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THE ANALYSIS OF METABOLIC CONTENT OF TRADITIONAL MILK COLLECTED FROM THREE REGIONS IN TURKEY BY NMR SPECTROSCOPY

Çağdaş Dağ[1,2,3,*], Oktay Göçenler[1], Cansu Deniz Tozkoparan[1]
[1] Department of Molecular Biology and Genetics, Koç University, Istanbul, Türkiye
[2] Nanofabrication and Nanocharacterization Centre for Scientific and Technological Advanced Research, Koç University, Istanbul, Türkiye
[3]Koç University Isbank Center for Infectious Diseases (KUISCID), Istanbul, Türkiye

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ABSTRACT
Milk is one of the primary animal-based foods in a healthy diet which provides vitamins, fats and particularly calcium to meet the daily requirements. Currently, there are a limited number of metabolomics studies on milk and more studies are required to establish reliable international standards and databases for milk metabolite profiles. In this study, metabolite analysis of three types of UHT milk which are whole-milk, semi-skimmed and lactose-free from Türkiye was investigated by NMR, with emphasis on the differences in metabolite concentrations related to lactose and energy metabolism. In all, 31 different metabolites were successfully identified and quantified with a single NMR experiment. Most of these metabolites are involved in energy and amino acid metabolism, and these findings show that NMR can easily detect perturbations of metabolites in these relevant pathways. In this study, the lactose levels of lactose-free labelled products also were analysed and compared with international threshold levels.
Keywords: Milk, NMR, metabolomic, lactose

TÜRKİYE'DE ÜÇ BÖLGEDEN TOPLANAN GELENEKSEL SÜTLERİN METABOLİK İÇERİĞİNİN NMR SPEKTROSKOPİSİ İLE ANALİZİ

ÖZ
Süt, insan beslenmesinin günlük ihtiyacını karşılamak için vitaminler, yağlar ve özellikle kalsiyum sağlayan sağlıklı bir diyetteki başlıca hayvansal gıdalardan biridir. Sütte bulunan metabolitler, sütün kimyasal özelliklerini ortaya çıkarmak, sütün kalitesini veya yabancı maddelerin tespitini belirlemek için çok önemlidir. Halihazırda, süt üzerinde sınırlı sayıda NMR bazlı metabolit profili çalışması bulunmaktadır ve NMR bazlı süt metabolit profilleri için güvenilir uluslararası standartlar ve veri tabanları oluşturmak için daha fazla çalışmaya ihtiyaç vardır. Bu çalışmada, laktoz ve enerji metabolizması ile ilgili metabolit konsantrasyonlarındaki farklılıklar vurgulanarak, Türkiye'de satılan

* *Corresponding author* / Yazışmalardan sorumlu yazar:
✉: cdag@ku.edu.tr ✆: (+90) 212 338 1000 🖷: (+90) 212 338 1000

Çağdaş Dağ; ORCID no: 0000-0003-1595-431X
Oktay Göçenler; ORCID no: 0000-0003-4310-0928
Cansu Deniz Tozkoparan; ORCID no: 0000-0001-9326-0642

konvansiyonel sütlerin metabolit analizi NMR spektroskopisi ile incelenmiştir. Toplamda, 31 farklı metabolit tek bir NMR deneyi ile tanımlandı ve nicel analizleri gerçekleştirilmiştir. Bu metabolitlerin çoğu, enerji ve amino asit metabolizmasında yer almaktadır. Elde edilen bulgular, NMR spektroskopinin bu ilgili yollardaki metabolitlerin bozulmalarını kolayca tespit edebildiğini göstermiştir. Bu çalışmada ayrıca, laktoz içermeyen etiketli ürünlerin laktoz seviyelerinin analizi ve uluslararası kabul görmüş seviyeler ile karşılaştırılmıştır.
Anahtar kelimeler: Laktoz, süt, NMR, metabolomiks

INTRODUCTION

Milk is a chemically complex colloidal bio-fluid secreted from the mammary glands of female mammals for feeding their infants. Milk is also a valuable animal-source food for humans that provides a high level of nutrients including fats, vitamins and minerals. Additionally, milk contains many bioactive compounds such as glycoproteins, antibodies, oligosaccharides, organic acids, biogenic amines, nucleotides, immunoglobulins, various vitamins and minerals iron, zinc, and iodine (Dror and Allen, 2011). Consequently, milk and milk-based products are commonly consumed all around the world, in a variety of forms such as cheese, butter, yoghurt, casein powder and ice cream. 83 % milk of dairy products is obtained from cows, 13% from buffaloes, 2% goats, 1% sheep, and 0.4% camels. (Foroutan et al., 2019).

Cow milk is rich in calcium and phosphorus, with an average content of 85-87% water, 3.8-5.5% fats, 2.9-3.5% protein, 4.6-5.0 % carbohydrates (mostly lactose), vitamins (riboflavin B2), 0.69% ash, and mineral substances (Erarslan, 2012; Kaskous, 2020). The content of milk varies depending on the environment, age, lactation stage, seasonal variation, diet, breed, and genetics thereby affecting the nutritive value and taste of milk on account of milk metabolites. Milk metabolites are intermediates and end products resulting from the metabolism of mammalian epithelial cells and peripheral blood (Kaskous, 2020 ; Dror and Allen, 2011).

Metabolomics technology allows rapid and efficient evaluation of metabolites in biological samples such as serum, plasma, and milk (Gowda et al., 2008). NMR spectroscopy, gas chromatography-mass spectroscopy, (GC-MS), liquid chromatography-mass spectroscopy (LC-MS) and ultra-performance liquid chromatography–quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) are examples of advanced technologies used for metabolomics (Yang et al., 2016). The detection of milk metabolites is one of the most effective ways of controlling the quality and safety of milk and its products (Sen et al., 2020). Metabolomic biomarkers are important molecules and can be used for identifying differences between milk varieties (Sen et al., 2020). Milk adulteration has been detected by using metabolomic biomarkers by Yang et al. (2016) who investigated the metabolomics profile of Chinese Holstein, Jersey, Buffalo, Goat, Camel, Horse, Yak (Bos mutus) milk samples by NMR spectroscopy and liquid chromatography-mass spectroscopy (LC-MS) using non-targeted metabolomics approaches (Yang et al., 2016). Sundekilde et al. (2011) showed that several metabolites like carnitine, choline, and citrate can be used as biomarkers for determining the differences between Danish Holstein cow and Jersey cow by NMR-based metabolomics (Sundekilde et al., 2011). Ozrenk and Selcuk Inci (2008) investigated the effects of the seasonal variation on milk components and yield. They have demonstrated the seasonal variation has an effect on the milk composition and lactation periods. Milk yield and composition are affected depending on seasonal nighttime length of the habitat where the dairy animal lives. The amount of fat and protein in milk in the areas with shorter nighttime is less than the regions with longer nighttime. The composition and yield of milk are so important that it also affects the processing ability of milk (Ozrenk and Inci, 2008).

NMR is a robust analytical technique used to investigate the biochemical properties of many substances. There are different uses of NMR in the dairy industry. This technology does not only provide information about milk and the dairy animal, but it is also used to facilitate the monitoring of processes during the conversion of milk into other dairy products in the industry (Maher and Rochfort, 2014). The quality of the data to be analysed by NMR is directly related to the extraction protocols used in the preparation of milk samples to be used in metabolomics studies. Yanibada et al. (2018) compared eight different preparation protocols for cow milk samples. Among the protocols used, the most effective preparation methods have been methanol extraction and ultrafiltration. (Yanibada et al., 2018).

Diluting or changing the content of high unit price or high demand products by adding low-cost foreign substances is a common fraud method known as adulteration in the food industry. Milk can be adulterated by addition of tap water, hydrogen peroxide, urea, detergents, sugars, melamine in order to increase the profitability of the original product (Francis et al., 2020). In another study, Northern Italian Friesian cows and other native breeds were fed the same diet on the same farm and the lactation stage was analysed by NMR (Tomassini et al., 2019). O'Callaghan et al (2018) investigated the metabolites contained in bovine milk by NMR spectroscopy (O'Callaghan et al., 2018). Klein et al (2013) used the combination of NMR and GC-MS techniques to specify bovine milk content at early and late lactation stages. They showed that milk metabolite composition changes with metabolic stress depending on lactation stages (Klein et al., 2013). Pregnancy and calving are preconditions for lactation. Lactation causes certain hormonal and metabolic changes in dairy cows, such as feeding behaviour that varies from one stage of lactation to another. In early lactation, which is called period 1, the dairy cows use a large proportion of their energy to produce milk. In this period, animal feed consumption should be increased to prevent nutrient deficiency (DeVries et al., 2003; Jorritsma et al., 2003). Between lactations, the period of time dairy cows has rested and have prepared for the next lactation is called the dry period. Cows are taken for a dry period 2 months before birth to preserve their milk yield (Madsen et al., 2008).

Lactose, which is a disaccharide composed of glucose and galactose and it's the most abundant carbohydrate in milk. Gastrointestinal problems are observed with milk consumption in individuals deficient in lactase, an enzyme which helps digest lactose. Lactose intolerance is observed in 70% of adults at some range worldwide. As a result, the demand for lactose-free dairy products is increasing as more adults become aware of the physiological discomfort of lactose consumption. Cunha et al. (2020) determined the quantification of lactose in milk samples by 1H NMR without the use of deuterated solvent (Cunha et al., 2020). Monakhova et al. (2012) investigated the labelling accuracy of milk, lactose-free milk, and other milk derivatives (soy, oat, rice) in the markets by 1H NMR spectroscopy (Monakhova et al., 2012). Söyler et al. (2021) investigated the advantages of bench-top NMR spectroscopy as a quality-control tool at different stages of milk production. First, bench-top NMR was used to monitor lactose hydrolysis. The artificial neural network model has been developed to classify the different components of milk samples by supporting benchtop NMR spectroscopy (Söyler et al., 2021). Laref (2020) has suggested that using the bioinformatics software following 1H NMR is more advantageous for metabolite identification (Laref, 2020).

In this study, metabolite analysis of conventional milk from Türkiye was performed, with particular focus on the differences in lactose levels investigated by NMR spectroscopy.

MATERIALS AND METHODS

Sampling

In this study, all UHT milk samples were chosen and collected from markets in Istanbul, Adana and Mersin regions. In order to be a homogenized sampling, samples were obtained from different provinces. In total, 18 different milk samples belonging to different characteristics and brands constitute the data set in the analysis. Three different types of UHT milk are used, which are reduced fat, full-fat and lactose-free. After the milk samples were brought to the laboratory in packages, they were divided into 6 tubes of 1.5

mL each in a sterile environment. 5 tubes were stored at -80°C for steps that may require repetition or control.

Sample preparation
Ultrafiltration method was used to remove proteins and fats from metabolites and thus to extract metabolites (Yanibada et al., 2018). Since there is no protein smaller than 3 kDa, ultrafiltration tubes with a cut-off size of 3 kDa were preferred. Marketed Ultrafiltration tubes are manufactured by impregnating with glycerol to ensure long-term durability before use. Glycerol is a molecule that gives a high intensity peak in 1-D NMR analysis. All ultrafiltration membranes were centrifuged at 3000 rpm for 15 minutes by adding 2 mL of distilled water to the sample chamber to remove glycerol from the membrane before the extraction process. This process was repeated 4 times to ensure complete removal of glycerol. After this process, the remaining water in the ultrafiltration tubes was removed and 1.5 mL of milk sample was added in the sample chamber of each ultrafiltration membrane.

Membrane units, to which milk samples were added, were centrifuged at 3000 rpm for 60 minutes at 4°C. At the end of this period, the condensed milk samples remaining in the sample chamber were mixed and homogenised with the help of an automatic pipette. After this process, the membranes were centrifuged again at 3000 rpm for 60 minutes. After the centrifugation process, which was carried out for a total of 120 minutes. Ultrafiltration devices are generally used in the concentration of proteins. Proteins are concentrated and remain in the upper part of the membrane. On the other hand, in metabolomics studies, since metabolites have smaller dimensions than the membrane size, they pass down the membrane. Therefore, the fraction that passes under the membrane is collected for further steps. For each sample, 500 microliters of the metabolite fraction were taken and transferred to another 1.5 mL volumetric tube. On top of it, a mixture containing 100 mM PBS and 5 mM trimethylsilylpropanesulfonic acid (DSS) dissolved in 55 microliters of deuterium was added. The final concentration in the NMR samples was 10 mM PBS 0.5 mM DSS. The sample mixture was added to a standard 5 mm NMR tube.

Data Collection
1D 1 H NMR data acquisition was carried out at the Koç University central research infrastructure directorate on a Bruker Avance Neo Ascend NMR spectrometer operating at 500.13 MHz equipped with a 5 mm BBO double-resonance probe and a SampleXpress sample changer. Topspin 4.0.9 (Bruker) and iconNMR were used to automate the data collection process. WATER pulse sequence was applied to suppress the residual water signal (Kim et al. 2011). Each spectrum consisted of 4096 scans of 32,768 complex data points with a spectral width of 9615.4 Hz. The 1D 1 H spectra were phased, baseline corrected, referenced to DSS automatically by iconNMR.

Data Analysis
For the NMR data analysis, DSS molecule was used both as a standard 0 for the calibration of ppm values and as an internal standard for the quantitative analysis of the metabolites. Chenomx 8.6 (Chenomx Inc, Canada) software used for all metabolite assignments and quantitative analysis. In all analyses, the different peaks of the molecules appearing in the spectrum were examined separately, and then the assignment of the molecule was carried out. Manual corrections were made for the assigned peaks in all spectra after the preliminary analysis performed with the automatic fit mode in the quantifications.

RESULTS AND DISCUSSION
NMR spectroscopy is a technique that is being used more and more in many fields day by day, since it has the ability to directly measure the amounts of components using atomic properties. 90% of the approximately 60 NMR spectrometers in Türkiye consist of 400 MHz and 500 MHz spectrometers. Although there are studies carried out with NMR spectrometers with different properties in different studies, it was not known how much success could be achieved from NMR analyzes at the level of spectrometers, which constitute the vast majority in Türkiye. In this

study, 31 different metabolites in milk were detected and quantified via a single NMR experiment without additional external calibration. Metabolites detected and quantified by NMR spectroscopy are given in Table 1. 31 metabolite assignments, there were 9 metabolites associated with amino acid metabolism and 6 metabolites from energy metabolism identified and their concentrations were calculated. Furthermore, 4 different types of sugar and 12 other important metabolite signals were detected in the NMR spectrum and quantitatively assigned. In Figure 1, the regions and peak heights of the metabolites detected are shown on one of the NMR spectra collected in this study. As can be seen from the spectrum, many metabolites can be detected with a single NMR analysis. One of the biggest advantages is that the signals of the metabolites are well dispersed over the spectrum.

Table 1. List of milk metabolites detected by NMR spectroscopy, calculated metabolite concentration (mM) with standard deviation and literature values (mM) (Foroutan et al., 2019

	Whole Milk	Semi-Skimmed Milk	Lactose Free Milk	Literature Values
Energy Metabolism				
Lactate	0.0730 ±0.0660	0.0910 ±0.0870	0.0970 ±0.084	0-0.1670
Pyruvate	0.0340 ±0.0170	0.0280 ±0.0190	0.0350 ±0.0100	0-0.0540
Citrate	7.8160 ±0.9280	7.9030 ±1.4090	8.1240 ±0.1720	3.6920-7.4350
Succinate	0.0860 ±0.0370	0.0830 ±0.0410	0.0610 ±0.0440	0.0160-0.0300
Fumarate	0.0080 ±0.0040	0.0120 ±0.0050	0.0050 ±0.0040	0.0060-0.0230
Acetate	0.3130 ±0.2160	0.3560 ±0.2700	0.3500 ±0.2200	0.0130-0.1130
Amino acid Metabolism				
Alanine	0.0530 ±0.0080	0.0530 ±0.019	0.0560 ±0.0030	0.0180-0.0780
Glutamate	0.4240 ±0.3000	0.3280 ±0.1450	0.3270 ±0.0180	0.1110-0.7400
Glycine	0.2460 ±0.9600	0.1900 ±0.1060	0.1200 ±0.0540	0.0620-0.0800
Valine	0.0210 ±0.0140	0.0200 ±0.0150	0.0240 ±0.0120	0.0060-0.0150
Isoleucine	0.0010 ±0.0010	0.0090 ±0.0020	0.0010 ±0.0009	0.0020- 0.0070
Leucine	0.0058 ±0.0035	0.0042 ±0.0043	0.0051 ±0.0028	0.0020-0.0060
Formate	0.0250 ±0.0200	0.0710 ±0.0890	0.0300 ±0.0020	0.0033-0.0460
Urea	3.6740 ±1.1710	4.9820 ±3.4030	2.8620 ±1.7260	0.1190-7.7000
Hippurate	0.0640 ±0.0190	0.0820 ±0.0280	0.0660 ±0.0130	0.0790-0.2670

Table 1. continue

	Whole Milk	Semi-Skimmed Milk	Lactose Free Milk	Literature Values
Saccharides				
Lactose	138.6760 ±17.8960	137.7600 ±40.2910	4.5250 ±4.1210	98.3570-153.2160
Glucose	1.2740 ±0.8170	1.7170 ±1.4680	116.6270 ±19.6550	0.2460-0.4780
Fucose	0.0390 ±0.0120	0.0230 ±0.0110	0.0330 ±0.0040	0.0060-0.0410
Galactose	1.0890 ±0.4000	1.5770 ±1.4120	109.1840 ±12.2770	0.0860-1.9600
Other Metabolites				
Creatine	0.2990 ±0.1710	0.3000 ±0.1980	0.3090 ±0.2670	0.3120-0.5430
Creatinine	0.1410 ±0.0770	0.1400 ±0.0520	0.1170 ±0.0250	0.0360-0.1250
Creatine-1-phosphate	0.0260 ±0.0210	0.0340 ±0.0190	0.0260 ±0.0260	0-0.1020
Choline	0.3630 ±0.1570	0.7220 ±0.7450	0.2660 ±0.2250	0.1520-0.4790
Betaine	0.0180 ±0.0110	0.0140 ±0.0030	0.0230 ±0.0060	0.0330-0.1150
O-phosphocholine	0.1090 ±0.0800	0.1730 ±0.1250	0.1590 ±0.0820	0-0.9410
sn-Glycero-3-phosphocholine	0.8170 ±0.2770	0.7870 ±0.2680	0.9230 ±0.3060	0.2910-1.2170
Dimethylamine	0.0220 ±0.0110	0.0060 ±0.0070	0.0230 ±0.0080	0.0070-0.0170
Isopropanol	0.0015 ±0.0007	0.0011 ±0.0007	0.0009 ±0.0001	0.0016-0.0022
Acetone	0.0290 ±0.0080	0.0290 ±0.0110	0.0120 ±0.0010	0.0090-0.4970
3-hydroxybutyrate	0.1060 ±0.1990	0.2140 ±0.4230	0.0440 ±0.0100	0.0120-0.1210
Dimethyl sulfone	0.0430 ±0.0140	0.0470 ±0.0270	0.0370 ±0.0090	0.0100-0.0590

Energy Metabolism
In all 3 different types of milk, we detected 6 main metabolites of the energy metabolism (Table 1). All detected metabolites were also quantitatively analysed using Chenomx software 9.0.1. Most of the detected metabolites were within the limits of the literature or close to the values stated in the literature but citrate (8.1240 mM) and acetate (0.3500 mM) levels were noticeably different from existing studies. Metabolites that their concentrations were above the literature levels, are especially associated with the lactation level. Citrate, which takes place in the Tricarboxylic acid (TCA) cycle in energy metabolism, has an important place in the energy cycle (Figure 2). However, in milk, citrate is an indicator of the mammary gland activity (Garnsworthy et al., 2006). All the samples used within the scope of the study consist of samples produced and packaged in the months of April and May. The high citrate concentration can be explained by the fact that milk obtained from cows in these months is milk produced at early lactation stage.

Lactose level
Lactose is a metabolite that shows wide variation according to seasons and lactation period. In our study, we determine the lactose level at commercial milk is between 96-207 mM. These results were in accordance with the literature (Foroutan et al., 2019). In a study conducted with commercial milk in the literature, the determined

lactose level is between 98-153 mM. In another NMR-based study, the lactose level of milk from the USA market was found in between 110-130 mM (Foroutan et al., 2019). Wu et al. (2016) investigated the lactose levels in the early and late periods of lactation in their study, they determined the lactose level as 220 mM in the early lactation period and 246 mM in the late lactation period (Wu et al., 2016).

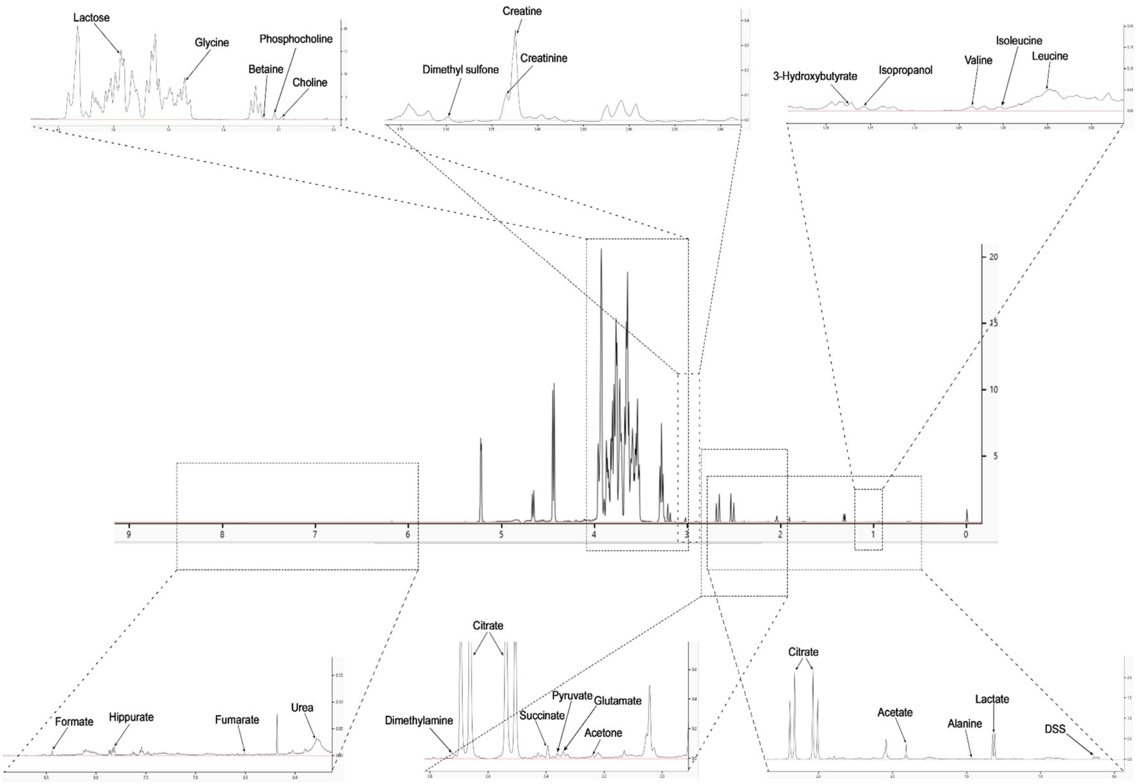

Figure 1. 1D ^{1}H NMR spectrum of the milk and detailed assignment regions on spectrum

One of the biggest concerns of whole milk is its adverse effects on lactose intolerant individuals. In recent years, lactose-reduced milks have been introduced to the market to make milk available to lactose intolerant consumers. For this reason, we also comparatively investigated the milk products labelled as reduced lactose and/or lactose free. The regulation of lactose-free and lactose-reduced products differ between regions or countries according to their food laws and regulations. In this regard, most of the countries consider the upper and lower limits of lactose in milk to be respectively, 0.5% and 0.01 %, to qualify the product as lactose free (Dekker et al., 2019). In accordance with regulations issued by authorities, the German dairy industry offers ≤0.1% lactose content into line with EU regulations (Morlock et al., 2014). The Spanish Agency of Food Safety and Nutrition (AESAN) has issued that a product labelled lactose-free is required to contain less than 0.01% lactose (0.299 mM) (Martínez Rodríguez al., 2021). The Ministry of Agriculture and Forestry in Türkiye, has declared that lactose-free labelled milk must have a lactose content of less than 0.1% (2.99 mM) (Guide for Turkish Food Codex Notification on milk, 2019/12). In this study, we determined the range of the lactose content of commercial lactose-free labeled milk products from Türkiye between 2.15-9.28 mM.

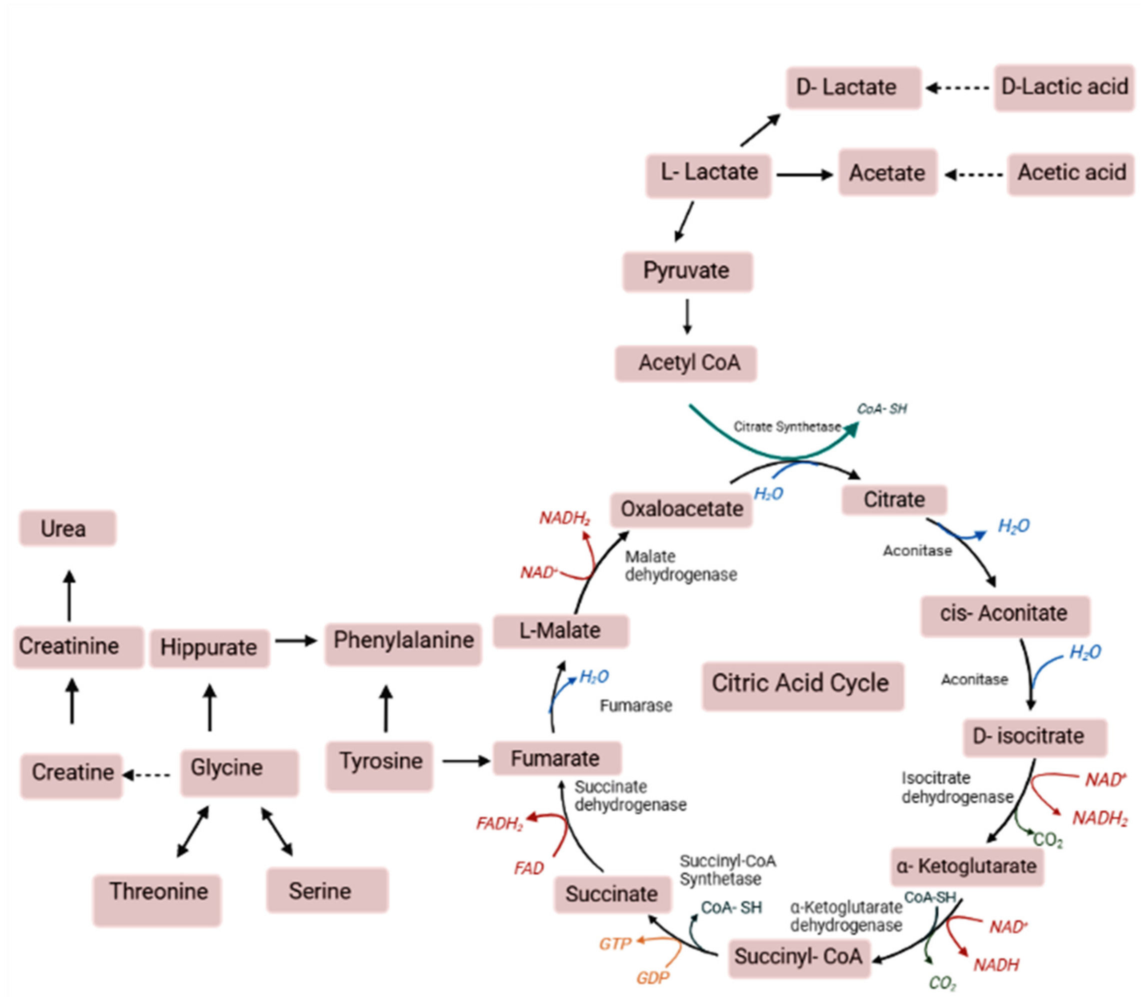

Figure 2. Illustration of combined energy and amino acid metabolisms

Amino acid Metabolism

Milk contains important essential amino acids. The concentration ranges of 9 amino acids have been determined through NMR spectroscopy among milk types such as whole, semi-skimmed and lactose-free milk. While it is very difficult to measure the amount of free amino acids in measurements performed with other techniques, another advantage of NMR spectroscopy is that it can directly measure the free amino acids remaining in the environment outside the proteins, without breaking down the proteins. The average concentration of alanine was measured 5.6 µM and 24 µM for valine in lactose-free milk. Concentration of amino acids 424 µM glutamate, 246 µM glycine and 5.8 µM leucine are detected more than average in whole milk. On the contrary, 9 µM isoleucine, 71 µM formate, 4.982 mM urea, 8.2 µM hippurate are detected more than average in semi-skimmed milk. The majority of the data obtained is consistent with the literature values for alanine, glutamate, urea and hippurate (Foroutan et al., 2019). However, the literature values for glycine, valine, leucine, isoleucine, and formate significantly different. The reason for this could be the selection of commercial milk, the nutrition of the animal from which the milk is obtained, its age, lactation stage or could be caused by differences in the experiment.

CONCLUSION

In this study single NMR experiment is used to determine metabolic content of milk with regard to its metabolite and lactose content for comparative analysis with the existing literature values. Based on our findings, concentrations of some of the metabolites in commercially sold milk in Türkiye, diverged from their literature values particularly amino acids citarate and lactose. It is concluded that the differences could be caused due to alterations of the lactation levels depending on seasonal nighttime/daytime length or conditions of the dairy cows in milk producing facilities. Lactose levels of milk labelled as lactose-free milk are also determined and lactose level in some lactose-free labelled products were also above the Turkish regulations. Although the maximum lactose levels for lactose free labeled products vary from country to country, the allowed level in Türkiye is 10 times higher than that of many countries. It is important that these levels are determined by international regulations.

In addition to experimental findings from NMR experiments, the utility of NMR is discovered for quality control and metabolomics analysis of milk. We believe that the data obtained within the scope of the study will contribute to the expansion of the Milk Composition Database (MCDB), thus increasing the controllability of milk. Accordingly, NMR-based metabolomics analyses have a very high reproducibility rate without requiring rapid sample preparation methods and detailed calibrations. With this study, we have shown that NMR is a very powerful and rapid technique for investigating milk metabolism. We believe that the use of NMR will be apreferred technique in the near future, especially in the analysis of routine lactose levels in the industry and in the measurement of the remaining lactose amount after lactose degradation.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the author.

AUTHORS CONTRIBUTIONS

CD design the experiments, OG performed the wetlab experiments; CD performed the NMR data collection and analyses; CD, OG and CDT contributed to the writing of the paper. All authors read and approved the manuscript.

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REFERENCES

Cunha, D. A., Valim T. C., Filgueiras P. R., Júnior V. L., Neto A.C. (2020). Lactose quantification in bovine milk by nuclear magnetic resonance without deuterated solvent (No-D qNMR). *Analytical methods: advancing methods and applications*, 12 (40): 4892-4898.

Dekker, P.J.T., Koenders, D., Bruins, M.J. (2019) Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, 11: 551.

DeVries, T. J., von Keyserlingk, M. A. G., Weary, D. M., Beauchemin, K. A. (2003). Measuring the Feeding Behavior of Lactating Dairy Cows in Early to Peak Lactation. *Journal of Dairy Science*, 86 (10): 3354-3361.

Dror, D. K., Allen, L. H. (2011). The importance of milk and other animal-source foods for children in low-income countries. *Food Nutrition Bulletin*, 32(3): 227-43.

Erarslan, A. (2012). A1 Tip İnek Sütü ve Süt Ürünlerinde Beta-Kazomorfin-7 İçin HPLC'ye Dayalı Tayin Yöntemin Geliştirilmesi, *Erciyes Üniversitesi* Yüksek Lisans Tezi. 122 sayfa.

Foroutan, A., Guo, A. C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran H., Budinski, Z., Mandal, R., Ametaj, B. N., Wishart, D. S. (2019). Chemical Composition of Commercial Cow's Milk. *Journal of Agricultural and Food Chemistry*, 67 (17): 4897-4914.

Francis, A., Dhiman, T., Mounya, K.S. (2020). Adulteration of Milk: A Review, *Journal of Science and Technology*, 5 (6): 37-41.

Garnsworthy, P. C., Masson, L. L., Lock, A. L., Mottram, T.T. (2006). Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. *Journal of Dairy Science*, 89 (5): 1604-12.

Gowda, G. A., Zhang, S., Gu, H., Asiago, V., Shanaiah, N., & Raftery, D. (2008). Metabolomics-based methods for early disease diagnostics. *Expert Review of Molecular Diagnostics*, 8(5): 617–633.

Guide for Turkish Food Codex Food Labelling and Consumer Information Regulation, *Turkish Food Codex Notification on Milk*, No. 2019/12.

Jorritsma, R., Wensing, T., Kruip, T.A., Vos, P.L., Noordhuizen, J.P. (2003). Metabolic changes in early lactation and impaired reproductive performance in dairy cows. *Veterinary Research*, 34 (1): 11-26.

Kaskous, S. (2020). A1- and A2-Milk and Their Effect on Human Health. *Journal of Food Engineering and Technology*, 9 (1): 15–21.

Klein, M. S., Almstetter, M. F., Nürnberger, N., Sigl, G., Gronwald, W., Wiedemann, S., Dettmer, K., Oefner, P. J. (2013) Correlations between milk and plasma levels of amino and carboxylic acids in dairy cows. *Journal of Proteome Research*, 12 (11): 5223-5232.

Laref, N. (2020). Efficiency of different bioinformatics tools in metabolite profiling of whole cow's milk using synthetically water-removed 1H NMR spectra: a comparative study. *Journal of Microbiology, Biotechnology and Food Sciences*, 10 (2): 241-244.

Martínez -Rodríguez, M., Samaniego-Vaesken, M. d. L., Alonso-Aperte, E. (2021). A New Food Composition Database of Lactose-Free Products Commercialized in Spain: Differences in Nutritional Composition as Compared to Traditional Products. *Foods*, 10: 851.

Madsen, T.G., Nielsen, M.O., Andersen, J.B., Ingvartsen, K.L. (2008) Continuous lactation in dairy cows: effect on milk production and mammary nutrient supply and extraction. *Journal of Dairy Science* 91 (5): 1791-801.

Maher, A.D., Rochfort, S.J. (2014). Applications of NMR in dairy research. *Metabolites*, 4 (1): 131-41.

Monakhova, Y., Kuballa, T., Leitz, J., Andlauer, C., Lachenmeier, D. (2012). NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. *Dairy Science & Technology*, 92: 109-120.

Morlock, G.E., Morlock, L.P., Lemo, C. (2014). Streamlined analysis of lactose-free dairy products. *Journal of Chromatography A*, 1324: 215-223.

Ozrenk, E., Selcuk Inci, S. (2008). The Effect of Seasonal Variation on the Composition of Cow Milk in Van Province. *Pakistan Journal of Nutrition*, 7: 161-164.

O'Callaghan, T. F., Vazquez-Fresno, R., Serra-Cayuela, A., Dong, E., Mandal, R., Hennessy, D., McAuliffe, S., Dillon, P., Wishart, D. S., Stanton, C., Ross, R. P. (2018). Pasture feeding changes the bovine rumen and milk metabolome. *Metabolites*, 8 (2): 27.

Sen, C., Ray, P.R., Bhattacharyya, M. (2020). A critical review on metabolomic analysis of milk and milk products. *International Journal of Dairy Technology*, 74 (1): 17-31

Söyler, A., Cikrikci, S., Cavdaroglu, C., Bouillaud, D., Farjon, J., Giraudeau, P., Oztop, M.C. (2021). Multi-scale benchtop 1H NMR spectroscopy for milk analysis. *Lebensmittel-Wissenschaft & Technologie*, 139: 110557.

Sundekilde, U.K., Frederiksen, P.D., Clausen, M.R., Larsen, L.B., Bertram, H.C. (2011). *Journal of Agricultural and Food Chemistry*, 59 (13): 7360-7367.

Tomassini, A., Curone, G., Solè, M., Capuani, G., Sciubba, F., Conta, G., Miccheli, A., Vigo, D. (2019). NMR-based metabolomics to evaluate the milk composition from Friesian and autochthonous cows of Northern Italy at different lactation times. *Natural Product Research.* 33 (8): 1085-1091.

Wu, J., Domellöf, M., Zivkovic, A. M., Larsson, G., Öhman, A., Nording, M. L. (2016). NMR-based metabolite profiling of human milk: A pilot study of methods for investigating compositional changes during lactation, *Biochemical and Biophysical Research Communications*, 469 (3): 626-632.

Yanibada, B., Boudra, H., Debrauwer, L., Martin, C., Morgavi, D.P., Canlet, C. (2018). Evaluation of sample preparation methods for NMR-based metabolomics of cow milk, *Heliyon*, 4 (10): e00856.

Yang, Y., Zheng, N., Zhao, X, Zhang, Y., Han, R., Yang, J., Zhao, S., Li, S., Guo, T., Zang, C., Wang, J. (2016). Metabolomic biomarkers identify differences in milk produced by Holstein cows and other minor dairy animals. *Journal of Proteomics,* 136: 174-82.