts for 50.2% of variation in goat milk yoghurt between storage day 14 and day 28. Fig. 1 (C1): Component 1 accounts for 12.3% of variation in goat milk yoghurt between storage day 0 and day 28, Component 2 accounts for 44.7% of variation in goat milk yoghurt between storage day 0 and day 28.

Table 1
Significant ($p < 0.05$) metabolites up-regulated in goat milk yoghurt during storage.

Metabolite	p-value	Fold change	Regulation	Functional Role
Storage interval: day 0 to 14 (14/0)				
D-Alanine	< 0.001	2.076	Upregulated	Amino acid
Nitroarginine	0.013	2.336	Upregulated	Vaso-constrictor (Nitro derivative of arginine)
Val-Cys	0.019	2.024	Upregulated	Di-peptide
Sebacic acid	0.026	2.262	Upregulated	Dicarboxylic acid
Met-Val	0.027	2.262	Upregulated	Di-peptide
N-alpha-acetyl-L-Lysine	0.030	2.968	Upregulated	N-acetylated amino acid
4-oxo-2-nonenal	0.035	1.309	Upregulated	Lipid Peroxide
Phenylalanine	0.048	1.741	Upregulated	α-amino acid
Storage interval: day 14 to 28 (28/14)				
L-Methionine	0.025	16.93	Upregulated	Amino acid
Propionic acid	0.002	3.974	Upregulated	Carboxylic acid
2-Hydroxybutyric acid	0.005	6.293	Upregulated	Organic acid
Pimelic acid	0.009	1.691	Upregulated	Dicarboxylic acid
Glycerophosphocholine	0.011	2.770	Upregulated	Glycolytic
D-Mannose-1-phosphate	0.013	21.305	Upregulated	Glycosylation
3,3-Dimethylglutaric acid	0.021	8.586	Upregulated	Dicarboxylic acid
D-Xylulose	0.022	4.269	Upregulated	Monosaccharide
N-pentanoic acid	0.033	6.746	Upregulated	Carboxylic acid
2,6-dihydroxybenzoic acid	0.034	3.214	Upregulated	Salicylic acid
Dodecanoic acid	0.054	2.731	Upregulated	Fatty acid
Storage interval: day 0 to 28 (28/0)				
Propionic acid	0.030	2.367	Upregulated	Carboxylic acid
Glycerophosphocholine	0.032	2.529	Upregulated	Glycolytic

were used for annotating the metabolites and to search for metabolites in the biosynthesis pathway.

3. Results and discussion

3.1. Differential analysis of goat milk yoghurt during storage

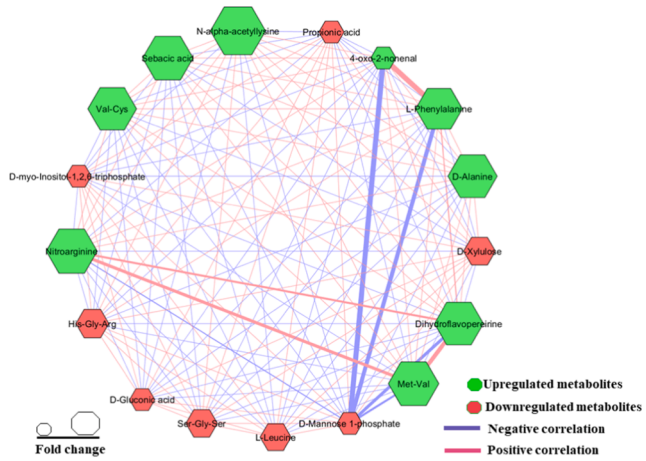
Metabolomic analysis of yoghurt depicted the presence of total 187 features, including 129 identified ones. The metabolites consisted of: 44 carboxylic acids and derivatives (38 amino acids, peptides and analogues, 6 di/mono carboxylic acids), 18 fatty acyls (15 fatty acid and conjugates, 1 fatty acid ester, 1 fatty acyl thioester and 1 fatty alcohol), 4 glycerophospholipids (2 glycerophosphocholines, 1 glycerophosphoethanolamine, 1 glycerophosphate), 15 organooxygen compounds (9 carbohydrates and carbohydrate conjugates, 3 carbonyl compounds and 3 alcohols and polyols), 4 benzene and substituted derivatives, 2 hydroxy acids and derivatives (1 α-derivative and 1 β-derivative) and 12 other metabolites (steroid and steroid derivatives, phenols and derivatives, indoles and derivatives, azoles, diazenes, pteridine and derivatives, cinnamic acids and derivatives, purine nucleotides etc.) (Table S1, Fig S1). Previously, using a GC–MS method, yoghurt from goat and sheep milk were compared and 56 metabolites were identified including mainly adipic acid, pimelic acid, butanoic acid and saccharides (Murgia et al., 2019)

Chemometric analysis namely, Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) exhibited the separation among different groups of yoghurt samples namely; GYD 0 and GYD 14 (Fig. 1 A), GYD 14 and GYD 28 (Fig. 1B), GYD 0 and GYD 28 (Fig. 1C) indicating that storage duration had significant ($p < 0.05$) effect. Similarity and differences in the samples were also detected by principal component analysis (Mitacek et al., 2019). PCA score plot revealed three principal components namely, PC1 (representing 52% variation between the groups), PC2 (representing 16.8% variation) and PC3 (representing 13.1% variation). To optimize the separation between the groups (storage intervals), the supervised OPLS-DA was applied. OPLS-DA models displayed a clear separation between the groups indicatinggood reliability within each group. As seen in Fig. 1 (A1, B1, C1), the two groups could be clearly discriminated by OPLS-DA as both the groups were separated on left and right sides of the origin point (x-axis). Two principal components (component 1 and component 2) were identified between each of three different groups. These components explained the percent of the variation between the two respective groups (For example, Fig. 1 (A1) depicts that component 1 accounts for 13.6% of the variation in goat milk yoghurt between storage day 0 and day 14, Component 2 accounts for 46.7% of the variation in goat milk yoghurt between storage day 0 and day 14). X- and Y-axis in the plots revealed the scores of first and second components, respectively. Fig (A2, B2, C2) displayed the results for permutation tests of the respective OPLS-DA models for goat milk yoghurt during storage. Since, OPLS-DA tends to over fit the data, permutation tests were applied to validate the models and understand whether the separation was statistically significant. R^2 (measure of model fit to original data) and Q^2 (measure of consistency between original and cross-validation predicted data) values of the permutation tests for goat milk yoghurt of different storage intervals namely, Day 0–14 ($R^2 = 0.992$ and $Q^2 = 0.662$), Day 14–28 ($R^2 = 0.954$ and $Q^2 = 0.717$) and Day 0–28 ($R^2 = 0.982$ and $Q^2 = 0.781$) were close

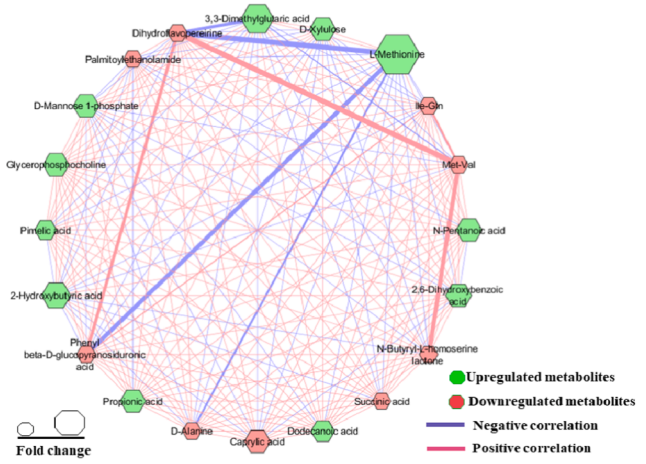
Table 2
Significant ($p < 0.05$) metabolites down-regulated in goat milk yoghurt during storage.

Metabolite	p-value	Fold change	Regulation	Functional Role
Storage interval: day 0 to 14 (14/0)				
D-mannose-1-Phosphate	0.031	0.544	Downregulated	Glycosylation
Leucine	0.004	0.632	Downregulated	Amino acid
Ser-Gly-Ser	0.005	0.547	Downregulated	Tri-peptide
D-gluconic acid	0.006	0.576	Downregulated	Carboxylic acid
His-Gly-Arg	0.012	0.376	Downregulated	Tri-peptide
D-Xylulose	0.015	0.534	Downregulated	Monosaccharide
D-myoinositol-1,2,6-triphosphate	0.018	0.700	Downregulated	Sugar Phosphate
Propionic acid	0.031	0.657	Downregulated	Carboxylic acid
Storage interval: day 14 to 28 (28/14)				
D-Alanine	<0.001	0.306	Downregulated	Amino-acid
Phenyl beta-D-glucopyranosiduronic acid	0.004	0.218	Downregulated	Uronic acid
Palmitoylethanolamide	0.014	0.344	Downregulated	Fatty acid amide
Dihydroflavopereirine	0.017	0.187	Downregulated	Alkaloid
Ile-Gln	0.026	0.169	Downregulated	Di-peptide
Met-Val	0.027	0.221	Downregulated	Di-peptide
N-Butyryl-L-homoserine lactone	0.039	0.652	Downregulated	Quorum sensing lactone
Octanoic acid	0.047	0.554	Downregulated	Fatty acid
Succinic acid	0.051	0.725	Downregulated	Dicarboxylic acid

No differentially downregulated metabolite was found in storage interval of 0–28 days



(A1)



(A2)

Fig 2. Correlation network analysis of differential metabolites ($p < 0.05$) for goat milk yoghurt during storage (A1) day 0 versus day 14; (A2) day 14 versus day 28. Green and red coloured octagon represents upregulated and downregulated metabolites, respectively. Bigger size of octagon represents greater fold change for metabolite. Red and blue coloured line represents positive and negative correlation among metabolites, respectively; while the increased width of the line represents the correlation strength. Heat map of hierarchal clustering analysis of differential metabolites ($p < 0.05$) for goat milk yoghurt during storage (B1) day 0 versus day 14; (B2) day 14 versus day 28; (B3) day 0 versus day 28. Coloured cells correspond to concentration value (samples in column and compounds in row). Data presented was normalized and subjected to T-test/ ANOVA and features were standardized to autoscaling. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to 1. This indicated that the separation of metabolites clusters between the different yoghurt groups during storage as displayed by OPLS-DA models was statistically significant (Xia et al., 2020).

Total 39 differential ($p < 0.05$) metabolites (22 upregulated and 17 downregulated) were observed in goat milk yoghurt stored at refrigeration temperature for 28 days. Table 1 and Table 2 shows significantly upregulated and downregulated metabolites of goat milk yoghurt, respectively during storage. Out of these, 17 differential ($p < 0.05$) identified metabolites including 9 upregulated and 8 downregulated, were reported in the storage interval of day 0–14 (day 14/0) and comprised of mainly peptides, amino- acids and carbohydrates, carboxylic acids, respectively. While, day 14–28 interval (day 28/14) was observed with 20 differential ($p < 0.05$) metabolites (11 upregulated and 9 downregulated). Upregulated metabolites in the storage interval of 14–28 days mainly consisted of fatty acyls and saccharides, whereas, downregulated metabolites were comprised of carboxylic acids and organooxygen compounds. Surprisingly, only 2 differential metabolites ($p < 0.05$) (both upregulated) were found in the storage interval of day 0–28 (day 28/0). There was no significant difference ($p > 0.05$) in 90 identified metabolites found in goat milk yoghurt during storage.

3.2. Functional annotation of metabolites and their enrichment analysis

The online KEGG and HMDB database were used to annotate the functions of metabolites identified in goat milk yoghurt during refrigerated storage of 28 days (Table S1). Also, SMPDB online database was used to link identified metabolites with specific pathways for the overview of their enrichment analysis. The analysis revealed that 63 SMPDB pathways were integrated (Table S2). Among top 20 SMPDB pathways, amino-acid and carboxylic acid metabolic pathways predominated. In the present study, 5 metabolites (glycine, L-glutamic acid, L-alanine, pyroglutamic acid and L-cysteine) and 7 metabolites (γ- amino butyric acid, glycine, L-glutamine, L-alanine, L-cysteine, adenosine diphosphate ribose) detected in yoghurt were involved in the differentially regulated ($p < 0.05$) SMPDB pathways namely, glutathione metabolism and glutamine metabolism, respectively.

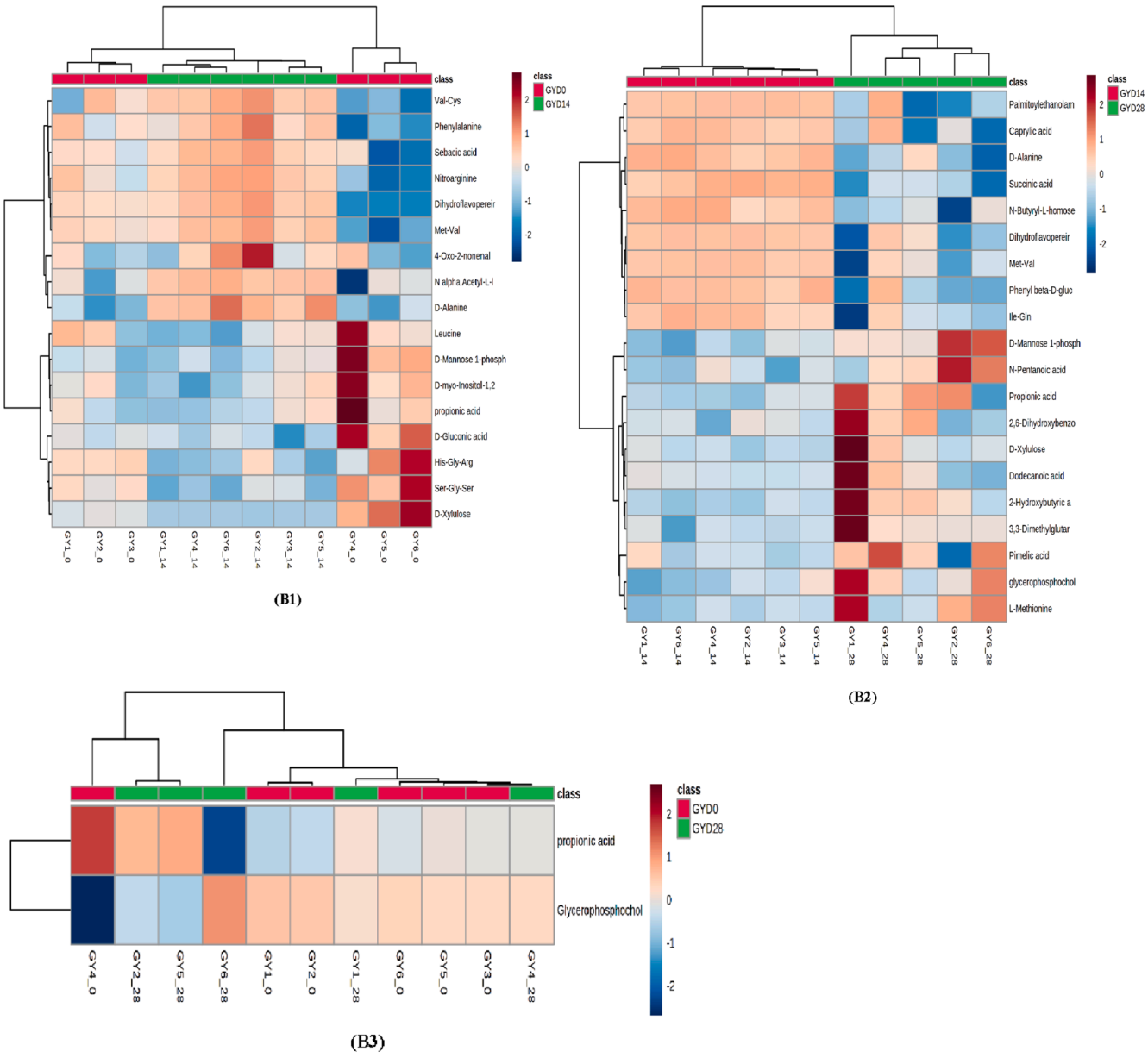


Fig 2. (*continued*).

KEGG online database was also used to link the metabolites for specific pathways to get the overview of KEGG pathway analysis of 129 identified metabolites of goat milk yoghurt. It contains manually drawn pathway maps which represent knowledge of the molecular interaction, reaction and relation networks. KEGG online database contains well-defined metabolite pathways for *Homo sapiens* but none for *Capra hircus*. In the KEGG online database, metabolite pathways for *Homo sapiens* were well defined with none for *Capra hircus*. Further, pathways for mapping the metabolites were integrated and total 43 pathways were obtained (Table S3). Nine pathways were differentially ($p < 0.05$) regulated wherein, 33 metabolites were involved (Fig S2). Pathways related to amino-acids, carboxylic acids and fatty acyl metabolism predominated. Thus, the results were consistent with identified metabolites in goat yoghurt (44 carboxylic acids and 18 fatty acyls).

3.3. Correlation network and hierarchal cluster analysis of differential metabolites

Correlation coefficient of differential metabolites was calculated to generate correlation matrix (File_2). As shown in Fig. 2 (A1), correlation network of differential metabolites of goat milk yoghurt during 0–14 days depicts the strong negative correlation of D-mannose-1-phosphate with a maximum number of upregulated metabolites (amino-acids, peptides, and carbonyl compounds) namely, 4-oxo-2-nonenal, L-phenylalanine, Met-Val and nitroarginine indicating that utilization of carbohydrates (as source of energy) by bacteria resulted in the production of amino-acids and peptides during initial storage interval. Strong positive correlation was observed only between upregulated metabolites such as Met-Val with nitroarginine. The positive correlations among downregulated/upregulated metabolites could be explained by their fold change and probable role of one in the biosynthesis of another metabolite. Day 14–28 storage indicated a stronger correlation among various differential ($p < 0.05$) metabolites of goat milk yoghurt (Fig 2 A2). Among upregulated metabolites, L-methionine showed a strong negative correlation with β-D glucopyranosiduronic acid and D-alanine. While Met-Val depicted a positive correlation with other downregulated metabolites, namely N-butyryl-L-homoserine lactone and Ile-Gln. Biosynthesis of methionine involves a series of steps,

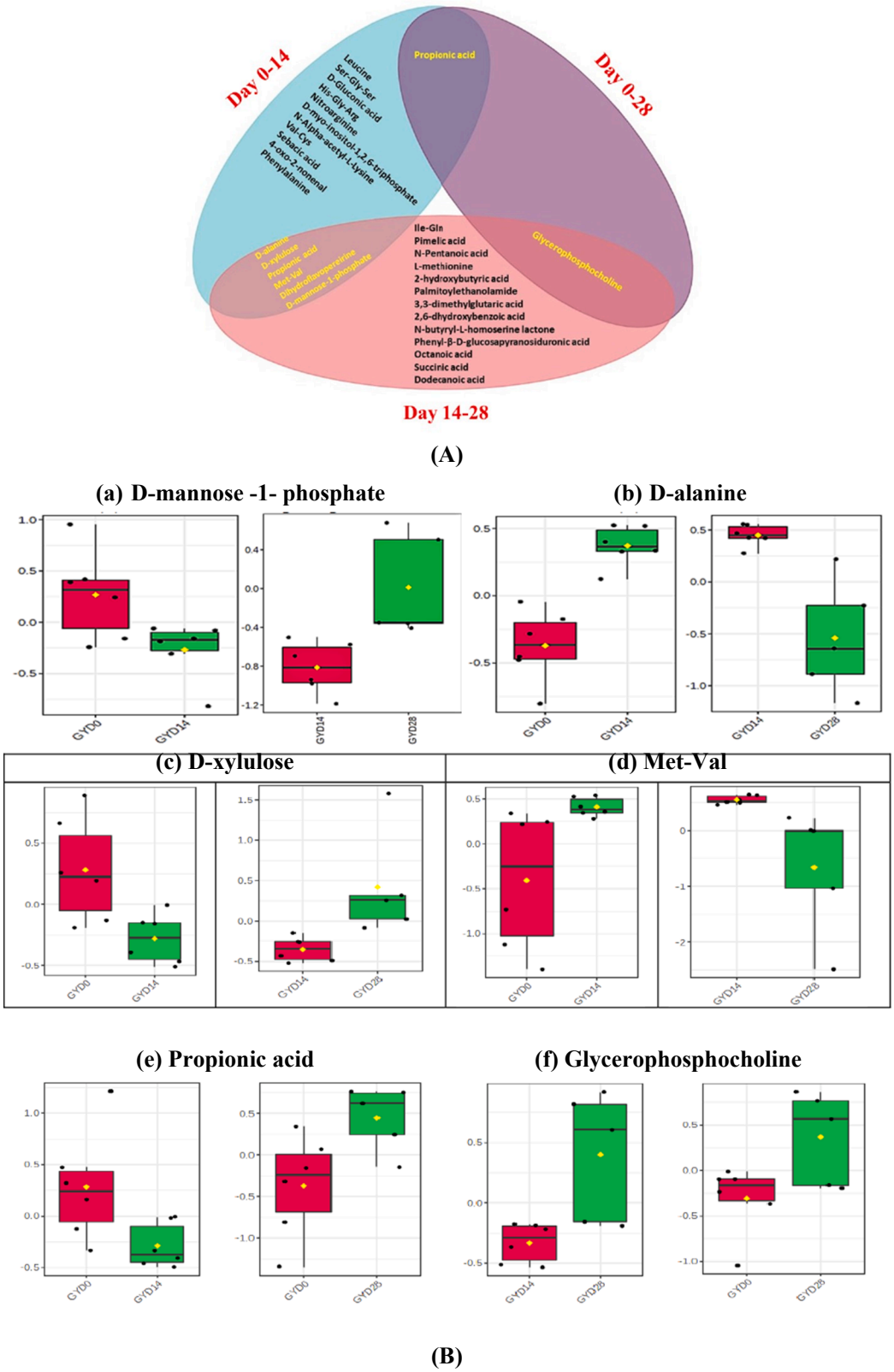


Fig 3. A. Venn diagram depicting common and exclusive identified metabolites ($p < 0.05$) in goat milk yoghurt during storage. B. Box and whisker plots of significantly identified metabolites ($p < 0.05$) in goat milk yoghurt that are common between day 0–14 and day 14–28 (a-e); day 0–14 and day 0–28 (f); day 14–28 and day 0–28 (f). C. Box and whisker plots of exclusive metabolites ($p < 0.05$) present in goat milk yoghurt during storage, day 0–14 (a-k) and day 14–28 (l-y). Storage interval of day 0–28 was not observed with significantly identified metabolites. Black dots in each plot represents the concentrations of selected feature from all samples. Notch indicates 95% confidence interval around median of each group. Non-overlapping of notch indicates differences between groups and mean concentration of each group is presented by yellow diamond in each box. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

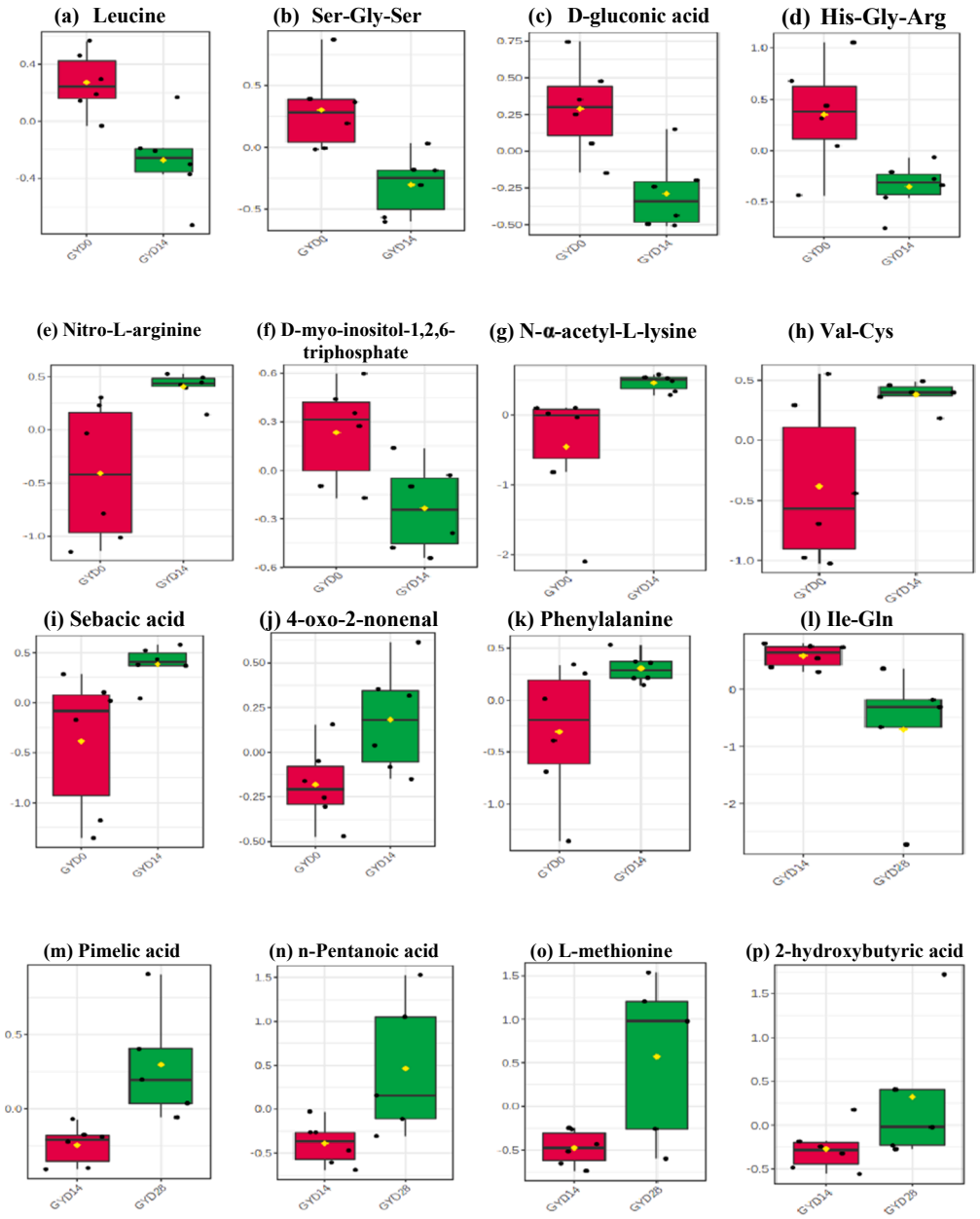


Fig 3. (*continued*).

including conversion of aspartate to homoserine isomers and derivatives, thus, suggesting the probable reason for the positive correlation between these two (Ferla & Patrick, 2014). The results of the correlation network clearly indicate that alteration of metabolites in goat milk yoghurt during storage are complex which implies that down or up-regulation of one metabolite might impact the regulation of one or more metabolites without any consistent trend Thus, the correlation network might depict the interactions of differential metabolites with similar physiological and molecular characteristics.

Hierarchal cluster analysis is used to categorize the metabolites possessing same characteristics and to recognize the variations between different metabolites groups. 39 identified metabolites ($p < 0.05$) of goat milk yoghurt were clustered in heat maps using Euclidean distance matrix of quantitative values. Differential metabolites identified during each storage interval were clustered in different heat maps for ease of categorization and comprehension (Fig 2 B1, B2, B3). The bright color corresponds to the higher concentration of a particular metabolite in the respective sample. Overall, the results of clustering the metabolites in heat maps revealed their correspondence with the results reported priorly.

3.4. Identification of common and exclusive metabolites during storage

Venn diagram was used to differentiate the common and exclusive metabolites of goat milk yoghurt during different storage intervals. As shown in Fig. 3 (A), six common differentially abundant metabolites were observed between the storage interval of day 0–14 and day 14–28, while only one metabolite was found common in both storage interval i.e., between day 14–28 and day 0–28 and between day 0–28 and 0–14. Thus, a total of 11 and 13 exclusive metabolites ($p < 0.05$) were observed in goat milk yoghurt during storage interval of day 0–14 and day 14–28, respectively.

The relative ionic abundance of common (Fig. 3B) and exclusive (Fig. 3C) metabolites of goat milk yoghurt is depicted in box and whisker plots. Among common differential metabolites ($p < 0.05$), D-alanine and Met-Val differed significantly ($p < 0.05$) with regard to ion abundance

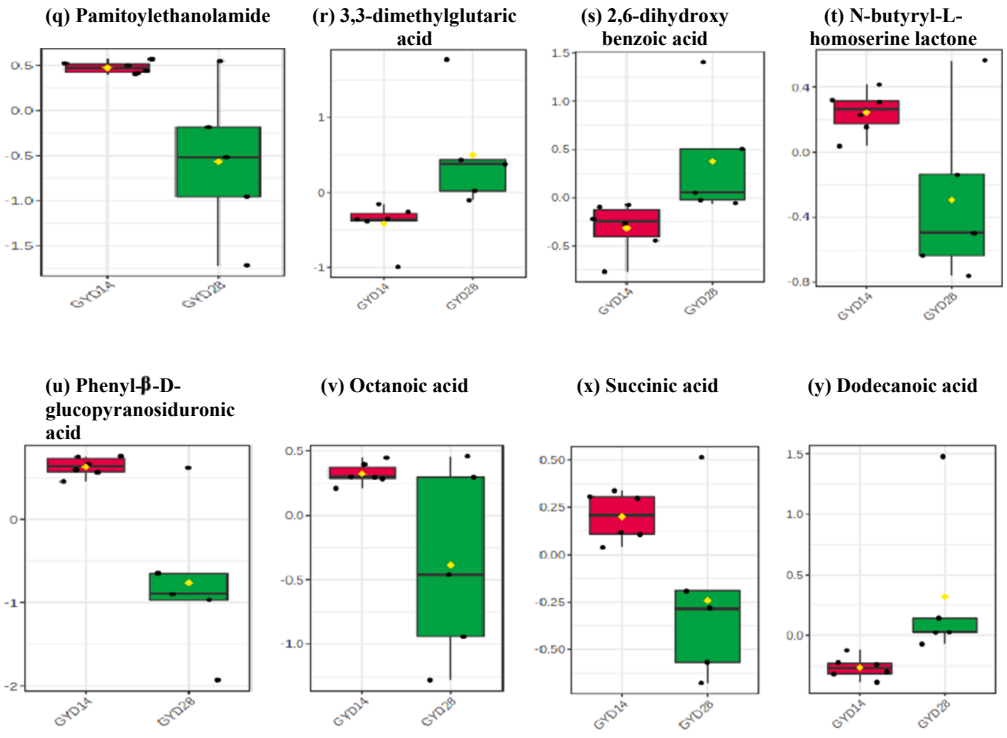


Fig 3. (*continued*).

while leucine, ser-gly-ser, nitroarginine, pimelic acid, phenyl-β-D-glucopyranoside, succinic acid and dodecanoic acid were observed with significantly ($p < 0.05$) different among exclusive metabolites. The relative ionic abundance of di-peptides (Met-Val, Val-Cys) was > 2-fold greater on day 14 than day 0, while carboxylic acids and sugars were lesser in concentration on day 14. This could be due to the utilization of lactose by yoghurt culture during the fermentation process. Since, lactic acid bacteria employed for fermentation depends solely on the source of carbohydrates for energy as it lacks the cytochrome system for electron transport or enzymes to operate TCA cycle (Tamime & Deeth, 1980). This also indicates the utilization of proteins by bacteria as energy source during initial storage days of yoghurt. Also, *S. thermophilus* is reported to produce proteinases establishing the fact that most of the proteolytic activity takes place during initial periods of storage (Tamime & Deeth, 1980). Mannose-1-phosphate, D-xylulose and propionic acid had relatively higher abundance by 21.3 fold, 4.26 fold, and 3.97 fold, respectively on day 28 than day 14, whereas spectral features containing di-peptides (Met-Val, Ile-Gln) were lesser on day 28. This might suggest that proteins are utilized primarily as an energy source by bacteria, while carbohydrates are a secondary source of energy. Lipolysis in yoghurt occurs to a very small extent because the lipases in the milk fat are inactivated at pasteurization temperature and reports suggest that most of the volatile acids formed in yoghurt are derived from non-fat components primarily, amino acids (Bao et al., 2016). This is also reflected in our results where fatty acyls (pimelic acid, glycerophosphocholine, n-pentanoic acid, dodecanoic acid and 2,6-dihydroxy benzoic acid) were observed at > 2 fold higher concentration at the expense of di-peptides on day 28.

All these metabolites not only have a significant role in developing the desirable characteristics of yoghurt but also modulating the human health system. For example, peptides containing valine are reported to be anti-hypertensive peptides thus, indicating that the product on day 14 might possess higher ACE inhibitory activity as compared to other storage intervals (Hagi et al., 2016). Sulphur containing amino acids (methionine) are reported to be anti-carcinogenic (Güzel-Seydim et al., 2000) which had 16.93 fold higher abundance on day 28. Propionic acid in the yoghurt rendered it to possess functional attribute by reducing the fatty acids content in liver and plasma and combating metabolic disorders such as diabetes and obesity (Al-Lahham et al., 2010). . Mannose-1-phosphate and D-xylulose are involved in synthesis of oligosaccharides (Caboni et al., 2019), thus goat milk yoghurt could be assumed as the richest source of oligosaccharides towards the end of the storage period. Glycerophosphocholine has been associated with choline (an essential nutrient for human body) bio-synthesis and is an intermediate product formed during metabolism of phosphatidylcholine, one of the two major phospholipids essential for cell membranes. These are also reported to modulate cardiovascular diseases and are identified as sensitive indicators of obesity related risks (Syme et al., 2016).

3.5. *Enrichment analysis and KEGG pathway impact analysis of exclusive metabolites*

Mapping and annotation of differential metabolites ($p < 0.05$) identified during different storage intervals was performed using KEGG, HMDB, and other available online databases (Table S4). Further, SMPDB enrichment analysis was performed for exclusive metabolites ($p < 0.05$) in goat milk yoghurt (day 0–14 and day 14–28). No differential exclusive metabolite was observed in the storage interval between day 0–28. Storage interval of day 0–14 revealed the enrichment of four major pathways, namely, vitamin K metabolism, phenylalanine, and tyrosine metabolism, propanoate metabolism and valine, leucine and isoleucine degradation (Fig 4 A1). Propionic acid, L-phenylalanine, and L-leucine were among the exclusive metabolites ($p < 0.05$) found to be majorly involved in these pathways. On the other hand, storage interval of day 14–28 revealed 23 enriched pathways, majorly including the fatty acid metabolic and carboxylic acid metabolism pathways (Fig 4 B1). This

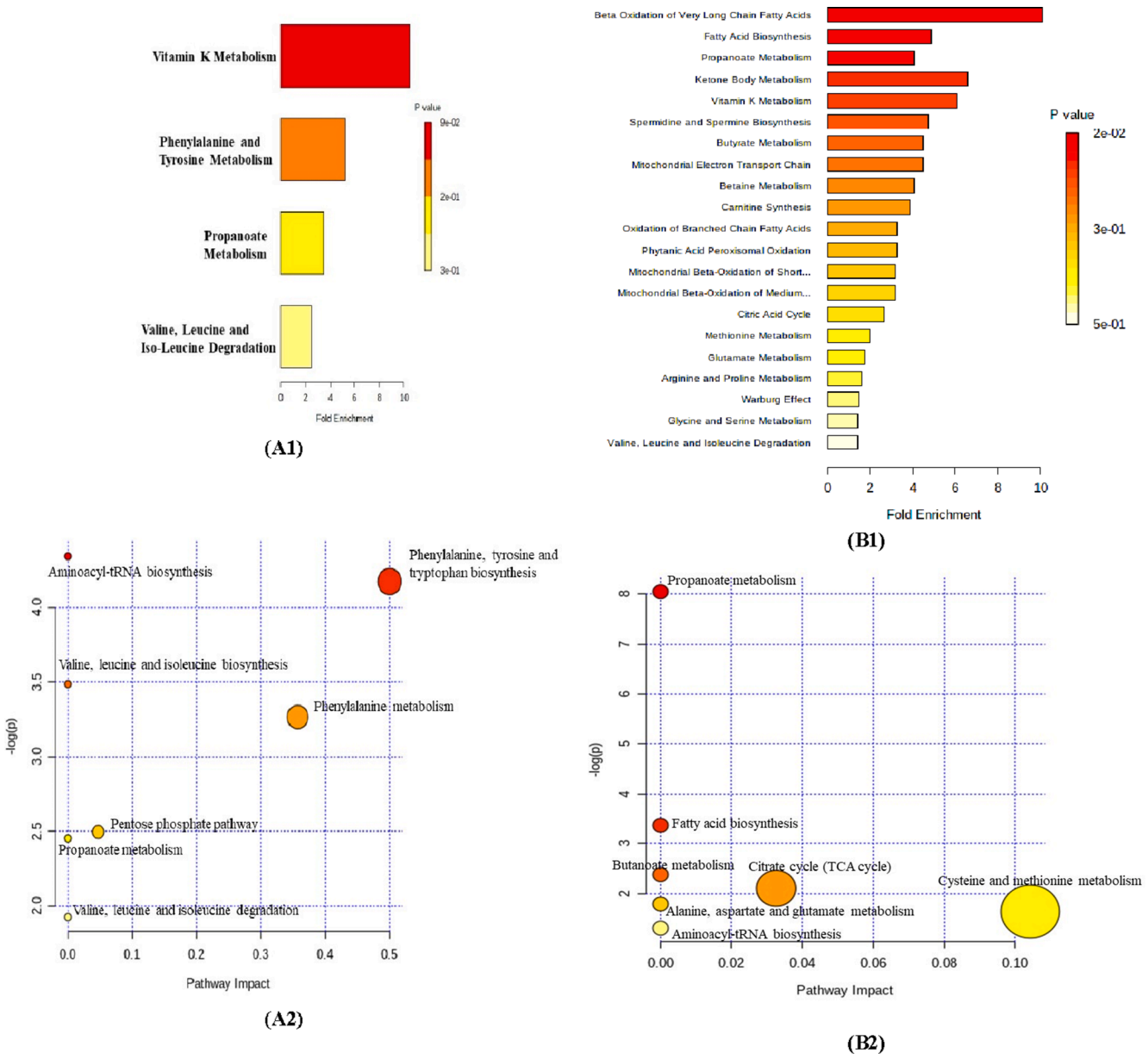


Fig 4. Bar-chart view of Enrichment Analysis: Small Molecular Pathway Database (SMPD)-associated (A1, B1) and Metabolomics view map (A2, B2) of of differentially ($p < 0.05$) exclusive identified metabolite sets of goat milk yoghurt during storage interval namely, day 0–14 (14/0) and day 14–28 (28/14), respectively. The X-axis represents the pathway impact, while y-axis represents -log (p) value. Larger size and darker colour indicate the major pathway enrichment and high pathway impact values, respectively. Important upregulated KEGG pathway maps of differentially ($p < 0.05$) exclusive identified metabolites of goat milk yoghurt during storage interval namely, day 0–14 (14/0) (C1: "Aminoacyl-tRNA biosynthesis", C2: "Phenylalanine, tyrosine and tryptophan biosynthesis", C3: "Valine, leucine and *iso*-leucine biosynthesis", C4: "Phenylalanine metabolism") and day 14–28 (28/14) (C5: "Propanoate metabolism", C6: "Fatty acid biosynthesis").

corroborates with our previous results of relatively higher ionic abundance of peptides and amino acids in storage interval of 0–14 days, while fatty acyls predominated during 14–28 days of storage interval. This might also be related to the post-acidification of yoghurt wherein; pH might not decrease at the same rate by which gets reduced at the end of storage. We speculate that amino-acids and peptides might offer some buffering capacity, thus resisting the change in pH of yoghurt during initial periods of storage.

Exclusive metabolites of goat milk yoghurt were further mapped to KEGG pathway analysis in order to explore the mechanisms leading to the alterations in their contents and to get the impact of the biological processes. Pathway impact revealed that day 0–14 storage interval witnessed the alterations in phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, pentose phosphate pathway, propanoate metabolism (Fig 4 A2), whereas, storage interval of day 14–28 revealed the alterations in citric acid cycle, cysteine and methionine metabolism, fatty acid biosynthesis (Fig 4 B2). These results suggest that differences in metabolic pathways could explain the differences in the presence of differentially exclusive metabolites during storage. These biochemical alterations might be used to comprehend the impact of storage duration on goat milk yoghurt composition and its post-acidification and aid in future investigations in potential and yet, unexplored nutritive value of goat milk yoghurt. Out of 12, four metabolic pathways (aminoacyl t-RNA biosynthesis, phenylalanine, tyrosine and tryptophan metabolism, valine, leucine and isoleucine biosynthesis, phenylalanine metabolism) were significantly altered (Fig 4 C1-C4) in

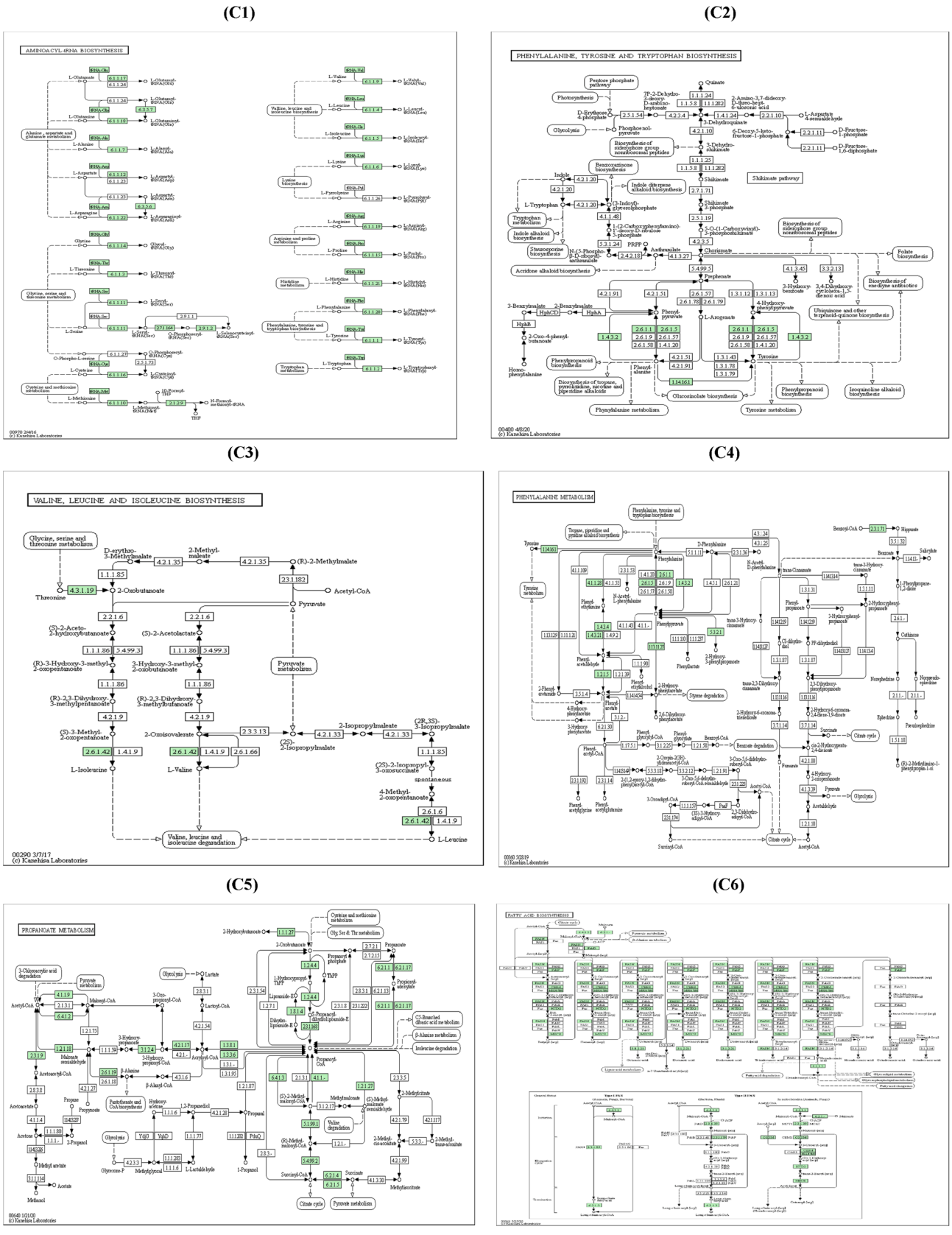


Fig 4. (*continued*).

the storage interval of 0–14 days, while two pathways (propanoate metabolism, fatty acid biosynthesis) were significantly altered during 14–28 days (Fig. 4, C5-C6). Alterations in key pathways were also consistent with our previously reported results of exclusive metabolites ($p < 0.05$) in goat milk yoghurt during storage. Different metabolic pathways during different storage intervals might also indicate the

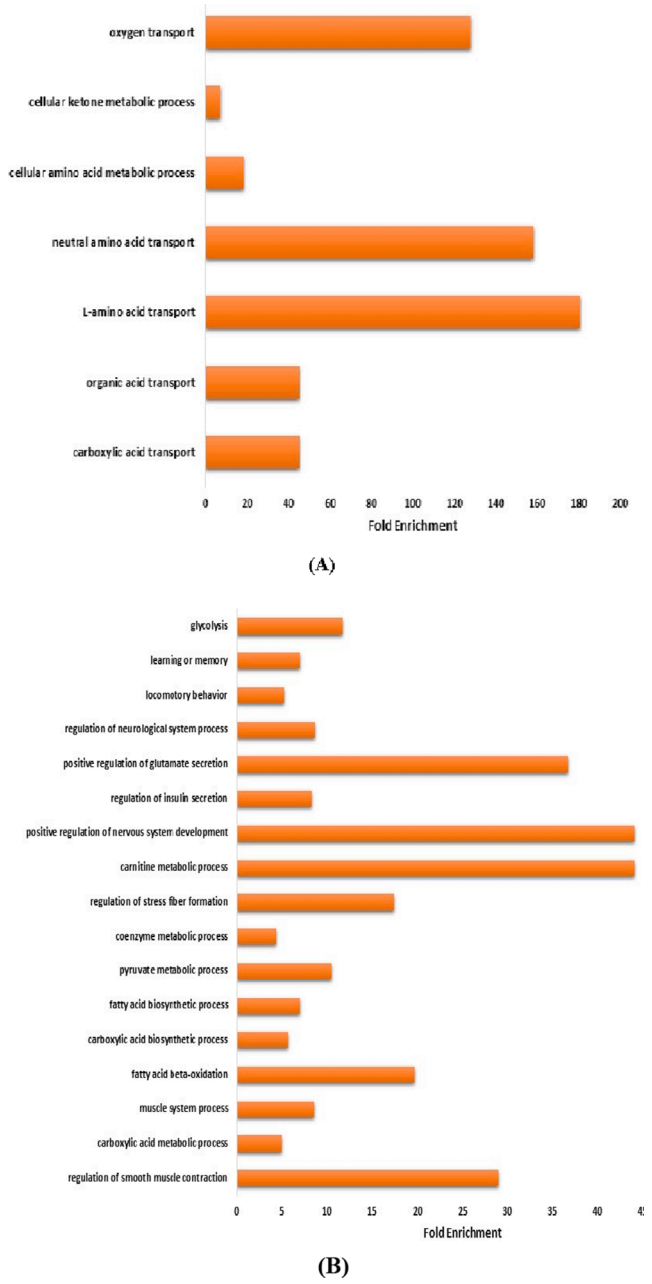


Fig 5. A. Enriched Gene Ontology (GO) terms of biological processes of genes interacted with metabolites in goat milk yoghurt during storage interval of day 0–14. B. Enriched Gene Ontology (GO) terms of biological processes of genes interacted with metabolites in goat milk yoghurt during storage interval of day 14–28.

preference of energy source utilization by the starter culture. More proteolytic activity during the initial storage period might have also been affected the sensory properties of yoghurt.

3.6. Metabolite-gene interaction analysis

Downstream analysis was performed to study metabolite-gene interaction network and predict genes interacting with differentially expressed metabolites ($p < 0.05$). On analysis, 18 and 130 genes ($p < 0.05$) were found to be interacting with metabolites in goat milk yoghurt during storage interval of 0–14 and 14–28 days, respectively (File_2). The common genes between 0 and 14 and 14–28 days were 5 (Fig S3 A). Upregulated metabolites, namely sebacic acid and phenylalanine, in storage interval of 0–14 days, showed 5 and 50 degree of connectivity, respectively while, upregulated metabolites of storage interval 14–28 days namely dodecanoic acid, L-methionine were found to have even more degree of connectivity (133 and 60, respectively). More degree of connectivity could be related to higher influence of metabolites on biological, cellular and molecular processes. Propionic acid, common metabolite in both storage interval, was found to be possessing highest degree of connectivity (199) (File_2). The details of common and exclusive predicted genes with their names have been provided in Fig S3B.

3.7. Functional annotation of genes interacting with differential metabolites

Gene ontology (GO) analysis revealed 20 and 387 biological processes of genes interacting with significant metabolites of goat milk yoghurt for storage interval of 0–14 and 14–28 days, respectively (File_3). The highest fold enriched GO terms during 0–14 days interval include L-amino acid transport, neutral amino acid transport, oxygen transport, organic acid transport, carboxylic acid transport (Fig. 5 A). During storage interval of 14–28 days, positive regulation of nervous development, carnitine metabolic processes, positive regulation of glutamine secretion, regulation in smooth muscle contraction, glycolysis were found to be the highest fold enriched GO terms (Fig. 5 B). Here, we could interpret that enriched GO terms might be regulated by the differentially regulated metabolites via interaction with the predicted genes. Also, these enriched GO terms could be related to the results of KEGG and impact pathway analysis, wherein amino-acid metabolic processes had a higher impact during 0–14 days storage interval, while carboxylic acid and fatty acid metabolism predominated during 14–28 storage interval. By integrating the exclusive metabolites with key biological processes associated with enriched GO terms, we tried to comprehend in-depth the metabolic mechanisms of goat milk yoghurt during storage.

4. Conclusion

In the present study, GC–MS based untargeted metabolomics was employed to study the metabolite changes occurring in goat milk yoghurt during storage (28 days at 4 ± 1 °C). In total 129 significantly different metabolites were identified in goat milk yoghurt. Storage interval of 14–28 exhibited higher number of differentially regulated metabolites ($p < 0.05$) than 0–14 days interval. Upregulated metabolites include amino-acids and peptides during 0–14 days (14/0), while; saccharides and carboxylic acids could be observed during 14–28 days (28/14). Correlation network analysis indicated that some differential metabolites were highly correlated, while heat map and box & whisker depicted the differences in the ion abundance/spectral features of upregulated and downregulated differential metabolites. Fold enrichment analysis and KEGG pathway analysis of exclusive metabolites ($p < 0.05$) identified six majormetabolic pathways including amino-acids metabolism (0–14 days) and fatty acid biosynthesis (14–28 days). The present data have provided comprehensive information regarding the spectral features and regulation of metabolites in goat milk yoghurt during storage. Such data could be referred for isolation of certain functional compounds in future and for improvement of -post-acidification changes in goat milk yoghurt during storage. This also provides reference values for future developments in nutritional research and improvement in goat milk based fermented products, thus ameliorating the dairy industry in terms of cost and quality.

CRediT authorship contribution statement

Heena Sharma: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Ranjith Ramanathan:** Methodology, Resources, Supervision, Project administration, Formal

analysis, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.110072.

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