Food Control 171 (2025) 111113

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Untargeted-metabolomics differentiation of unripened cow milk cheese produced from different sources of rennet

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ARTICLE INFO

Keywords:
Cow milk cheese
Rennet (enzyme)
Untargeted metabolomics
Unripened cheese
Statistical analysis

ABSTRACT

The objective of this study was to investigate the effect of different rennet sources including calf, pig, and microbes on cheese prepared from cow's milk using untargeted metabolomics approach. A total, of 89 compounds, including amino acids, aldehydes, saccharides, sulfur containing, cholesterol derivatives, alcohols, carboxylic acids, fatty acids, and lipids, were identified in different cheese samples. Comparison of metabolic profiles revealed that cheese produced with calf rennet exhibited higher levels of saccharides compared to pig rennet cheese. Conversely, pig rennet cheese showed significantly higher concentration of propionic acid, whiles the level of dimethyl disulfide, and was observed to be higher in cheese obtained from calf rennet. Pathway analysis highlighted metabolic pathways associated with amino acid metabolism (glycine, serine, and threonine), glyoxylate and dicarboxylate metabolism, and valine, leucine, and isoleucine biosynthesis. Overall, this study provides a detailed metabolic profile that distinguishes among cheese samples produced using different sources of rennet.

Table of Abbreviations

Abbreviation	Full Form
ANOVA	Analysis of variance
AQ	Aqueous layer
CL	Chloroform layer
FFAs	Free fatty acids
GC-MS	Gas chromatography mass spectrometry
HCA	Hierarchical cluster analysis
MPP	Mass profiler professional
MSA	Acetic acid
MSC	Calf rennet
MSI	Metabolomics standards initiative
MSM	Microbial rennet
MSP	Porcine rennet
MSTFA	N-methyl-N-trimethylsilyl) trifluoroacetamide
MUFA	Monounsaturated fatty acids
NIST	National institute of standards and technology
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
QQQ	Triple quadrupole
rpm	Revolutions per minute
VIP	Variable importance in projection

(*continued on next column*)

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1. Introduction

Cheese is a popular dairy product not only due to its high nutritional value but also owing to the diversity of flavors, textures, varieties, tastes, and shapes (Ah & Tagalpallewar, 2017; Gharibzahedi et al., 2018). The total annual production of cheese is estimated to be about 20 million

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https://doi.org/10.1016/j.foodcont.2024.111113
Received 16 September 2024; Received in revised form 29 November 2024; Accepted 20 December 2024
Available online 22 December 2024

tons out of which more than 80% is produced from cow milk (Seçkin, Yilmaz, & Tosun, 2017). Cheese making is a dynamic process in which various technical steps such as heat treatment, homogenization, and milk coagulation can affect the final product's characteristics (Kumar, Grover, Sharma, & Batish, 2010). Milk coagulation is an important step in the production of cheese making and has a pronounced effect on its, yield, texture, flavor, and taste (Faccia, Gambacorta, Martemucci, Difonzo, & D'Alessandro, 2019).

Various types of rennet are used as coagulants for the production of cheese and are available in both liquid and powder forms (Harboe, Broe, & Qvist, 2010). Rennet is a mixture of enzymes extracted from the animal intestine, microbial culture, and plant as well. Traditionally the calf rennet is extensively used in modern industries (Liu et al., 2021). However, due to continuous increases in global cheese consumption, the dairy industry is unable to meet the growing demands in cheese production using calf rennet (Johnson, 2017; Liu et al., 2021). To fulfill this need, several other milk coagulants are also introduced which include chicken, fungi, porcine (McSweeney & Sousa, 2000; Uniacke-Lowe & Fox, 2017), microbial, plant-based (Harboe et al., 2010) and recombinant chymosin produced with genetically modified microorganisms (Júnior, Tribst, & Cristianini, 2017). However, there are some limitations with the use of rennet and its substitute. Consumer preferences play a significant role in the choice of rennet. Animal-derived rennet is unsuitable for vegetarians and may raise ethical concerns for those adhering to specific dietary restrictions or religious guidelines (Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022). The growing demand for plant-based and ethical food products has further accelerated the need for alternative rennet sources. These considerations have pushed the dairy industry to explore microbial and plant-based rennet as viable options to meet diverse consumer expectations while maintaining product quality and flavor consistency. It is well-known that the use of rennet with different ratios between chymosin and pepsin gives rise to different proteolytic patterns in cheese (Uniacke-Lowe & Fox, 2017). However, as the age of the animal increases there is an increase in pepsin content as compared to chymosin and it being a highly active, non-specific proteolytic enzyme causes bitter flavor in cheese (Guinee & Wilkinson, 1992).

Metabolomics is a powerful analytical tool that involves the comprehensive study of metabolites, which are small molecules produced within living organisms (Oliver, Winson, Kell, & Baganz, 1998). This technique allows for the quantitative and qualitative analysis of metabolites, providing insights into metabolic pathways, biochemical profiles, and functional interactions within biological systems. This allows detailed profiling of both polar and non-polar metabolites, making it possible to identify and quantify hundreds to thousands of compounds simultaneously. In food science, both targeted and untargeted metabolomics analysis plays a crucial role in understanding the chemical composition, quality, safety, and nutritional aspects of food products. It is widely used to authenticate food products, monitor food quality during processing, and study the effects of food processing on the metabolic profiles of raw materials and finished products. By elucidating the metabolic fingerprints associated with different food products, metabolomics helps in differentiating products based on their origin, production methods, and processing conditions (Li, S. et al., 2021; Chin, E., & Slupsky, C. M., 2013).

Metabolomics has recently gained significance in modern food science, often referred to as "foodomics" (Capozzi & Bordoni, 2013; Cifuentes, 2009). This methodology addresses various systemic issues related to the safety, quality, and nutritional aspects of food (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Savorani, Rasmussen, Mikkelsen, & Engelsen, 2013; Savorani, Tomasi, & Engelsen, 2010). The study conducted by Pisano et al., showed that the use of metabolomics effectively distinguished the variations among Mozzarella cheese samples produced from different raw materials (Pisano, Scano, Murgia, Cosentino, & Caboni, 2016). Several studies have been performed to analyze the odor-active volatile compounds responsible for the distinctive aromas in cheese, for instance, the nutty flavor compounds in cheddar cheese (Avsar et al., 2004), while another the characterization of the aroma profile of cheeses similar to Gouda (Van Leuven, Van Caelenberg, & Dirinck, 2008). The hydrophilic compounds, which are soluble in water and contribute to flavor, are responsible for the distinct taste profiles found in cheeses such as Gouda cheese (Andersen, Ardö, & Bredie, 2010), Comte cheese (Salles, Septier, Roudot-Algaron, Guillot, & Etievant, 1995), Cheddar cheese (Andersen et al., 2010), and goat's milk cheese (Engel et al., 2000). However, due to the diversity of cheese varieties, it is challenging to use solely a scientific method in this industry; as a result, years of experience-based artisanal skills are still necessary for maintaining quality throughout the cheese-making process. Moreover, various metabolites are affected not only by the nutritional value of dairy products but also reflect key metabolic pathways (Sharma, Ozogul, Bartkiene, & Rocha, 2023).

The objective of this study is to differentiate unripened cow milk cheese produced from various rennet sources, including calf, porcine, and microbial rennet, using an untargeted metabolomics approach. Addressing these differences is particularly relevant in light of growing consumer demands for ethical, vegetarian, and religiously compliant food products, emphasizing the need for detailed metabolic profiling to support transparency, traceability and informed consumer choices in cheese production. The study employs a comprehensive GC-MS-based metabolomics workflow to profile both polar and non-polar metabolites present in these cheese samples. By analyzing the metabolic composition, the study aims to identify distinctive biochemical markers that are associated with each rennet type, allowing for a deeper understanding of the metabolic changes that occur during the cheese-making process. This study fills this gap by exploring the metabolic differences in unripened cheese made with different types of rennet. Multivariate statistical analyses, such as PCA and PLS-DA, were used to identify differential metabolites contributing to sample classification and highlighting potential biomarkers. While no previous study has explored the metabolic differentiation of unripened cow milk cheese using different rennet types. The results of this study will not only provide valuable insights into the biochemical dynamics of cheese production but will also contribute to the development of more precise methods for traceability, quality control, and authentication of cheese products. Ultimately, the findings will support the dairy industry in ensuring the authenticity and consistency of cheese products, especially in terms of differentiating cheeses made with various rennet sources, which is crucial for addressing consumer preferences, ethical considerations, and regulatory requirement. Nonetheless, this work supports the future exploitation of foodomics to effectively investigate and support cheese traceability, quality, and authenticity issues.

2. Materials and methods

2.1. Materials

The reagents and chemicals utilized in this study were all of analytical-grade standard. Solvents such as chloroform, (Merck, Darmstadt, Germany), hexane, and methanol (Sigma Aldrich, Burlington, MA, United States) were used for the extraction process, while ribitol was used as an internal standard in this study; Pyridine (Lab-Scan, Bangkok, Thailand), MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide), and methoxyamine hydrochloride were purchased from Acros Organic, (New Jersey, USA). Potassium chloride, calcium chloride, sodium chloride and acetic acid were purchased from Sigma–Aldrich (Burlington, MA, United States). Deionized water (Milli-Q) was obtained from a Milli-Q assembly (Billerica, MA, USA) throughout the study.

2.2. Cheese-making procedure

Cheese samples were prepared from commercial pasteurized cow

milk without the addition of starter culture, Cheese samples were prepared using the reported protocol. The existing protocol was optimized and slightly modified to ensure consistency across different rennet types (García-Gómez, Vázquez-Odériz, Muñoz-Ferreiro, Romero-Rodríguez, & Vázquez, 2020). Three commercially available rennet were used: microbial rennet from *mucor miehei* animal rennet from calf rennet, and porcine rennet purchased from Sigma Aldrich (St. Louis, MO, USA) and acetic acid. Four cheese batches were manufactured. The first batch was coagulated with calf rennet (MSC); the second batch was coagulated with porcine rennet (MSP) the third batch was coagulated with microbial rennet (MSM) and the fourth batch was coagulated with acetic acid (MSA). The flow diagram for the production of prepared cheeses is depicted in Fig. 1.

2.3. Extraction of cheese metabolites

The cheese samples were first ground, then frozen with liquid nitrogen, and subsequently subjected to freeze drying process. The protocol for metabolite extraction was carried out with slight modifications in previously reported method to enhance the extraction of polar and non-polar metabolites from the complex cheese matrix, thereby enhancing the efficiency of the extraction process and providing a more comprehensive metabolite profile for untargeted GC-MS analysis (McSweeney, 2004). Metabolites were extracted from freeze-dried cheese (200 mg) with 1.25 mL of methanol and 0.6 mL of chloroform. 100 μL of an internal standard solution containing 0.2 mg/mL ribitol was used for the experiment. Samples were sonicated for 30 min to obtain the rupture of matrix micelles and then vortexed in the solution for 1 min. Later, 3.8 mL of chloroform and 0.9 mL of aqueous potassium chloride (14.8 g/L) were added. The sample was centrifuged at 16, 000×*g*, for 20 min at 4 °C. The aqueous and non-aqueous layers were then dried completely at room temperature under a vacuum (Eppendorf Concentrator 5301, Hamburg, Germany).

2.4. Sample derivatization

After drying, each layer from aqueous and non-aqueous cheese extract was re-dissolved in 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) and then vortexed and incubated at 37 °C for 3 h by mixing at 750 rpm in a Thermomixer comfort (Eppendorf, Germany) to initiate the methoxyation reaction. One hundred μL of MSTFA was added as a second derivatizing agent and then again for 1 h at 37 °C to induce the silylation reaction. After 1 h, 100 μL of hexane was added to the sample tubes, centrifuged, and transferred into GC vials for analysis.

2.5. GC-MS analysis

Gas chromatography analysis was carried out using an Agilent Technologies 7890A gas chromatography system (Agilent Technologies, Santa Clara, CA, USA), equipped with a 7000 triple (QQQ) system (Agilent Technologies, USA) and a GC autosampler 120 (Agilent Technology, PAL-LHX-AG12). The fused silica capillary column, with dimensions of 30 m × 0.25 mm (Phenomenex, Torrance, CA, USA), was employed. This column was chemically bonded with a stationary phase comprising 5% diphenyl cross-linked with 95% dimethylpolysiloxane. For GC-MS analysis, 2 μL of the derivatized sample were injected. The derivatized cheese sample was injected in split mode, with a split flow rate of 15 mL/min and a split ratio of 10:1, using helium as the carrier gas at a constant flow rate of 1 mL/min throughout the column. The injector and source temperature was maintained at 300 °C. The oven temperature program was as follows: initially maintained at 50 °C, then increased to 250 °C at 7 min and retained at 250 °C for 8 min. Subsequently, the temperature was further increased to 300 °C for 15 min. Ions were produced at a voltage of 70 eV with an electron ionization source. Data is obtained in full scan mode over the mass range *m/z* 50–650 with a scan time of 1.6 scan/s.

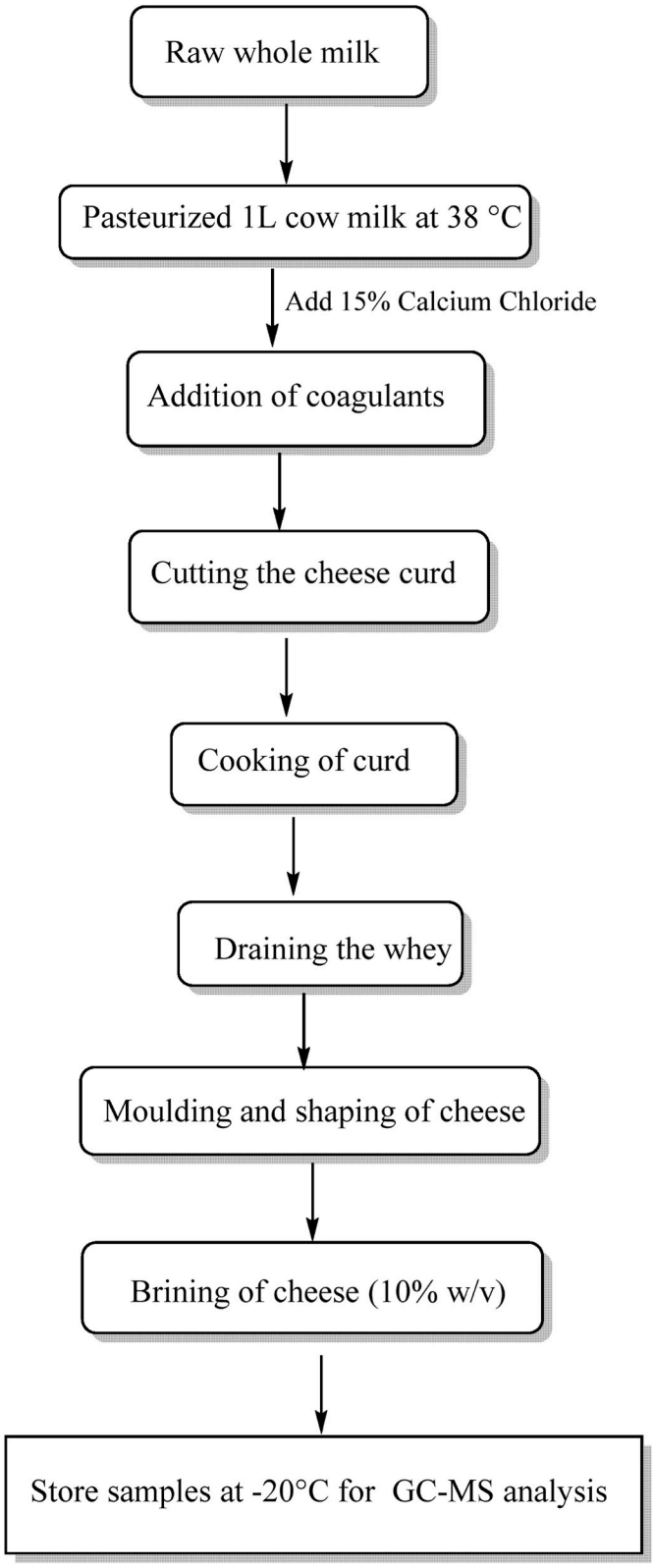

Fig. 1. Flow diagram illustrating the process of cheese samples preparation.

2.6. Data processing and metabolite identification

The raw data was processed using Agilent Mass Hunter Qualitative Analysis (B.04.00 version), with slight modifications based on the specifications outlined in a previous study (Musharraf et al., 2017) These modifications included adjustments to the peak integration

algorithm to optimize signal detection under our experimental conditions. Additionally, the spectral deconvolution parameters were refined to better handle the complexity of the cheese matrix. Subsequently, the mass profiler professional (MPP) software was employed to conduct further analysis of the feature spectra. The minimum absolute abundance of the mass spectrometry signal was set at 5000 counts, and all data were subjected to chromatogram alignments and compound filtering. A frequency-based data reduction filter was applied, discarding features absent in at least 75% of replications within a given treatment. For putative identification at level 2 according to Metabolomics Standards Initiative (MSI) guidelines, the obtained mass spectra were then compared with those already present in the NIST mass spectral library (Wiley registry).

2.7. Statistical analysis

MetaboAnalyst (online version 5.0) was used to perform multivariate data analysis techniques, including principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA). For detailed statistical analysis, the initial raw data was arranged into a matrix structure, where samples were placed into rows and metabolites arranged in columns. The raw data was normalized by using the sum method, the log transformation, and Pareto scaling before analysis. To identify major potential metabolites of cheese production features were screened using analysis of variance (ANOVA) with *p*-values less than 0.05 and variable importance in the projection (VIP) values ≥ 1. For visualization of the major differential metabolites' abundance, one-way hierarchical cluster analysis (HCA) was carried out with the complete linkage rule, and a heatmap was generated.

PLS-DA was chosen for its capability to handle the high-dimensional and collinear data typical of metabolomics studies. Unlike unsupervised methods like PCA, PLS-DA enables supervised group separation, allowing the identification of key metabolites responsible for distinguishing between cheeses samples produced with different rennet types. Its interpretability and focus on maximizing between-group variance make it particularly well-suited for this study. To address potential overfitting associated with PLS-DA, we conducted permutation tests (N = 20) and evaluated R2 and Q2 values to assess the model's predictive ability. The high R2 (0.997, 0.897) and Q2 (0.879, 0.679) values, along with consistent clustering within Hotelling's 95% confidence ellipse, confirm the model's robustness.

To identify the biochemical pathways associated with the metabolites, topology and enrichment analysis were performed using same software. The analysis was based on the hypergeometric test and relative betweenness centrality in pathway topology analysis. The hypergeometric test was employed to determine the overrepresentation of specific metabolic pathways, while relative betweenness centrality was used to assess the importance of individual metabolites within the metabolic network. Pathway enrichment was evaluated by comparing the identified metabolites to known metabolic pathways in the MetaboAnalyst database. Pathway enrichment scores were considered significant at a p-value of less than 0.05.

3. Results

3.1. Features extraction and identification

An untargeted metabolomics approach was carried out to evaluate the alterations in metabolites by using different sources of rennet. Feature extraction was performed on raw data using Mass Hunter workstation software by chromatogram deconvolution which results in a total of 1456 features. Among them, 234 features were identified (68 and 166 metabolites identified in an aqueous and non-aqueous layer, respectively), based on MS level 2 identification with a similarity index greater than 70% and a match score of more than 700 using the NIST mass spectral library. Identified features belong to various classes of compounds, including amino acids, aldehydes, sugars, disulfide, cholesterol derivatives, alcohols, carboxylic acids, hydrocarbons, ketones, fatty acids, lipids, terpene alcohol, amine and other metabolites. All the significant identified metabolites from aqueous and non-aqueous were mentioned in Tables 1 and 2, respectively.

3.2. Multivariate analysis of metabolites from aqueous and non-aqueous layer

The data was analyzed using many univariate and multivariate analyses. The ANOVA Tukey's post hoc test is univariate analysis provides a preliminary insight into metabolites that could play a potentially significant role in distinguishing between conditions within the study plot. This helps to identify the specific metabolite that carries significance among the different groups. A one-way ANOVA analysis was performed on data which was obtained from cheese samples originating from different sources of rennet. (MSC, MSP, MSM, and MSA denoted calf rennet, pig rennet, microbial rennet, and acetic acid, respectively), and a total of 89 significant metabolites were observed ($p < 0.05$), which were causing variation among all groups. Out of these significant metabolites, 36 metabolites were from the aqueous layer and 53 belong to the non-aqueous layer of all cheese samples. Pattern recognition approaches, which include the supervised PLS-DA and the unsupervised PCA models, were employed to identify the variations in metabolites among various cheese samples. All samples were found within Hotelling's 95% confidence ellipse in Figs. 2A and 3A. The total variance elucidated by the first two principal components in the PCA was 45.4% and 25.3% for the aqueous layer, and 34.2% and 25.7% for the non-aqueous layer, respectively. The cheese samples produced with various types of enzymes were clearly separated from one another and there was a clear aggregation of biological replicates within each of the groups, suggesting acceptable reliability within the different groups. Overall, these findings indicate that PCA effectively distinguished the samples within each group with no outlier in the data.

Figs. 2B and 3B depict the dynamic biochemical alterations in fresh cheese, and a specific discriminant model was created through the application of the supervised PLS-DA statistical method. In Figs. 2B and 3B, the supervised PLS-DA statistical technique was employed to establish a precise discriminant model based on the dynamic biochemical variations observed in fresh cheese. The first and second principle components revealed 39.8% and 26.1% of the total variance of the aqueous layer and 24.8% and 23.6% for the non-aqueous layer, respectively. PLS-DA exhibited more clearly the variations observed within each group. Notably, the PLS-DA score maps for both aqueous and non-aqueous layers demonstrated excellent separation among different cheese groups, with R2 values of 0.997, 0.897, and Q2 values of 0.879, and 0.679, respectively. These results showed that the use of three different types of rennet during the cheese-processing procedure had a significant impact on the composition of metabolites in cheese samples. The significant features with higher variable importance in projection (VIP) scores are considered more important in explaining the variation and contributing to the discrimination between samples in the PLS-DA model.

Furthermore, the top fifteen metabolites with (VIP >1) score values for aqueous and non-aqueous layer samples are shown in Figs. 2C and 3C. Permutation tests (N = 20; Figs. 2D and 3D) indicated no overfitting. In this study PLS-DA models exhibited high R2 and Q2 values, indicating excellent fitness and predictive capability, and could be further used for identifying different metabolites contributing to the clustering of samples. Based on dynamic variation in the abundance of identified metabolites, Hierarchical Cluster Analysis (HCA) was performed and the results are presented in the Heatmap, Fig. 4 (aqueous) and Fig. 5 (non-aqueous). Notably, the calf rennet sample and cheese obtained from pig rennet exhibited clear distinctions. The data indicated that the variations in metabolite abundance in the cheese samples were influenced by the different types of rennet and these metabolites could

Table 1
The list of identified aqueous metabolites significantly different among cheese samples showing up/down-regulation with $p < 0.05$.

S. No.	Feature No.	Feature Name	Mass	Retention time (min)	VIP Score	*p*-value
1	1	Glycine	75.0	5.7	2.112	9.79E-04
2	2	Threonine	119.1	6.0	2.080	8.83E-07
3	4	Propanediol	76.0	6.5	0.599	2.16E-06
4	7	Glutamine	146.1	7.0	1.811	2.04E-04
5	8	4-Hydroxy-4-methylpentanone	116.1	7.2	1.125	1.42E-10
6	13	Hydroxypropanoic acid	90.0	7.6	0.698	4.53E-04
7	15	Neopentyl pentanoate	172.2	8.4	0.996	1.77E-06
8	12	*N*-Methylphenethylamine	135.2	9.4	0.459	1.18E-05
9	16	Isopropyl methyl cyclohexanone	154.2	9.5	0.927	1.83E-04
10	17	O-benzyl hydroxylamine	123.1	9.7	0.839	1.12E-02
11	18	Hydroxy-methyl benzoic acid	152.1	10.2	0.786	4.24E-05
12	19	Linalool	154.2	11.3	0.596	1.11E-05
13	20	Disulfide, dimethyl	94.2	11.7	0.294	3.41E-02
14	24	Propionic acid	74.0	14.1	0.138	1.73E-05
15	29	Benzoxazol-2-amine	134.1	16.9	0.510	1.21E-02
16	30	Myo-inositol	180.1	18.3	0.470	9.33E-07
17	32	3-Hydroxy-2,2-dimethylpropyl isobutyrate	174.2	18.8	0.494	5.64E-05
18	35	Trihydroxypentanal	134.1	19.6	0.782	4.88E-06
19	37	Threonic acid	136.1	20.2	1.643	3.20E-11
20	38	Citronellyl hexanoate	254.4	21.3	0.487	1.33E-06
21	39	Lactose	342.3	22.5	1.184	6.39E-08
22	40	Mannitol	182.1	23.2	1.571	1.91E-10
23	41	Mannose	180.1	23.4	1.834	1.13E-05
24	42	Diethoxyhexane	174.2	25.4	0.749	4.34E-06
25	43	Eicosanoic acid	312.5	27.3	0.886	9.79E-06
26	44	Octanediol	146.2	28.6	0.707	8.78E-06
27	45	Hexenedioic acid	144.1	28.9	1.424	3.00E-06
28	53	Galactopyranosiduronic acid	475.5	32.2	0.836	1.10E-04
29	54	Talose	180.1	32.6	0.591	2.13E-04
30	49	Galactopyranoside	180.1	32.8	0.713	3.59E-08
31	50	Hexadecyloxyethanol	286.5	33.7	1.191	4.04E-04
32	56	Melibiose	342.3	33.8	0.674	1.70E-02
33	51	Gluconic acid	196.1	34.3	0.533	1.45E-07
34	52	Maltose	342.3	35.2	0.475	2.61E-06
35	59	Arabinose	150.1	39.2	0.977	9.95E-06
36	60	6-O-(6-deoxy-alpha-L-mannopyranosyl)-D-glucose	326.3	41.3	0.698	5.38E-05

be considered as potential marker metabolites.

3.3. Metabolomics pathway analysis

The metabolites identified in the cheese made from cow milk using rennet from different sources were further analyzed with the online software MetaboAnalyst. This analysis aimed to identify the biochemical metabolic pathways associated with the identified metabolites, using the hypergeometric test and relative betweenness centrality in pathway topological analysis. The metabolic pathway analysis provide valuable insights into the potential metabolic pathways, as shown in Fig. 6A (aqueous layer) and Fig. 6B (non-aqueous layer). Tables S1 and S2 present a list of various catabolic and anabolic pathways that are influenced by altered metabolism in different cheese production processes attributed to enzymes. To prepare fresh cheese from different rennet, nine main pathways were found, with a statistical significance of ($p < 0.05$). Among them five pathways were found to be in the aqueous phase named glyoxylate and dicarboxylate metabolism, galactose metabolism, glycine, serine, and threonine metabolism, biosynthesis of unsaturated fatty acids, nitrogen and galactose metabolism. The remaining four from non-aqueous named as biosynthesis of unsaturated fatty acids, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism and valine, leucine and isoleucine biosynthesis. The annotated visual representation of the metabolic pathways influenced by different rennet sources is presented in Fig. S1 for the aqueous layer and Fig. S2 for the non-aqueous layer. Among all nine identified pathways four pathways: glyoxylate and dicarboxylate metabolism, glycine, serine, and threonine metabolism, biosynthesis of unsaturated fatty acids, and nitrogen metabolism explicitly have an impactful effect on the overall quality of cheese due to the differential metabolites profile displayed by each cheese variety, which could be attributed to enzyme activity. The most significant metabolic pathways were chosen based on the impact values and *p*-values obtained from topology and enrichment analysis.

4. Discussion

In the present study, an untargeted metabolomics differentiation approach was used to characterize various samples of unripened cow milk cheese produced by the enzyme rennet of varying origins. The origin of rennet is considered to be an important factor not only in the production of cheese (Balcones, Olano, & Calvo, 1996) but also in contributing to the flavors of the cheese (Urbach, 1997). Cheese flavor is a highly complex phenomenon. Each type of cheese possesses a distinct flavor profile characterized by numerous specific compounds, varying in concentration and belonging to different chemical classes. During cheese production and maturation, various biochemical reactions occur, leading to the formation of cheese aroma. The compounds responsible for the cheese flavors are derived from three main milk components: lactose, lipids, and proteins (Karali, Georgala, Massouras, & Kaminarides, 2013). Most previous studies have focused on ripened cheese (Hayaloglu & Karabulut, 2013), with few reports are available on the characteristics of soft, unripened cheese intended for immediate consumption. Differentiation between various types of sheep milk cheese using calf rennet and pig rennet was achieved through metabolic analysis of fatty acid content and volatile compounds during the ripening process (Aquilanti, Santarelli, Babini, Osimani, & Clementi, 2013; Di Giacomo, Casolani, & Del Signore, 2014; Gardini et al., 2006). However, there has been no study conducted on samples of cow milk cheese produced using different types of rennet. Cheeses obtained from different types of rennet are differentiated by around 89 aqueous and non-aqueous metabolites. The box and whisker's plots illustrating the

Table 2
The list of identified non-aqueous metabolites significantly different among cheese samples showing up/down-regulation with $p < 0.05$.

S. No.	Features No	Features Name	Mass	Retention time (min)	VIP Score	*p*-value
9	36	Tert-butanol	74.1	16.6	0.561	5.90E-03
2	7	Hexanoic acid	116.1	7.2	0.183	3.60E-03
1	3	Valine	117.0	5.7	2.223	2.00E-04
7	26	Citronellol	156.2	12.3	2.736	3.00E-04
26	88	Fucose	164.1	36.9	1.099	9.00E-04
23	85	Fructose	180.1	35.3	0.723	2.90E-03
21	80	Glucopyranoside, methyl	194.1	33.5	0.777	9.43E-05
25	87	Hydroxydodecanoic acid	216.3	35.7	0.693	1.37E-02
15	72	Hexadecenal	238.4	28.5	0.024	7.78E-06
8	35	Trimethyltetradecane	240.5	16.0	0.022	6.20E-03
42	118	Hexanoic acid, 3,7-dimethyl-octenyl ester	254.2	49.3	0.149	3.06E-06
3	8	Nonadecatriene	262.5	7.4	0.865	1.72E-05
5	16	Octadecatrienoic acid	278.4	10.0	2.204	4.00E-04
24	86	Oleic Acid	282.5	35.5	1.346	5.01E-09
14	71	Stearic acid	284.5	27.3	0.428	2.80E-03
16	73	Monomyristin	302.4	28.9	0.800	3.55E-05
4	14	Eicosatrienoic acid	306.5	9.1	2.610	1.00E-04
12	58	Eicosenoic acid	310.5	22.9	0.320	2.00E-04
17	75	Isopropyl stearic acid	326.6	30.6	0.516	4.00E-04
20	79	Dihydroxypropyl palmitate	330.5	32.8	1.188	1.00E-04
18	76	Docosatrienoic acid	334.5	30.9	2.593	1.00E-04
13	60	Heptadecyl chloropropanoate	347.0	23.4	0.653	2.00E-04
32	100	Glyceryl 2-linolenate	352.5	42.9	0.490	1.78E-06
19	77	Glyceryl monolinoleate	354.5	31.1	2.246	1.17E-02
28	95	9-Octadecenoic acid, 2,3-dihydroxypropyl ester	356.2	41.3	0.102	3.40E-03
31	99	2-Vaccenoyl-glycerol	356.5	42.8	2.213	6.61E-08
22	82	2,3-dihydroxypropyl stearate	358.2	34.3	1.858	8.40E-03
10	44	Glycerol diacetate laurate	358.5	18.6	0.240	1.46E-02
29	97	Butoxyethyl oleate	382.6	41.5	0.411	8.59E-07
27	94	Cholesterol	386.7	40.8	1.179	3.98E-07
6	20	Glyceryl diacetate 1-linolenate	436.6	10.8	2.248	2.00E-04
36	106	Ethyl iso-allocholate	436.6	43.8	0.693	8.68E-07
46	126	Octadecanoic acid, 2,3-bis(acetyloxy) propyl ester	442.6	54.7	0.378	5.21E-06
11	56	Methyl dimethoxy octadecenoate	444.7	21.3	1.643	3.00E-04
40	112	Dodecyl cis-9,10-epoxyoctadecanoate	466.8	46.2	0.919	3.31E-07
45	125	9-Octadecenoic acid, tetradecyl ester	478.8	53.9	0.411	3.60E-08
35	105	Palmitic acid,2-(tetradecyloxy) ethyl ester	496.8	43.7	0.566	6.57E-03
30	98	Glyceryl 1,3-dimyristate	512.8	42.6	2.263	3.00E-04
39	111	Octadecanoic acid, 3-hydroxy-2-(1-oxotetradecyl)-, methyl ester	524.4	46.0	0.295	9.07E-06
53	144	9-Octadecenoic acid, octadecyl ester	534.9	72.4	1.116	1.63E-03
38	109	Decanoic acid, 1,2,3-propanetriyl ester	554.8	45.7	0.589	4.68E-06
43	119	Oleic acid, eicosyl ester	563.0	49.6	0.974	1.67E-10
33	103	9-Octadecene, 1-[2-(octadecyloxy) ethoxy]	564.9	43.4	1.513	4.74E-07
47	132	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	568.9	59.9	2.270	3.52E-07
49	135	2-hydroxypropane-1,3-diyl dipalmitate	568.9	62.6	2.793	4.27E-12
51	141	2-[9-Octadecenyloxy] ethyl -9-octadecenoate	577.0	68.2	0.657	1.35E-04
50	140	9-Octadecenoic acid, 2-(octadecyloxy) ethyl ester	579.0	67.1	1.909	4.11E-06
34	104	Oleic acid, 3-(octadecyloxy) propyl ester	593.0	43.5	0.067	3.65E-04
48	133	9-Octadecenoic acid, 2-hydroxy-3-[(1-oxohexadecyl) oxy] propyl ester	594.5	60.8	2.068	5.16E-07
41	117	2-Hydroxy-3-(palmitoyloxy) propyl oleate	594.9	49.0	0.252	2.18E-09
44	124	Glycerol 2-acetate 1,3-dipalmitate	610.9	53.4	0.131	1.60E-02
52	143	2-Hydroxy-3-(stearoyloxy) propyl -9-octadecenoate	623.0	71.3	0.735	2.23E-02
37	107	Distearin	625.0	44.0	0.627	4.88E-04

major metabolites of cow milk cheese show significant differences between calf and pig rennet, as presented in Fig. 7 (aqueous) and Fig. S3 (non-aqueous). Moreover, box and whisker's plots of microbes and acetic acid are presented in Fig. S4 for the aqueous layer and in Fig. S3 for the non-aqueous layer. These metabolites were predominantly fatty acids and lipids, as cheese is widely recognized for its high content of these components. The fatty acid and lipid profiles of cheeses varied depending on the type of rennet used, although oleic, palmitic, and myristic acids were consistently being the most predominant. Oleic acid was notably the most abundant fatty acid in cheese samples made with calf rennet. In this study, the up-regulated content of oleic acid could influence the aroma of the cheese. According to Hanus et al., oleic acid is one of the most significant fatty acids known for its beneficial properties, including anti-cancer and anti-atherogenic effects, making it a favorable choice in dietary considerations (Hanuš, Samková, Křížová, Hasoňová, & Kala, 2018). The study also found that calf cheese samples had significantly higher levels of monounsaturated fatty acids (MUFA), while acetic acid (MSA) and microbial (MSM) cheese samples showed similar amounts. The presence of high levels of saturated fatty acids (SFA) in dairy products has been associated to negative health effects, contributing to the occurrence of various diseases such as type 2 diabetes, cancer, cardiovascular disease, and obesity (Ruiz-Núñez, Dijck-Brouwer, & Muskiet, 2016; Thorning et al., 2016).

However, previous studies suggested that the relationship between the development of these conditions and SFA may be more complex (Huth & Park, 2012; Lordan, Tsoupras, & Zabetakis, 2017). Foods are comprised of a combination of unsaturated and saturated fatty acids. However, enzymatic hydrolysis of triglycerides into fatty acids derivatives and glycerol including mono, and diglycerides plays a crucial role in flavor development in some varieties of cheese (McSweeney & Sousa, 2000). Fatty acids not only directly contribute to cheese flavors but also act as substrates in various biochemical reactions that produce several organic compounds such as alcohols, aldehydes, and lactones, thereby enhancing the cheese aroma (Thierry et al., 2017). Our findings

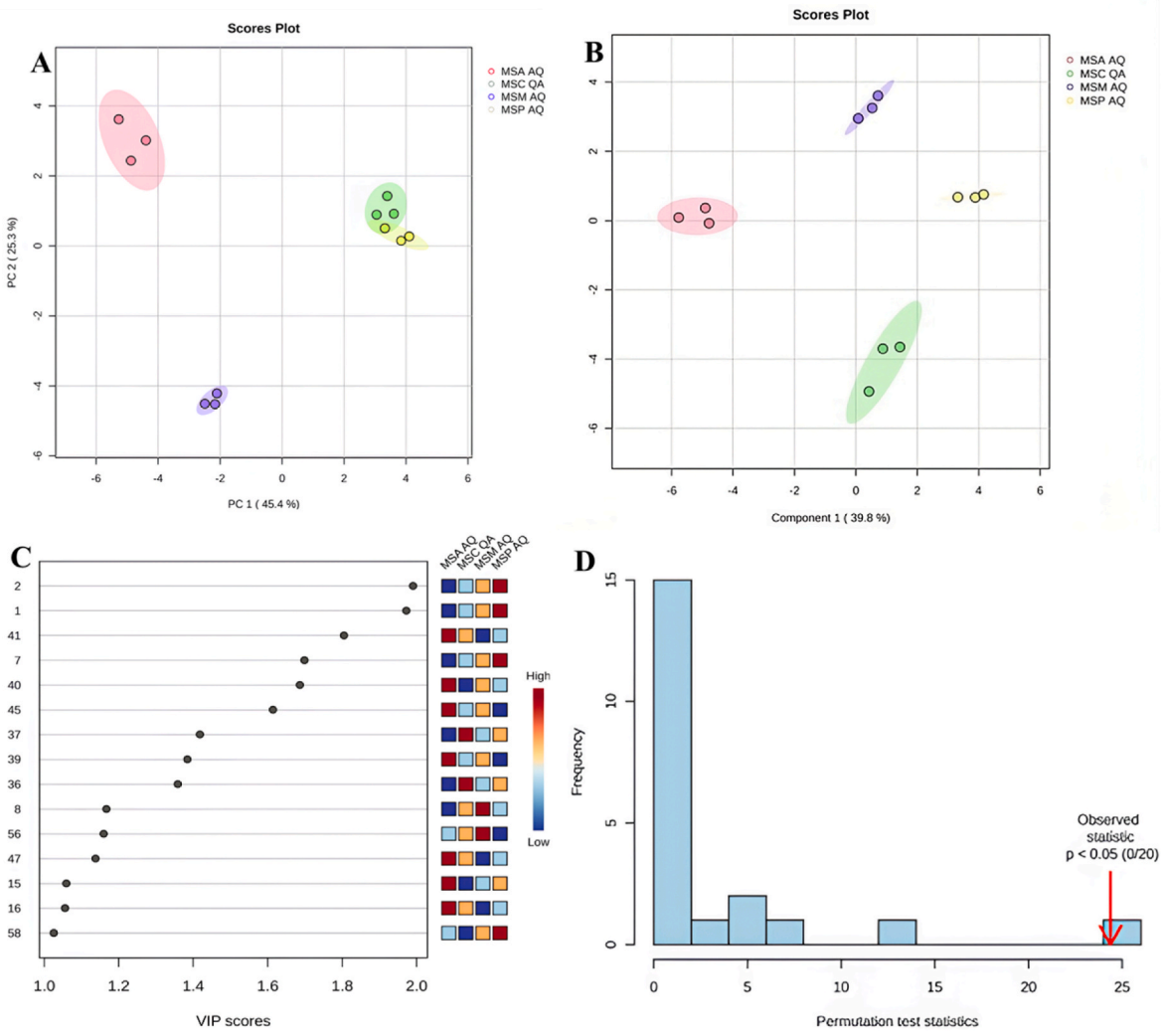

Fig. 2. A) Principal components analysis (PCA) scores plot (**B**) PLS-DA, Partial least squares discriminant analysis scores plot exhibiting the discrimination among the four groups. MSC (AQ), MSP (AQ), MSM (AQ), and MSA (AQ) denote calf rennet, pig rennet, microbial rennet, and acetic acid cheese samples for aqueous layer (AQ) (**C**) VIP (variable importance in projection) values by PLS-DA modeling and (**D**) permutation test of the PLS-DA model with 20 times.

from VIP analysis highlighted several medium and long-chain free fatty acids (FFAs) including eicosatrienoic acid, octadecatrienoic acid, hydroxydodecanoic acid, hexanoic acid, eicosanoic acid, and oleic acid. The content and composition of fatty acid groups, such as saturated, monounsaturated, and polyunsaturated fatty acids in cheese, can exhibit notable differences depending on the type of milk utilized and the specifics of the manufacturing process (Ceylan, Tokgöz, Akgül, & Atasoy, 2024; Paszczyk & Łuczyńska, 2020). Cheese obtained from calf rennet showed a high abundance of oleic acid and dipalmitoylglycerol compared to the other cheese. In contrast, cheese obtained with pig rennet showed significant up-regulation of metabolites including, glyceryl diacetate 1-linoleate, glyceryl monolinoleate, octadecatrienoic acid (polyunsaturated fatty acid), methyl-dimethoxy octadecenoate (fatty acid methyl esters), cholesterol, glyceryl monolinoleate, 2-hydroxy-3-(s-tearoyloxy) propyl-9-octadecenoate, palmitic acid, 2-(tetradecyloxy) ethyl ester are shown in Fig. S3. These metabolites were found to be a potential marker of pig rennet cheese. Significant changes in carbohydrates were observed, with galactopyronoside, maltose, lactose, trihydroxypentanal, and threonic acid identified as the most prominently up-regulated metabolite in calf rennet cheese samples compared to those in pig rennet cheese. Moreover, the presence of different saccharides among the up-regulated metabolites in samples led us to hypothesize a lower consumption of these molecular components by microorganisms, the presence of which was depleted in the original milk by the heat treatment.

Another up-regulated metabolite was gluconic acid, present in dairy products due to the oxidation of glucose, compared to all other samples. Dimethyl disulfide level was found to be higher in cheese produced using calf rennet as compared to cheese made with pig rennet. It is formed during the breakdown of sulfur-containing amino acids, such as cysteine and methionine, by microbial action during cheese ripening. It is typically produced by bacteria, yeast, or molds present in the cheese, particularly those involved in proteolysis and sulfur metabolism. The previous studies of the cheese samples revealed the presence of dimethyl sulfide, dimethyl trisulfide, dimethyl disulfide, and carbon disulfide (Bertuzzi, McSweeney, Rea, & Kilcawley, 2018; Marangoz & Bostan, 2020). Interestingly, O'Callaghan et al., observed similar concentrations of dimethyl sulfide in Cheddar cheese made from outdoor and indoor feeding systems (O'Callaghan et al., 2017). The observed variations among the cheeses may be attributed to variations in their oxidation-reduction potential, which can be influenced by the manufacturing procedure and the specific starter cultures used (Caldeo & McSweeney, 2012). Proteolysis contributes directly to cheese flavors by releasing peptides and amino acids. It initially breaks down caseins into large and medium-sized peptides through the action of curd and proteolytic enzymes. Amino acids serve as substrates for transamination,

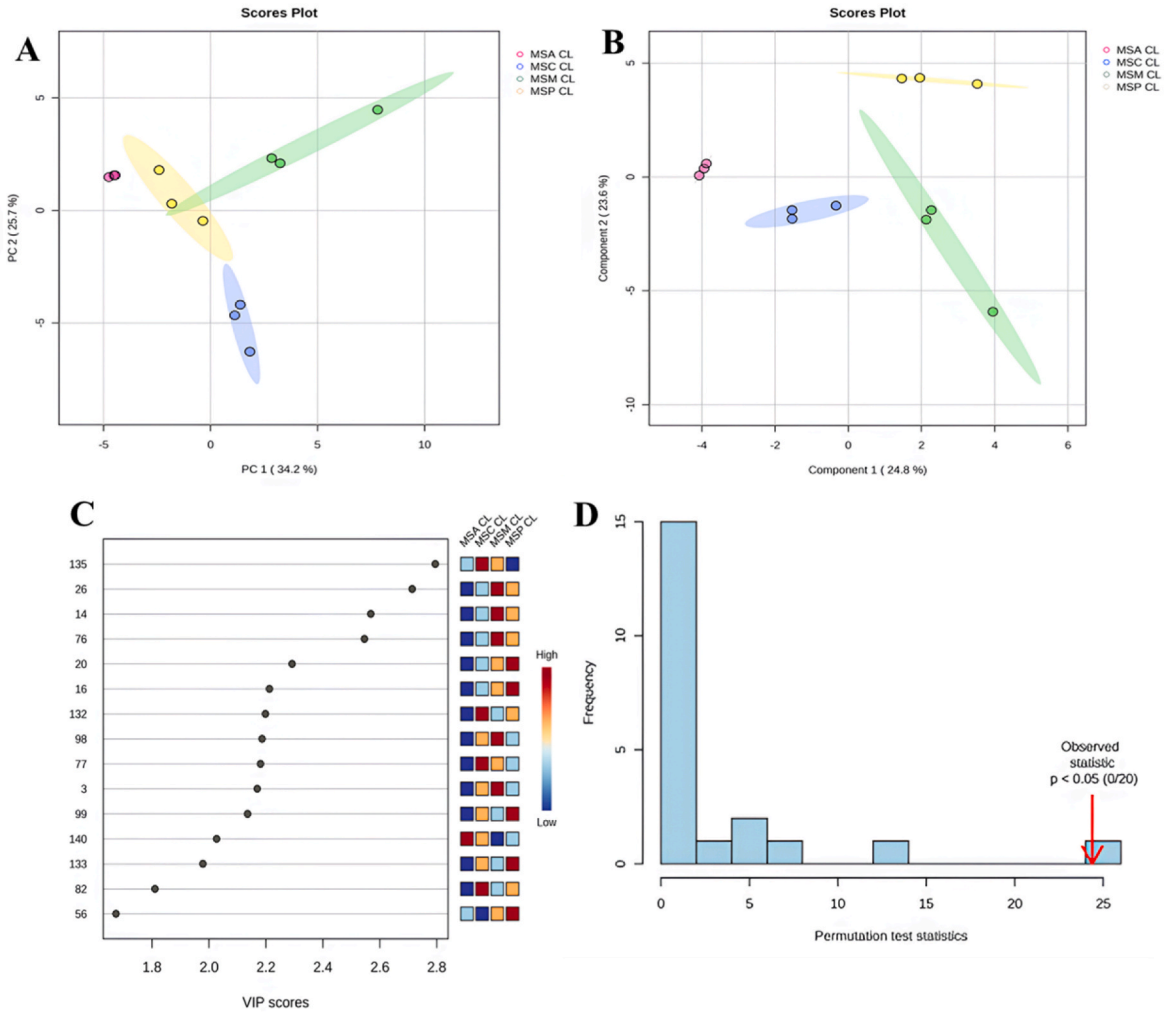

Fig. 3. (**A**) Principal components analysis (PCA) score plot (**B**), PLS-DA scores plot exhibiting the discrimination among the four groups. MSC (CL), MSP (CL), MSM (CL), and MSA (CL) denote calf rennet, pig rennet, microbial rennet, and acetic acid cheese samples for non-aqueous layer (CL) (**C**) VIP (variable importance in projection) values by PLS-DA modeling and (**D**) permutation test of the PLS-DA model 20 times.

dehydrogenation, decarboxylation, and reduction, producing a diverse array of flavor compounds. In this study, three amino acids were identified as distinct metabolites in the cheese, comprising glycine, threonine, and glutamine. It was reported that the presence of glutamine was found to be associated with bitterness (Schirone, Tofalo, Mazzone, Corsetti, & Suzzi, 2011). Glycine, threonine, and glutamine were identified as the most up-regulated metabolites from pig rennet. However, these amino acids were down-regulated compared to all other types of cheese samples. The sensory quality of cheese may be reduced as a result of these amino acid variations. It has been suggested that the gradual breakdown of casein in cheese results in the formation of polypeptides, which are subsequently hydrolyzed to produce amino acids (Zhang, Zheng, Feng, Zhou, & Ma, 2022; Zhang, Zheng, Zhou, & Ma, 2022). These variations may be due to proteolysis and enzymatic processes, in addition to the effect of rennet and starter culture (Puchades, Lemieux, & Simard, 1989; Subramanian, Alvarez, Harper, & Rodriguez-Saona, 2011; Zhang, Zheng, Zhou, & Ma, 2022). Additionally, similar variations in amino acid concentrations have been observed in Cheddar cheese (Subramanian et al., 2011).

The abundance of eicosanoic acid in cheeses from pig rennet is higher among all. This metabolite is crucial to the flavor characteristics of cheeses. This acid originates as a consequence of the breakdown of fatty acids, amino acids, and lactic acid during the production of cheese produced by the coagulant or microorganism-associated enzymes (McSweeney, 2004). A higher level of propionic acid was detected in cheese made using pig rennet in comparison to MSA, MSC, and MSM cheese samples. The acidity in the taste could be attributed to the esterification process occurring between fatty acids and short-chain alcohols in Mongolian cheese during storage, leading to the formation of esters (Zhang, Zheng, Feng, et al., 2022). In pig rennet cheese, melibiose and octanediol were observed as up-regulated metabolites compared to calf rennet cheese. Melibiose could be hydrolyzed into galactose and glucose under the influence of α-galactosidase (Karali et al., 2013) and previously reported in French Brie cheese (Zhang, Zheng, Feng, et al., 2022), and it was used as a source of carbon for *E. coli* (Zhang, Zheng, Feng, et al., 2022). Octanediol might be generated from the aldehydes produced during the breakdown of amino acids. Undergoing this conversion into the respective alcohols occurs through a decomposition pathway involving the action of alcohol dehydrogenase (Karali et al., 2013). Additionally, various metabolites not only affect the quality of dairy products but also offer insights into crucial metabolic pathways (Sharma et al., 2023). These compounds were the key factors in distinguishing cheeses produced with different types of rennet. From the foregoing discussion, it can be concluded that an untargeted GC-MS based metabolomics differentiation can applied not only for samples of cow milk cheese produced with various types of rennet but also for cheese samples produced from other sources.

In the current study, the metabolic pathways of glyoxylate and

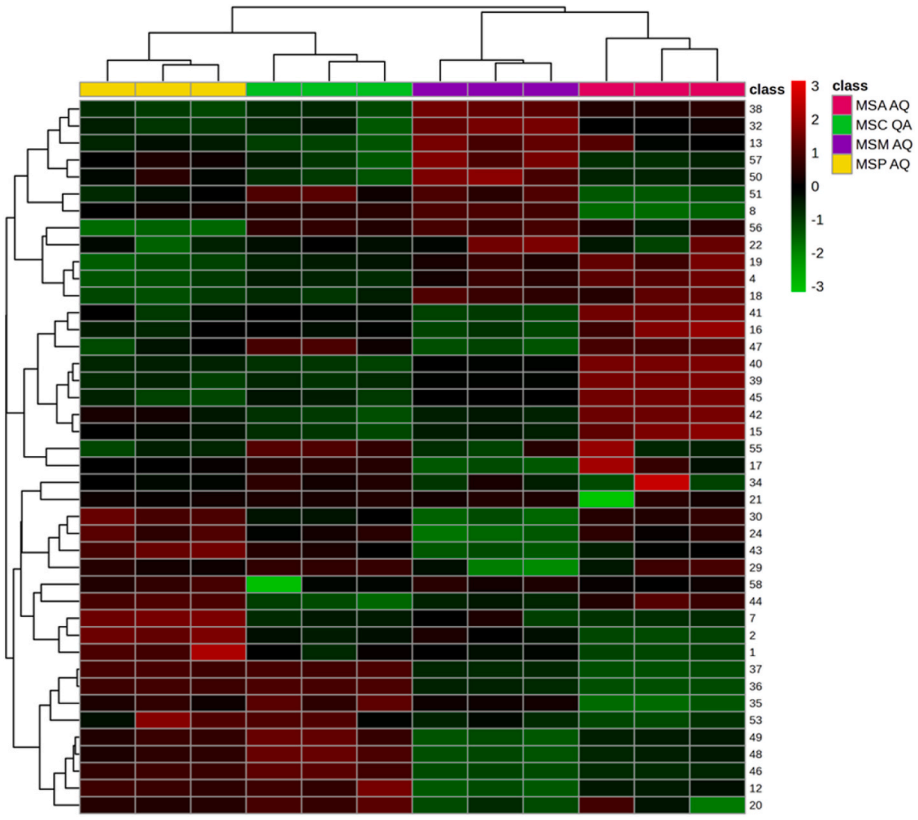

Fig. 4. Heatmap generated from hierarchical clustering analysis (HCA) of aqueous layer (AQ) metabolite variations among unripened cheese samples, showing discrimination among the four groups. MSC (AQ), MSP (AQ), MSM (AQ), and MSA (AQ) denote cheese samples made with calf rennet, pig rennet, microbial rennet, and acetic acid, respectively, for the aqueous layer.

dicarboxylate metabolism, glycine, serine, and threonine metabolism, biosynthesis of unsaturated fatty acids, nitrogen metabolism, and galactose metabolism, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism and valine, leucine and isoleucine biosynthesis were particularly affected. The observed changes in the relative abundance of various metabolites in cheese are consistent with these predicted pathways. The metabolic pathways identified were closely related with the breakdown of lipids and amino acids. This underscores the significant impact that proteolytic and lipolytic activities, occurring during cheese production, can significantly impact on the formation of flavors (Yvon & Rijnen, 2001; Zhang, Zheng, Feng, et al., 2022). As reported in previous studies, the glyoxylate and dicarboxylate metabolic pathway is associated with the production of volatile fatty acids like acetate, propionate, and butyrate, which are important for cheese flavor development (Chen et al., 2023). These metabolites are produced during fermentation and contribute to the complex flavor profile of cheese. In cheese, nitrogen metabolism is closely related to the production of many aroma compounds. This relationship arises from the generation of aroma compound precursors through the process of casein hydrolysis (Li et al., 2022). Among the most abundant amino acids in cheese and can be converted into α-keto acids and then α-ketoglutarate, ultimately contributing to the production of glutamic acid, a substance known for its umami taste. As a result, the availability of peptides and amino acids, which are derived from nitrogen sources, significantly influences the development of cheese flavor. The degradation of casein by milk coagulating enzymes and proteinases is a key biochemical process in this context, ultimately impacting the sensory properties of the cheese (Yuceer et al., 2009). Moreover, the metabolism of glycine, serine, and threonine was strongly associated with the composition of amino acids, playing a crucial factor in influencing the taste changes. These amino acids can participate in glycolysis and the TCA cycle to support microbial metabolism, which is essential during cheese production (Ganesan & Weimer, 2017). Noteworthy pathways include glycine, serine, and threonine metabolism, emphasizing the pivotal role of glycine and threonine in these processes. The biosynthesis of unsaturated fatty acids in cheese involves various metabolites and pathways, including unsaturated fatty acid biosynthesis, linoleic acid metabolism, and fatty acid biosynthesis. Cheese enriched with fatty acids and conjugated linoleic acid has been shown to affect lipid metabolism and fat profile. The metabolites involved in the biosynthesis of unsaturated fatty acids serve as substrates for the enzymes involved in the desaturation and elongation of fatty acids, ultimately leading to the production of unsaturated fatty acids such as octadecanoic acid, and eicosonoic acid.

The metabolic profiling of cheese made with acetic acid has revealed a diverse array of metabolites, indicating the complexity of the biochemical transformations during cheese production. These metabolites include alcohols, ketones, esters, amines, and acids that contribute significantly to the flavor, aroma, and overall quality of the cheese. The compound 4-Hydroxy-4-methylpentanone and isopropyl methyl cyclohexanone are ketone that contributes to the characteristic aroma of the cheese. Ketones are primarily produced via lipid oxidation or amino acid catabolism. Their presence aligns with findings from other studies, where ketones such as 2-heptanone was considered one of the main compounds accountable for fresh cheese aroma (Cincotta et al., 2021). Further oxidation and breakdown of long-chain unsaturated fatty acids can result in the production of aroma compounds including aldehydes, alcohols, and ketones, which give fermented items their aromas (Shahidi & Hossain, 2022). The aroma is one of the main elements influencing the sensory attributes of food products, and also affects cheese purchasing behavior and preference of consumers (Yavuz, Kasavi, & Öner, 2021). Neopentyl pentanoate, an ester is known for their fruity and sweet aroma, which balances the sharpness of acetic acid-derived cheeses. Hexenedioic acid could arise from the breakdown of unsaturated fatty acids. Hydroxy-methyl benzoic acid, potentially a derivative of phenolic

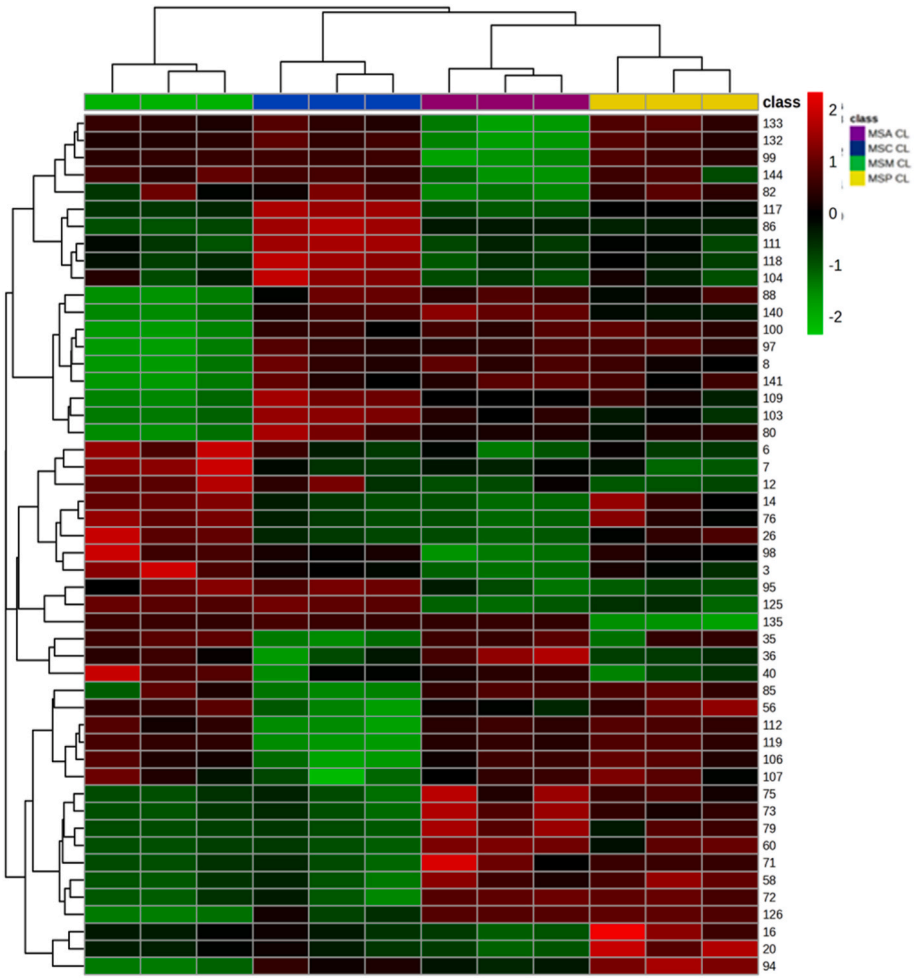

Fig. 5. Heatmap generated from hierarchical clustering analysis (HCA) of non-aqueous layer (CL) metabolite variations among unripened cheese samples, showing discrimination among the four groups. MSC (CL), MSP (CL), MSM (CL), and MSA (CL) denote cheese samples made with calf rennet, pig rennet, microbial rennet, and acetic acid, respectively, for the non-aqueous layer.

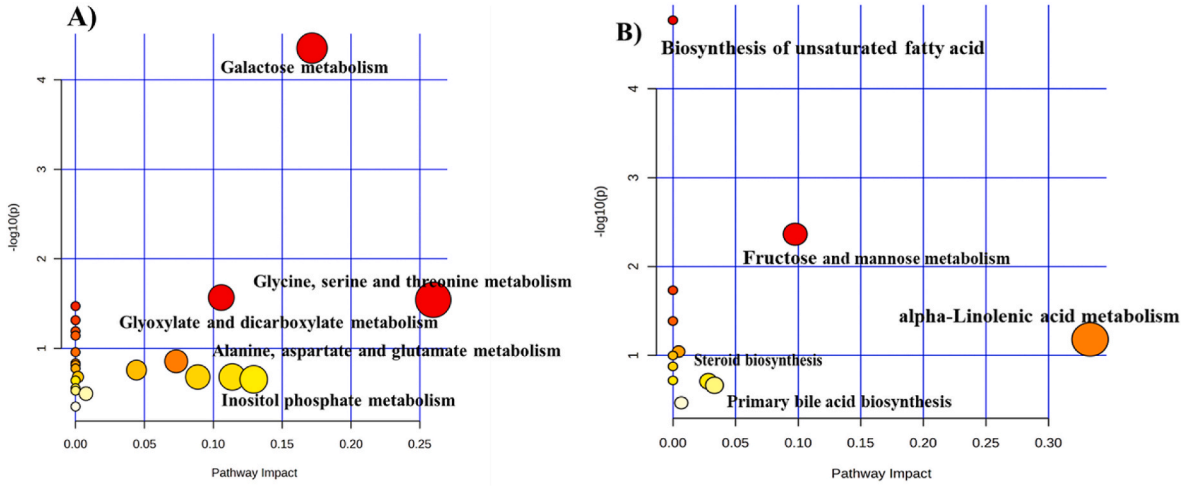

Fig. 6. A) Aqueous and **B)** Non-aqueous layer metabolites are subjected to metabolic pathways associated with differential metabolite variations from the cheese produced by different sources of rennet.

compounds, might contribute to the antioxidant properties of the cheese (Fox, Guinee, Cogan, & McSweeney, 2017). All these metabolites are down-regulated in cheese made with calf, pig and microbial rennet as shown in Fig. S4.

In this study, the content of hydroxypropanoic acid, associated with an acidic taste, was found to be up-regulated in cheese samples made with microbial rennet compared to others. This may be attributed to the esterification of free fatty acids with short-chain alcohols (Eş,

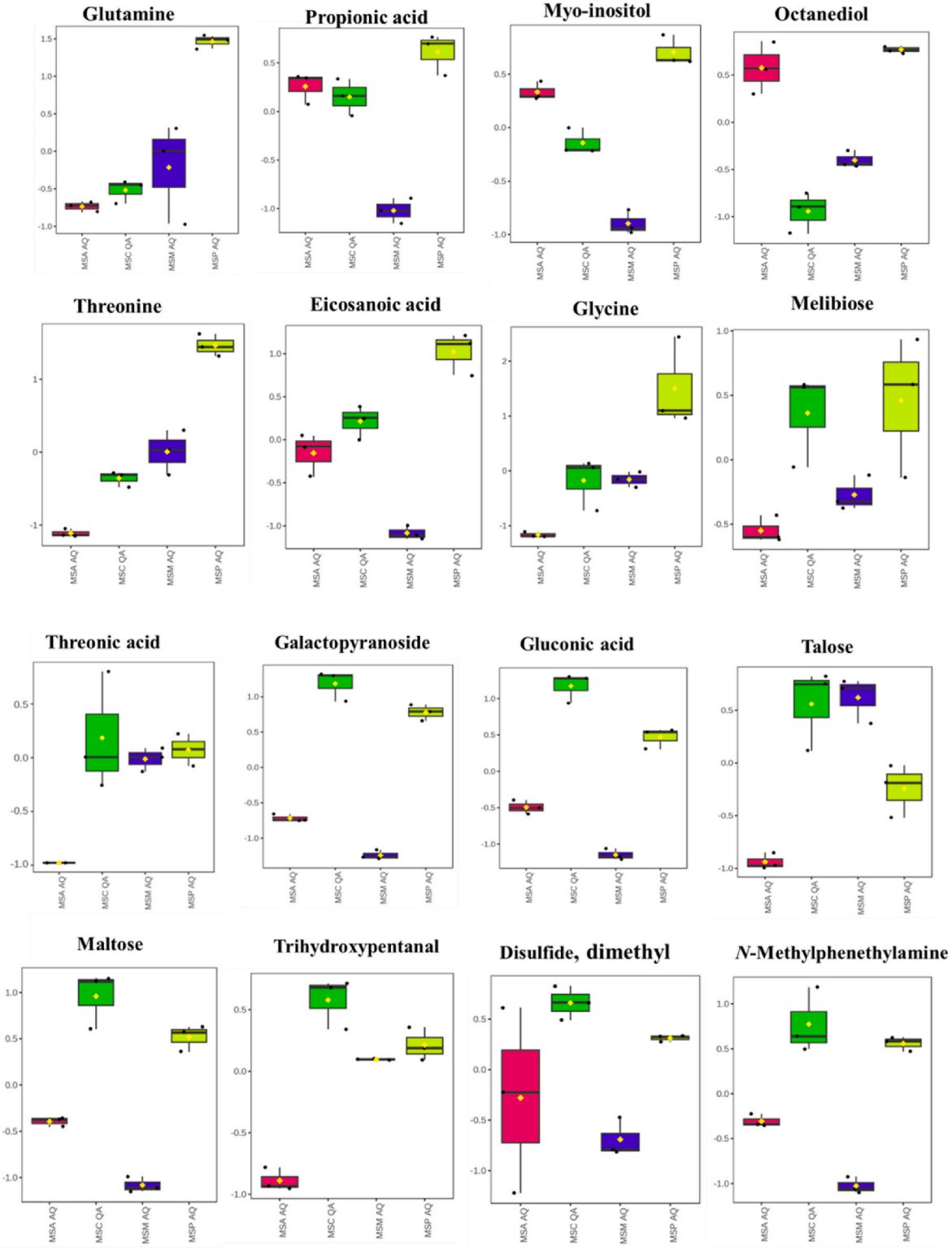

Fig. 7. Box plot showing up and down-regulated metabolites in the aqueous layer identified in one-way ANOVA (non-parametric), post hoc Bonferroni statistical hypothesis testing (p-value <0.05).

Khaneghah, Hashemi, & Koubaa, 2017; Sabia, Gauly, Napolitano, Cifuni, & Claps, 2020). Cheese made with microbial rennet exhibits higher levels of 3-hydroxy-2,2-dimethylpropyl isobutyrate and citronellyl hexanoate compared to other samples. These compounds add fruity and floral notes, enhancing the overall flavor complexity. Citronellyl hexanoate, which is known for its sweet and citrusy aroma, has been identified in various fermented products (McSweeney & Sousa, 2000). These metabolite were up-regulated in cheese made with microbial rennet as compared to cheese made with pig, calf rennet, or acetic acid. The biochemical differences offer insights into how enzymes impact the flavors and overall quality of cheese. The obtained data from this study provide a baseline metabolomic profile for unripened cheese, which could be further expanded in future research to monitor storage-related changes or quality issues. Additionally, the metabolite

differentiation identified in this study enhances understanding of cheese composition, offering potential applications in quality control, nutritional evaluation, and traceability across the cheese production process.

The insights gained from this study have practical implications for cheese manufacturers and regulatory bodies. By identifying metabolites linked to different rennet sources, manufacturers can tailor production processes to achieve specific flavor and texture profiles. Moreover, these findings facilitate traceability and authenticity verification, enabling regulatory bodies to ensure accurate labeling and compliance with dietary or ethical standards, such as Halal and vegetarian certifications.

5. Conclusion

We conducted comprehensive metabolites screening of cow milk cheese samples that were prepared from different sources of rennet, which offered new insights into the relationships between the variations in compounds associated with different coagulation sources. Cheeses based on calf and pig, microbial rennet, and acetic acid, discrimination were all made possible by the use of supervised PLS-DA and unsupervised PCA-indicated metabolite differences between the four groups. The study revealed nine significant metabolic pathways, including glyoxylate and dicarboxylate metabolism, glycine, serine, and threonine metabolism, biosynthesis of unsaturated fatty acids, nitrogen metabolism, and galactose metabolism, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism and valine, leucine and isoleucine biosynthesis. This study provides a foundation for understanding how rennet influences flavor, texture, and overall quality. These findings are particularly valuable for manufacturers aiming to meet diverse consumer preferences and dietary requirements. The findings of the current study have certain limitations. The small sample size restricts the generalizability of the results, necessitating future studies with larger datasets. Additionally, the exclusion of plant-based and other alternative coagulants limits the scope of the study. Including these coagulants in subsequent analyses would enhance our understanding of their metabolic and sensory impacts on cheese production. Future studies should focus on the targeted identification of metabolites and the use of a larger sample dataset to confirm these findings through more robust chemometric models. This work supports the future application of foodomics to effectively investigate and address issues related to cheese traceability, quality, and authenticity. Additionally, future research should explore the effects of rennet in ripened cheeses and expand to cheeses made from other milk sources, such as goat or sheep milk, to further elucidate the metabolic and sensory variations in cheese production.

CRediT authorship contribution statement

Azra Akbar: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Amna Jabbar Siddiqui:** Writing – review & editing, Visualization, Validation, Supervision, Investigation. **Ali Raza:** Writing – review & editing, Methodology. **Anamta Zia:** Writing – review & editing. **Khadijah Nakyinsige:** Writing – review & editing, Methodology. **Kawalya Hakiimu:** Methodology. **Syed Ghulam Musharraf:** Writing – review & editing, Visualization, Supervision, Investigation, Conceptualization.

Funding

The authors acknowledge the joint financial support from the Higher Education Commission of Pakistan under the funding program for the Center of Excellence (CoE-75) and under reverse linkage project facilitated by the Islamic Development Bank (IsDB) between Pakistan and Uganda.

Declaration of competing interest

The authors declare that there are no competing interests.

Acknowledgments

The authors are thankful to Hussain Iqbal and Afshan Iqbal for their technical support in GC-MS analysis. Thanks to Adeeba Khadim for her assistance with the cheese preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.111113.

Data availability

Data will be made available on request.

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