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The Role of Fatty Acids in the Development of Obesity and Diabetes

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ARA	Arachidonic acid
BMI	Body mass index
C-section	Cesarean section
CLA	Conjugated linoleic acid
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
EFA	Essential fatty acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC-MS	Gas chromatography with mass spectrometry
GC-FID	Gas chromatography with flame ionization detection
HDL	High-density lipoprotein
HoP	Holder pasteurization
HM	Human milk
LA	Linoleic acid
ALA	Alpha-linolenic acid
LCPUFA	Long chain polyunsaturated fatty acid
LCFA	Long chain fatty acid
LDL	Low-density lipoprotein
MCFA	Medium chain fatty acid
MUFA	Monounsaturated fatty acid
OA	Oleic acid
PCA	Principal component analysis
QDA	Quadratic discriminant analysis
RECOOP HST	Regional Cooperation in the Fields of Health, Science, and Technology
SFA	Saturated fatty acid
SCFA	Short chain fatty acid
sn	Stereospecific numbering
TLR	Toll-like receptor
TFA	*Trans* fatty acid
TAG	Triglyceride
WHO	World Health Organization

1. INTRODUCTION

The increasing global prevalence of obesity and diabetes, including gestational diabetes, has become a significant public health concern. According to the World Health Organization, over 890 million people are obese, while more than 422 million individuals suffer from diabetes (WHO, 2024). Additionally, gestational diabetes affects approximately 14% of pregnancies globally, posing significant health risks for both mothers and their offspring (CDC, 2022; Schaefer-Graf et al., 2018). Consequently, understanding early-life factors that contribute to these disorders—particularly those related to maternal and infant nutrition—has become a crucial area of research.

Human milk, widely regarded as the ideal source of nutrition for infants, plays a key role in early metabolic programming. It provides essential nutrients that influence various aspects of infant development, including growth, neurodevelopment, and immune function (Boquien, 2018). Among these nutrients, fatty acids hold particular importance due to their roles in energy provision, brain and vision development, and the regulation of metabolic pathways (Munblit et al., 2019; Ramiro-Cortijo et al., 2020). Research has shown that variations in the fatty acid composition of human milk, which can be influenced by maternal health conditions such as obesity and gestational diabetes, may predispose infants to metabolic diseases like obesity and diabetes later in life (Farpour-Lambert et al., 2018; Wang et al., 2017; Chertok et al., 2017; Zhong et al., 2022).

The roles of different fatty acids—namely, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs)—are of significant interest in this context. Elevated levels of certain SFAs and n-6 PUFAs, have been linked to increased inflammation and insulin resistance (Capurso and Capurso, 2012; Kim et al., 2007; Willemsen, 2016), while n-3 PUFAs are recognized for their anti-inflammatory properties and benefits to insulin sensitivity (Wendell et al., 2014). Maternal health conditions, including, normal weight, obesity and gestational diabetes, have been shown to significantly alter the fatty acid composition of human milk, potentially influencing the long-term metabolic health of infants (Bravi et al., 2021; Leghi et al., 2020; Peila et al., 2020; Samuel et al., 2022).

Geographical differences in maternal diets further contribute to variability in the fatty acid composition of human milk (Hatem et al., 2024). Diets rich in certain SFAs and n-6 fatty acids, which are common in many Western countries, tend to promote a pro-inflammatory environment that can contribute to metabolic dysfunction (Z. Zhang et al., 2022; Medzhitov, 2010; Simopoulos, 2002). Conversely, diets high in n-3 fatty acids, often found in coastal regions with high fish consumption,

are associated with more favorable metabolic outcomes for both mothers and their infants (Bobiński and Bobińska, 2020; Kasamatsu et al., 2023). Therefore, the nutritional quality of maternal diets has significant implications for the health of future generations.

Despite substantial research linking maternal nutrition to infant health, gaps remain in understanding the specific role those fatty acids in human milk play in the early development of obesity and diabetes. Most studies focus on individual nutrients or specific maternal health conditions, leaving a need for comprehensive research that examines how maternal and neonatal factors collectively influence the fatty acid profile of human milk and, subsequently, infant health.

My dissertation aims to address these gaps by investigating the fatty acid composition of human milk in mothers with varying health statuses, including those who are of normal weight, obese, and affected by gestational diabetes. Furthermore, the study explores additional influencing factors such as maternal nationality, infant sex, delivery mode, and the impact of Holder pasteurization. The objective is to analyze how these conditions affect the fatty acid profile of human milk, with particular focus on samples collected from Hungarian and Ukrainian mothers to provide a broader, cross-cultural perspective on this critical issue.

Through this research, we seek to deepen our understanding of the complex interactions between maternal health, dietary habits, and fatty acid composition. By doing so, this study aims to inform future nutritional guidelines and public health strategies that are designed to enhance the health outcomes of both mothers and infants, ultimately contributing to the broader body of knowledge on maternal and infant health.

2. OBJECTIVES

The objective of this dissertation is to explore the role of fatty acids in the development of diseases such as obesity and diabetes. This study utilizes breast milk as a sample to examine the effect of different health-related parameters on the fatty acid profile. The investigation focuses on identifying the key factors that affect the fatty acid composition of human milk, including maternal health status, body mass index, nationality, infant sex, delivery methods, and the process of Holder pasteurization.

The specific aims are as follows:

- To investigate the impact of maternal health conditions, such as normal weight, obesity, and gestational diabetes, on the fatty acid profile of breast milk.
- To investigate the fatty acid composition in breast milk samples from distinct geographic regions, specifically Ukraine and Hungary.
- To identify and evaluate the variability and significant differences in the fatty acid composition of breast milk concerning:
  a) infant sex
  b) delivery mode (caesarean section vs. vaginal birth)
  c) the influence of Holder pasteurization

3. LITERATURE OVERVIEW

3.1. Breastfeeding benefits and alternatives

Human milk (HM) is universally recognized as the gold standard in infant nutrition, providing a perfectly balanced mix of nutrients, immune defenses, and developmental benefits (Boquien, 2018). HM is notably richer in bioactive compounds essential for newborn growth, providing significant advantages over infant formulas and alternative sources such as cow's milk, goat's milk, and soy-based formulas, which often lack these crucial components and fall short in capturing HM's bioactive richness and functionality (O'Connor et al., 2003; Vass et al., 2020a, 2020b).

The World Health Organization (WHO) has established a global health consensus recommending exclusive breastfeeding for the first six months of an infant's life, followed by continued breastfeeding with the introduction of appropriate complementary foods for up to 24 months or beyond. The benefits of breastfeeding extend beyond providing essential nutrients, it plays a crucial role in laying the foundation for lifelong health (WHO, 2023).

Human milk is primarily composed of approximately 87% water, 7% carbohydrates, 3.8% fat, 1% protein, 0.2% minerals, with the remainder consisting of vitamins and biologically active. The fat in HM provides about 50% of the energy required by infants, while carbohydrates contribute around 40% (Ballard and Morrow, 2013; Grote et al., 2016; Rao, 2013). Breastfeeding offers numerous benefits for both infants and mothers (Fischer Fumeaux et al., 2019).

For infants, HM dynamically adapts to meet their evolving health and nutritional needs, ensuring optimal development throughout all stages of infancy (Favre et al., 2002; Tijerina-Sáenz et al., 2009; Tully et al., 2001). Compared to formula-fed peers, breastfed infants achieve better nutritional status and development, a difference that is especially crucial for preterm infants, as it helps reduce the incidence of severe neonatal conditions (Johnson et al., 2014; Ullah et al., 2018).

Beyond its nutritional benefits, HM contains bioactive compounds that serve various physiological functions. These compounds influence the immune system, act as antimicrobial agents, regulate hormones, and include enzymes that contribute to overall health (Ballard and Morrow, 2013; Park and Nam, 2015). Breastfeeding has been shown to increase intelligence quotient scores by an average of 3.4 points, decrease the risk of future overweight or obesity by 26%, and lower the incidence of certain cancers (Horta et al., 2015; Søegaard et al., 2024). Furthermore, breastfeeding positively impacts long-term vision health and proper tooth alignment (S. Liu et al., 2018; Abate et al., 2020).

During breastfeeding, mothers experience the release of oxytocin, a neurochemical often referred to as the "bonding hormone". Oxytocin significantly enhances maternal emotional well-being, alleviating symptoms of postpartum depression and anxiety, and boosting maternal self-esteem and confidence in parenting (Entwistle et al., 2010; Pope and Mazmanian, 2016; Smith et al., 2016).

Additionally, breastfeeding is associated with several long-term health benefits for mothers, including reduced risks of breast cancer, ovarian cancer, and hypertension, as well as a lower risk of type II diabetes post-delivery. Furthermore, breastfeeding aids in the expedited return of the uterus to its pre-pregnancy size, reduces postpartum bleeding, helps prevent anemia, and facilitates weight loss (Much et al., 2014; Zhang et al., 2021).

In situations where breastfeeding is not possible or sufficient due to various medical or physiological reasons such as illness, cesarean delivery, or premature mammary gland development (Geddes et al., 2013; Henderson et al., 2008), alternatives like infant formula and pasteurized donor milk becomes essential for infant nutrition (Gribble, 2014).

Infant formula is commonly used when breastfeeding is not an option (Medicine et al., 2004). It is designed to mimic human milk as closely as possible, providing essential nutrients necessary for infant growth and development. For instance, regulations in the European Union mandate the inclusion of docosahexaenoic acid (DHA) in infant formulas, which is particularly important for preterm infants who require higher levels of DHA to support optimal growth and neurodevelopment. However, infant formula lacks the immunological and bioactive components naturally present in human milk, which play a crucial role in bolstering the infant's immune system and overall health (EU Commission, 2015; Innis, 2008; Martin et al., 2016). Research indicates that infants who are exclusively formula-fed may have an increased risk of infections and certain chronic conditions compared to those who are breastfed (Dieterich et al., 2013).

Pasteurized donor milk (DM) is another valuable alternative, offering both the nutritional and immunological advantages of breast milk compared to formula milk, especially for preterm or medically fragile infants. Although some nutrients are lost due to heat treatment, it is still highly recommended as an alternative food and is considered better than infant formula (Perrin, M.T., 2018). This option is particularly vital for preterm infants, who are more vulnerable due to their underdeveloped gastrointestinal systems. Such infants are at a heightened risk for serious health issues, including necrotizing enterocolitis and sepsis. Human milk banks play a crucial role by distributing this DM. To guarantee the safety of the milk provided, all DM must be pasteurized to eliminate any present microbial agents (Hartmann, 2017). Importantly, it also preserves the essential macronutrients

necessary for infant development (Kronborg et al., 2015; Peila et al., 2016; Wesolowska et al., 2019).

3.2. General composition of human milk

Human milk is a complex biological fluid uniquely designed to meet the nutritional needs of infants (Figure 1). It comprises approximately 87% water, which is crucial for maintaining hydration and facilitating nutrient delivery. Carbohydrates, primarily lactose, make up about 7% of HM, providing 40% of the total energy and aiding in the absorption of minerals such as calcium and the growth of beneficial intestinal bacteria (Guo, 2020; Kim and Yi, 2020).

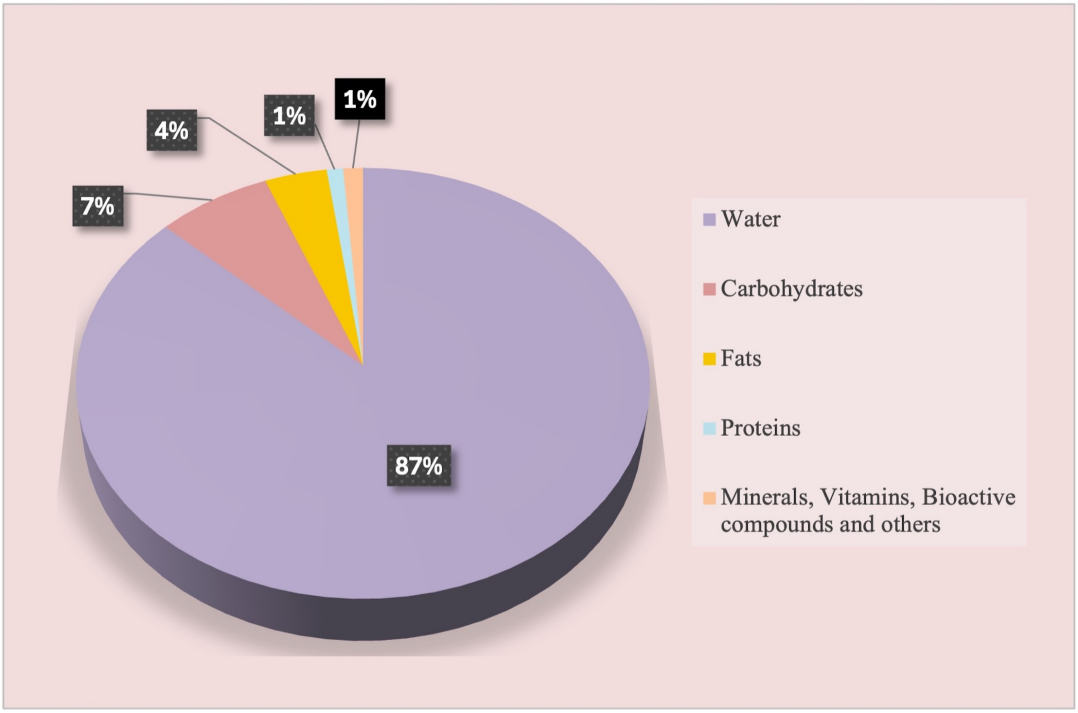

Figure 1. Chemical composition of human milk *(original figure)*

Fats rank as the second most prevalent solid nutrient in breast milk, making up approximately 4% of its composition and providing about 50% of the energy required by infants (George et al., 2021). These fats play a vital role in brain development, hormone production, and the absorption of fat-soluble vitamins such as A, D, E, and K (Kim and Yi, 2020; Koletzko, 2017; Lee et al., 2016; Visentainer et al., 2018). Among the macronutrients found in human milk, fat content is the most variable. Factors such as the time of day, lactation stage, and maternal BMI can all influence the fat concentration in breast milk (Daniel et al., 2021; Italianer et al., 2020; Mitoulas et al., 2002; Mizuno et al., 2009).

Proteins constitute around 1% of HM, with a higher portion being easily digestible whey proteins.

These proteins possess antimicrobial properties that help protect infants from infections (Guo, 2020; Kim and Yi, 2020).

Human milk is also rich in essential vitamins and minerals, including vitamins A, C, D, E, and K, as well as calcium, phosphorus, magnesium, and trace elements like zinc and iron. These nutrients are crucial for promoting growth, bone development, and immune function (Dror and Allen, 2018).

Beyond these macronutrients, HM contains a wide array of bioactive compounds, including hormones, growth factors, and enzymes, all of which play a significant role in supporting infant development and health (Ballard and Morrow, 2013).

The fats present in HM serve not only as a primary energy source but also contain essential fatty acids critical for brain development and the formation of neural structures (Maheshwari, 2022). Therefore, the complex lipid composition of human milk is essential for meeting an infant's nutritional needs and promoting long-term health. Understanding the composition of these fats, particularly the fatty acid profile, is crucial for appreciating the full range of benefits that human milk offers in terms of nutrition and development.

3.3. Fatty acids composition of human milk

Human milk contains various lipid classes, with triglycerides (TAGs) being the most predominant, comprising approximately 98% of the total lipid content. These TAGs carry about 88% of the fatty acids (FAs) found in HM, and the composition of these fatty acids is crucial in determining the nutritional value and physical properties of human milk fat. In addition to TAGs, human milk also contains about 1.3% cholesterol and 1.1% phospholipids. Other lipid components present in smaller quantities include free fatty acids, sterols, diglycerides, monoglycerides, glycolipids, carotenoids, and fat-soluble vitamins (Donda and Maheshwari, 2022; Koletzko, 2017; Parodi, 2004). Recent research has identified over 800 different lipid species in human milk (Zhong et al., 2022).

Triglycerides is composed of three fatty acids esterified to a glycerol molecule. The physical and biochemical characteristics of a TAG molecule depend on the specific positions of its fatty acids. These positions are identified using the stereospecific numbering system (sn), with the outer positions labeled as sn-1 and sn-3, and the central position labeled as sn-2 (Berry et al., 2019). Pancreatic lipase specifically targets and hydrolyzes the ester bonds at the sn-1 and sn-3 positions of triglycerides. In infant formulas made with vegetable oils, palmitic acid (C16:0) is typically esterified at these positions. This configuration leads to poor absorption of fat and calcium, resulting in harder stools for infants. In contrast, human milk predominantly contains palmitic acid (C16:0) esterified at the sn-2 position,

which facilitates better absorption of fat and calcium. Additionally, sn-2 palmitate-enriched formulas have been developed to mimic this aspect of human milk, thereby enhancing fat and calcium absorption in infants (Martin et al., 2016; Zhang et al., 2023).

Fatty acids are fundamental components of milk fats and play crucial roles in health outcomes for newborns, including optimal growth, neurocognitive development, and reduced risks of metabolic and cardiovascular diseases in adulthood (Munblit et al., 2019; Ramiro-Cortijo et al., 2020).

Human milk contains over 200 different fatty acids, the most common ones are presented in Table 1 and Table 2. Among these 40% are saturated fatty acids with no double bonds, 42% monounsaturated fatty acids with one double bond, and 18% polyunsaturated fatty acids with multiple double bonds. Among the polyunsaturated fatty acids, 15% are n-6 fatty acids, and 3% are n-3 fatty acids (Ćwiek et al., 2023; Schwingshackl and Hoffmann, 2012).

Table 1. Saturated and monounsaturated fatty acids present in human milk *(original table)*

Type	Fatty acid	Common name	Systematic name
SFA	C4:0	Butyric acid	Butanoic acid
	C5:0	Valeric acid	Pentanoic acid
	C6:0	Caproic acid	Hexanoic acid
	C8:0	Caprylic acid	Octanoic acid
	C10:0	Capric acid	Decanoic acid
	C12:0	Lauric acid	Dodecanoic acid
	C13:0	Tridecylic acid	Tridecanoic acid
	C14:0	Myristic acid	Tetradecanoic acid
	C15:0	Pentadecylic acid	Pentadecanoic acid
	C16:0	Palmitic acid	Hexadecanoic acid
	C17:0	Margaric acid	Heptadecanoic acid
	C18:0	Stearic acid	Octadecanoic acid
	C20:0	Arachidic acid	Icosanoic acid
	C21:0	Heneicosylic acid	Heneicosanoic acid
	C22:0	Behenic acid	Docosanoic acid
	C24:0	Lignoceric acid	Tetracosanoic acid
MUFA	C14:1 n-5	Myristoleic acid	(9Z)-tetradec-9-enoic acid
	C16:1 n-7	Palmitoleic acid	(Z)-hexadec-9-enoic acid
	C16:1 n-7	Palmitelaidic	(E)-hexadec-9-enoic acid
	C17:1	Heptadecenoic acid	(Z)-heptadec-10-enoic acid
	C18:1 n-7	cis-Vaccenic acid	(E)-octadec-11-enoic acid
	C18:1 c	Oleic acid	(Z)-octadec-9-enoic acid
	C18:1 t	Elaidic acid	(E)-octadec-9-enoic acid
	C20:1 n-9	Gondoic acid	(11Z)-icos-11-enoic acid
	C22:1 n-9	Erucic acid	(Z)-docos-13-enoic acid

Table 2. Polyunsaturated fatty acids present in human milk *(original table)*

Type	Fatty acid	Common name	Systematic name
PUFA	C18:2 n-6	Linoleic acid (LA)	(9Z,12Z)-octadeca-9,12-dienoic acid
	C18:2 t	Linoelaidic acid	(9E,12E)-Octadeca-9,12-dienoic acid
	C18:3 n-3	alpha-Linolenic acid (ALA)	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid
	C18:3 n-6	gamma-Linolenic acid (GLA)	(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid
	C18:4 n-3	Stearidonic acid	(6Z,9Z,12Z,15Z)-octadeca-6,9,12,15-tetraenoic acid
	C20:2 n-6	*cis*-11,14-Eicosadienoic acid	(11Z,14Z)-icosa-11,14-dienoic acid
	C20:3	*cis*-11,14,17-Eicosatrienoic acid	(11Z,14Z,17Z)-icosa-11,14,17-trienoic acid
	C20:3 n-9	*cis*-5,8,11-Eicosatrienoic acid	(5Z,8Z,11Z)-icosa-5,8,11-trienoic acid
	C20:3 n-6	Dihomo-gamma-linolenic acid (DGLA)	(8Z,11Z,14Z)-icosa-8,11,14-trienoic acid
	C20:4 n-3	Eicosatetraenoic acid	(2E,4E,6E,8E)-icosa-2,4,6,8-tetraenoic acid
	C20:4 n-6	Arachidonic acid (ARA)	(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid
	C20:5 n-3	Eicosapentaenoic acid (EPA)	(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid
	C22:4 n-6	Adrenic acid	(7Z,10Z,13Z,16Z)-docosa-7,10,13,16-tetraenoic acid
	C22:5 n-3	Docosapentaenoic acid (DPA)	(7Z,10Z,13Z,16Z,19Z)-docosa-7,10,13,16,19-pentaenoic acid
	C22:6 n-3	Docosahexaenoic acid (DHA)	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid

The composition of FAs in human milk is determined by three primary sources: the release of fatty acids from maternal fat stores, endogenous synthesis within the mammary gland, and direct uptake from maternal plasma. The latter is particularly significant as it reflects the lactating woman's current diet (Del Prado et al., 2001; Garg et al., 2005; Innis, 2013; Koletzko et al., 2001).

Essential fatty acids (EFAs) are fatty acids that the human body cannot synthesize and must be obtained from dietary sources to maintain normal physiological functions (Chipponi et al., 1982; Goodhart and Shils, 1980; Vaughan and Geissler, 2009). Children require an adequate supply of EFAs, such as linoleic acid (LA, C18:2 n-6) and alpha-linolenic acid (ALA, C18:3 n-3), as well as n-3 and n-6 fatty acids, including docosahexaenoic acid (DHA, C22:6 n-3) and arachidonic acid (ARA, C20:4 n-6), to support their growth and metabolism. Additionally, monounsaturated fatty acids (MUFAs) and some medium chain fatty acids (MCFAs) are important for their overall health and development. The first six months of life are critical for neural development, during which the infant's brain grows rapidly, reaching about half the mass of an adult brain. This significant growth underscores the importance of adequate nutrition, including EFAs, for optimal brain development and function. Adequate intake of these fatty acids is directly correlated with the maternal diet and significantly influences infant development (Dobbing and Sands, 1973; Knickmeyer et al., 2008; Tau and Peterson, 2010).

The development of an infant's nerve cells requires a substantial amount of DHA (Knickmeyer et al., 2008; Levitt, 2003). Clinical studies from various continents confirm a strong correlation

between maternal DHA intake and its concentration in HM, underscoring the importance of adequate DHA consumption during pregnancy (Antonakou et al., 2013; Koletzko et al., 2014; Liu et al., 2016; Sherry et al., 2015). Moreover, the maternal dietary ratio of n-6 to n-3 fatty acids should ideally be from 1:1 to 5:1 to maintain physiological balance. However, modern diets often disrupt this ratio, highlighting the necessity for dietary adjustments to support optimal health outcomes (Küllenberg et al., 2012; Larsson et al., 2004; Liu et al., 2016).

Unsaturated fatty acids in human milk can exhibit either a *cis* or *trans* configuration at the double bond, as illustrated by examples of 18-carbon saturated, *cis*, and *trans* monounsaturated fatty acids in Figure 2. Each configuration has distinct physiological effects. Fatty acids in their natural form typically exhibit the *cis* configuration, such as oleic acid (C18:1 n-9c) shown in Figure 2, where the hydrogen atoms are located on the same side of the double bond. This *cis* configuration introduces a bend in the fatty acid chain, which contributes to the positive health benefits associated with *cis*-unsaturated fatty acids. In contrast, *trans* fatty acids, such as elaidic acid (C18:1 n-9*t*), have hydrogen atoms on opposite sides of the double bond, resulting in a straighter and more rigid structure that closely resembles that of saturated fats. Due to this structural similarity, *trans* fatty acids are generally linked to adverse health outcomes (Hatem et al., 2024; Oteng and Kersten, 2020; Valenzuela and Morgado, 1999).

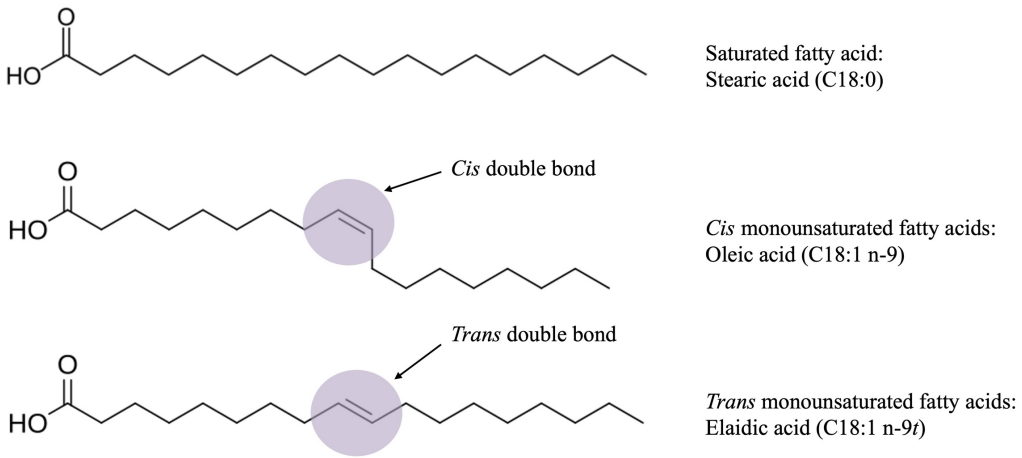

Figure 2. The effect of double bond configuration on molecular structure: examples of 18-carbon saturated, cis, and *trans* monounsaturated fatty acids *(original figure)*

Trans fatty acids (*TFA*s) can be further categorized into those that are industrially produced and those derived from ruminant animals, with the former being more harmful. Research indicates that industrially produced *TFA*s are linked to various health issues, including insulin resistance, obesity, diabetes, and chronic inflammation, affecting both adults and children. Studies have shown that industrially produced *TFA*s can negatively impact the levels of long chain polyunsaturated fatty acids (LCPUFAs) in newborns and infants, potentially hindering their growth and development. Since diet is the sole source of *TFA*s in the human body, the content of these fats in breast milk plays a critical role in determining the *TFA* intake of breastfed infants, which can, in turn, influence the levels of LCPUFAs necessary for proper neurodevelopment and overall health (Hatem et al., 2024).

3.3.1. Saturated fatty acids in human milk

Saturated fatty acids (SFAs) are unbranched, linear chain organic acids characterized by their chemical structure, which consists of carbon-carbon single bonds and a terminal carboxylic acid group (aliphatic monocarboxylic acids). These fatty acids typically contain an even number of carbon atoms (Livingstone, 2014).

Based on the number of carbon atoms, saturated fatty acids can be divided into several groups. Short chain fatty acids (SCFAs), which have fewer than six carbon atoms, include acetic acid (C2:0), propionic acid (C3:0), butyric acid (C4:0), and valeric acid (C5:0). These SCFAs are crucial for the optimal growth of offspring (Nogal et al., 2023). The concentration of SCFAs in breast milk varies significantly among individual lactating mothers, ranging from 13 to 4300 μmol/L (Prentice et al., 2019). Some researches indicate that butyric acid (C4:0) in human milk can inhibit gut pathogens in infants and influence fat production and adipocyte metabolism, either directly or indirectly, through anti-inflammatory mechanisms (Xi et al., 2024; Prentice et al., 2019).

Medium chain fatty acids (MCFAs, C6-C12) in human milk are primarily synthesized in the mammary glands from the carbohydrate-rich diet of pregnant women. Key MCFAs include lauric acid (C12:0), capric acid (C10:0), caprylic acid (C8:0) and caproic acid (C6:0) (Annison et al., 1967; Carey and Dils, 1972; Cowie et al., 1951; Lundsgaard et al., 2021; Nasser et al., 2010). It is well established that MCFAs are rapidly absorbed in the infant's stomach, serving as an important energy source for this organ (Szewczyk and Hanczakowska, 2010). Studies have shown that the percentage of MCFAs in HM increases with the degree of prematurity and lower birth weight of the infant relative to gestational age (Bier et al., 2002; Moltó-Puigmartí et al., 2011). MCFAs are known for their antiviral and antibacterial properties, which help protect infants against infections (Sprong et al., 2001; Nejrup et al., 2017). Additionally, MCFAs play a crucial role in shaping the gut microbiota during

early infancy, which is essential for the development of the immune system and overall health (Martin and Sela, 2013). MCFAs constitute approximately 10–35% of the total fatty acids in human milk (Wei et al., 2019). Caprylic acid (C8:0) and capric acid (C10:0) are particularly notable for their ease and speed of absorption, enhancing fat absorption and exhibiting antibacterial properties in infants (He et al., 2020; Huang et al., 2011). Research also suggests that MCFAs facilitate the conversion of eicosapentaenoic acid (EPA, C20:5 n-3c) to docosahexaenoic acid (DHA, C22:6 n-3c) and support vital metabolic functions (Bobiński and Mikulska, 2015; Jan et al., 2004; Legrand and Rioux, 2010). However, the diet of the mother can significantly impact the content of MCFAs in breast milk. Obese mothers who consume a high-fat diet may have lower levels of MCFAs in their milk (Marín et al., 2005). A study from Poland demonstrated a positive correlation between maternal intake of lauric acid (LA, C12:0), *trans* fatty acids, and alpha-linolenic acid (ALA, C18:3 n-3), and the content of lauric acid (LA, C12:0) in their breast milk (Bobiński et al., 2015).

Long chain fatty acids (LCFAs, C>13) in HM, such as myristic acid (C14:0), palmitic acid (C16:0), arachidic acid (C20:0), and behenic acid (C22:0), exhibit immunomodulatory effects. Palmitic acid (C16:0) is a predominant SFA owing an average relative amount of 25% in HM, it is typically located at the sn-2 position of triglycerides. It is known that positional configuration of fatty acids within triglycerides is crucial (Koletzko, 2017). As I mentioned earlier, this positioning facilitates the action of pancreatic lipase, enhancing fat and calcium absorption in infants (Nelson et al., 1996). It is found that palmitic acid (C16:0) stimulates inflammatory and metabolic responses through the Toll-like receptor (TLR) signaling pathway. Diets high in palmitic acid (C16:0) are linked to reduced fatty acid oxidation, impaired insulin signaling, and increased fat mass (Kien et al., 2005; Rogero and Calder, 2018). Moreover, palmitic acid (C16:0) enhances the expression and secretion of pro-inflammatory cytokines and damaging the insulin signaling pathway (Capurso and Capurso, 2012; Kim et al., 2007).

During lactation, the content of stearic acid (18:0) remains stable at about 6% (Giuffrida et al., 2022). Significant correlations have been noted between dietary SFA intake, high-fat dairy product consumption, and SFA content in HM (Bravi et al., 2016). Long chain saturated fatty acids enter the mammary alveolar cells from plasma, sourced from diet or lipid stores (Neville and Picciano, 1997; Rudolph et al., 2007).

Saturated fatty acids can be found in both animal fats and plant oils, with rich dietary sources including butterfat, meat fat, and tropical oils such as palm oil, coconut oil, and palm kernel oil. Research has demonstrated that consuming SFAs with carbon chain lengths between 8 and 16 atoms can elevate serum low-density lipoprotein (LDL) cholesterol levels, commonly known as 'bad'

cholesterol, which is associated with an increased risk of cardiovascular disease. For example, palmitic acid (C16:0) has been shown to raise cholesterol levels, partly because it suppresses the expression of LDL receptors. Myristic acid (C14:0) is even more potent in raising LDL cholesterol concentrations compared to palmitic acid (C16:0). In contrast, the cholesterol-raising effects of lauric acid (C12:0), capric acid (C10:0), and caprylic acid (C8:0) are less pronounced. Interestingly, stearic acid (C18:0) is unique among SFAs as it does not raise serum LDL cholesterol concentrations. The underlying mechanism remains unclear, but it is suggested that stearic acid (C18:0) is rapidly converted into oleic acid (C18:1 n-9c) in the human body, which may explain its neutral effect on cholesterol levels (Grundy, 2004).

3.3.2. Monounsaturated fatty acids in human milk

Monounsaturated fatty acids (MUFAs) in HM are more stable compared to SFAs, but the proportions of MUFAs and SFAs remain relatively constant (Devaraj et al., 2023). Oleic acid (OA, C18:1 n-9c), which constitutes about 90% of MUFAs in HM, plays a significant role in the nutrition of both infants and breastfeeding mothers. For infants, OA is a critical component of tissues and cellular membranes and is essential for the development of brain myelin (Şahín et al., 2006). During the process of myelination, OA is rapidly incorporated, increasing its concentration in the brain lipid content as the central nervous system continues to develop (Rioux and Innis, 1992).

Oleic acid (OA, C18:1 n-9c) also lowers the melting point of triglycerides, aiding in the formation, transport, and metabolism of milk fat globules (Giuffrida et al., 2022; Jensen, 1996). The content of OA in HM can be influenced by the lactating mother's diet (Şahín et al., 2006). For instance, Asian mothers typically have higher levels of OA (1.5 g/L) compared to European mothers (1.3 g/L) (Butts et al., 2018). Oleic acid (C18:1 n-9c) is the most abundant fatty acid in human adipose tissue, and its concentration in HM is positively correlated with its levels in maternal fat tissue and plasma (Giuffrida et al., 2022; Kokatnur et al., 1979).

Monounsaturated fatty acids have increasingly been recognized for their anti-inflammatory effects (Ravaut et al., 2020; Rocha et al., 2017). The Mediterranean diet, which is rich in MUFAs primarily from olive oil, comprises approximately 60% of its total fat content as MUFAs, mainly oleic acid (C18:1 n-9c), providing about one-third of the total caloric intake (Ravaut et al., 2020). This diet is associated with a range of health benefits, including significant reductions in plasma triglyceride levels, with reductions reaching up to 60% (Watts et al., 2013). Additionally, it has been shown to reduce LDL cholesterol by 22% (Garg, 1998). The Mediterranean diet also exerts anti-inflammatory

effects on adipose tissue and the liver (Yarla et al., 2018). Unlike diets high in SFAs, the Mediterranean diet enhances lipid profiles without compromising insulin sensitivity (Bos et al., 2010), aids in reducing body weight (Estruch and Ros, 2020), and offers protection against type II diabetes (Muscogiuri et al., 2022). Furthermore, it promotes favorable changes in the gut microbiota, supporting both metabolic and cardiovascular health (Meslier et al., 2020).

Human milk contains approximately 2-5% *trans* fatty acids (TFAs), primarily *trans*-vaccenic acid (C18:1 n-7*t*) and *trans*-elaidic acid (C18:1 n-9*t*), with their levels being significantly influenced by maternal diet (Bousset-Alféres et al., 2022; Samur et al., 2009). *Trans* fatty acids cannot be synthesized by the human body, meaning that their presence in the body is entirely dependent on dietary intake (Keikha et al., 2017). As I mentioned earlier, *TFA*s can be classified into two categories: natural *TFA*s, which are considered beneficial and typically make up about 5% of the total fatty acids, and industrial *TFA*s, which are harmful and can constitute up to 50% of the total fatty acids in certain products. Natural *trans* fats, such as *trans*-vaccenic acid (C18:1 n-11*t*), are commonly found in milk and dairy products, and they have also been detected in HM. Mammals metabolize *trans*-vaccenic acid (C18:1 n-11*t*) into rumenic acid (C18:2 n-9*t*), a specific isomer of conjugated linoleic acid (CLA, C18:2 n-9c), which is characterized by conjugated double bonds, where a single bond separates the two double bonds. This conversion has been linked to several positive health outcomes (de Souza Santos da Costa et al., 2016; Hatem et al., 2024; Kuhnt et al., 2016; Mcguire and Mcguire, 2000). Notably, CLA has been shown to improve immune function by reducing the production of pro-inflammatory mediators, potentially lowering the risk of conditions such as atopic dermatitis, eczema, and food allergies in infants (Thijs et al., 2011).

In contrast, industrial *TFA*s are produced through a process that typically involves subjecting unsaturated fatty acids to high temperatures and pressures, often in the presence of a catalyst. This process partially hydrogenates the double bonds in the fatty acids, effectively lowering their melting point. Additionally, some of the *cis* double bonds are converted into a *trans* configuration during this process, altering the structure and stability of the fatty acids. *Trans*-elaidic acid (C18:1 n-9*t*), the primary industrial *trans* fatty acid found in food products, has been detected in significant amounts in HM (Hatem et al., 2024; Kuhnt et al., 2011; Oteng and Kersten, 2020). *Trans*-elaidic acid (C18:1 n-9*t*) and *trans*-linolelaidic acid (C18:2 n-9*t*, n-12*t*) are commonly found in industrial foods, such as hydrogenated vegetable oils and grilled or fried foods (Craig-Schmidt, 2006; Mosley et al., 2006). Industrial *TFA*s are detrimental to health, elevating "bad" LDL levels while lowering "good" high-density lipoprotein (HDL) levels and increasing inflammation (Pipoyan et al., 2021). High levels of

industrial *TFA*s in the body can disrupt the desaturation process of LA and ALA. This disruption can lead to diminished levels of ARA and DHA in the body, negatively affecting the development of the infant's brain and vision (Aumeistere et al., 2021; Cohen et al., 2011; Craig-Schmidt, 2006; Mennitti et al., 2015; Mosley et al., 2006). Another study indicated that the intake of bad *TFA*s in infants could contribute to the development of insulin resistance, potentially leading to negative long-term health outcomes as the child matures (Osso et al., 2008). It is essential for lactating mothers to be aware of their dietary intake of *TFA*s to ensure optimal health outcomes for themselves and their infants.

3.3.3. Polyunsaturated fatty acids in human milk

Polyunsaturated fatty acids, characterized by having more than one double bond in their hydrocarbon chains, are classified as n-3 and n-6 fatty acids based on the position of the first double bond from the methyl end (Bazinet and Layé, 2014). These fatty acids perform crucial biochemical roles. Especially the long chain polyunsaturated fatty acids (LCPUFAs) are particularly crucial. An adequate supply of these fatty acids during intrauterine, postnatal, and infant development is crucial for the proper maturation of the nervous system, retina, and other essential structures (Bobiński and Bobińska, 2020). The most important LCPUFAs include alpha-linolenic acid (ALA, C18:3 n-3c) and linoleic acid (LA, C18:2 n-6c) act as precursors for n-3 and n-6 fatty acids, respectively. Since the human body cannot synthesize these essential fatty acids, they must be obtained through dietary sources (Hoppenbrouwers et al., 2019; Miles and Calder, 2017; Moghadasian and Shahidi, 2017). They are vital for the health of pregnant women and their infants, enhancing fetal development, reducing preterm birth risks, and supporting postnatal growth, immune development, and nervous system maturation (Duttaroy and Basak, 2020; Hurtado et al., 2015; Kilari et al., 2010; van Elten et al., 2015). These LCPUFAs are crucial for the development of the infant's brain and retina during the last trimester of pregnancy and the first few months after birth (He et al., 2020). During pregnancy, there is a significant accumulation of LCPUFAs in fetal tissues. These fatty acids are transported to the fetal circulation via specialized transport systems (Uauy et al., 2000b). As a result, the concentrations of certain LCPUFAs, such as DHA and ARA, are substantially higher in fetal tissues, often reaching levels that are 20 to 30 times greater than those found in the mother's tissues (Haggarty, 2002, 2004). Immediately after birth, due to intense metabolism and the rapid increase in the infant's weight, these reserves begin to deplete quickly. Consequently, the primary source of these EFA becomes the mother's milk (Bobiński and Bobińska, 2020).

Eicosapentaenoic acid (EPA, C20:5 n-3c) is derived from ALA and is further converted into docosahexaenoic acid (DHA, C22:6 n-3c). Arachidonic acid (ARA, C20:4 n-6c) is synthesized from

LA through a similar metabolic pathway (Gibson et al., 2011; Hoppenbrouwers et al., 2019; Innis, 2003). However, the conversion efficiency of these precursors into LCPUFAs in humans is quite low. In the body, these precursors undergo elongation, desaturation, and beta-oxidation, leading to the formation of LCPUFAs with additional double bonds and carbon atoms (Figure 3) (Plourde and Cunnane, 2007). This conversion process occurs in the hepatic endoplasmic reticulum and is facilitated by Δ6 and Δ5 desaturases and elongase enzymes, with LA and ALA competing for these enzymes (Saini and Keum, 2018). Although humans can convert ALA into EPA and DHA, the efficiency of this process is limited, being most efficient during the perinatal period and decreasing significantly with age (Brenna, 2002). Therefore, DHA directly obtained from the maternal diet is a more effective source for supporting fetal and neonatal neural development than ALA, which requires conversion to DHA (Greiner et al., 1997). Additionally, an excessive intake of LA can inhibit the endogenous synthesis of DHA, leading to a greater proportion of n-6 LCPUFAs, which can reduce the availability of DHA for incorporation into developing brain tissues in infants (Gibson et al., 2011; Lefkowitz et al., 2005).

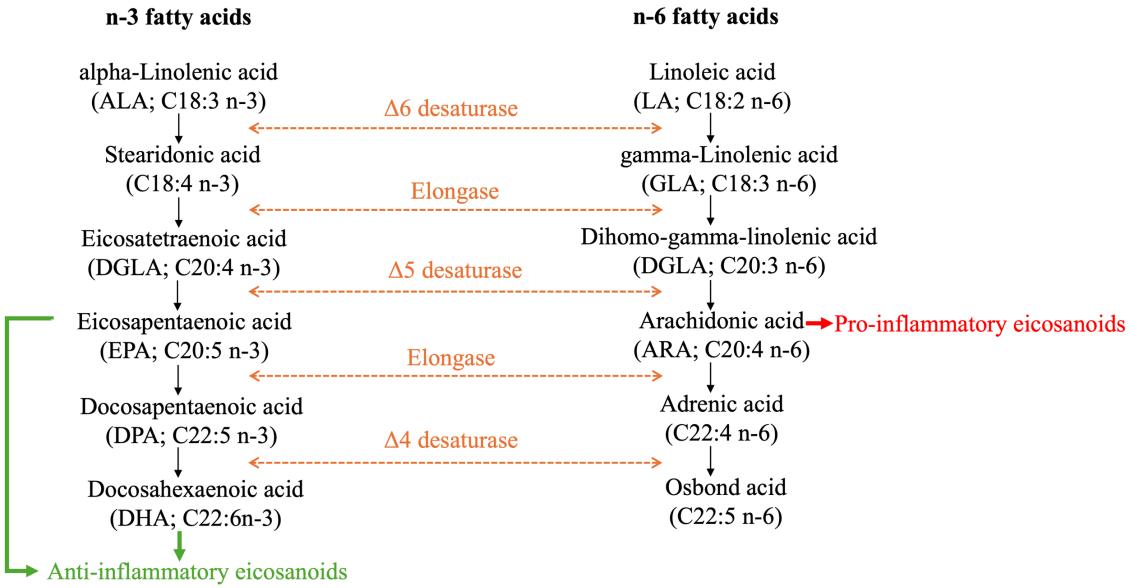

Figure 3. Metabolism of n-3 and n-6 fatty acids *(original figure)*

Unlike adults, most infants lack the ability to synthesize sufficient levels LCPUFAs from EFAs. During pregnancy, these crucial fatty acids are transferred to the fetus via the placenta, supporting fetal development. After birth, the transfer of these fatty acids continues through human milk (Lavandera et al., 2017; Uauy et al., 2000a). DHA, essential for nervous system development, comprises about 10% of the brain's fatty acids (Petersohn et al., 2024). Mature human milk contains

approximately 0.46% ARA and 0.36% DHA (Giuffrida et al., 2022). To ensure adequate DHA intake, the European Union mandates that all infant formulas sold contain DHA levels between 0.33% and 1.14% of total fats (Hopperton et al., 2022).

Within the spectrum of PUFAs, n-3 fatty acids are known for their potential to diminish inflammation and alleviate allergy symptoms (Wendell et al., 2014), while n-6 PUFAs are typically linked to promoting inflammatory responses and the onset of allergic conditions (Willemsen, 2016). Some studies suggest that a balanced n-3 to n-6 ratio is beneficial for the optimal growth and development of infants, highlighting the importance of a balanced maternal diet or the selection of appropriate infant formula (Hyejin Kim et al., 2017).

Adequate consumption of EFAs by lactating mothers is crucial, as research has demonstrated a positive correlation between maternal EFA intake and the concentrations of these fatty acids in breast milk (Makrides et al., 1995; Xiang et al., 2005). However, only a portion of the EFAs consumed by the mother is transferred directly into her milk. The rest is stored in tissues, primarily adipose tissue, where it is utilized for the synthesis of LCPUFAs (Demmelmair et al., 1998; Villalpando et al., 2001).

Human milk comprises about 7.2% *trans* PUFAs, primarily in the form of *cis*-9, *trans*-11 conjugated linoleic acid (CLA, C18:2 n-9c, C18:2 n-11*t*) (Martysiak-Żurowska et al., 2018). CLA enhances the infant's immune system and helps prevent the onset of allergic asthma (Jaudszus et al., 2016). It has been observed that when lactating mothers consume foods rich in CLA, there is a significant increase in the CLA content of their breast milk, thereby meeting the CLA needs of their infants (Moutsioulis et al., 2008).

Some studies have indicated that industrial *trans* fatty acids interfere with the metabolism of LCPUFAs during the perinatal period, reducing the availability of these essential LCPUFAs. This disruption may negatively affect the nervous system and overall somatic development in infants, as these fatty acids are crucial for proper neural and physical growth (Decsi and Boehm, 2013).

Higher maternal intake of *TFA*s during pregnancy may negatively affect the availability of LCPUFAs in the newborn. This is illustrated by the inverse relationship observed between elevated *TFA* levels and reduced concentrations of essential LCPUFAs, such as ARA, EPA, and DHA. These LCPUFAs are crucial not only for the development of the infant's nervous system but also for adequate energy provision during early growth stages. Therefore, to minimize the harmful *trans* fatty acid content in HM, it is essential for breastfeeding mothers to make appropriate dietary adjustments, particularly focusing on reducing their intake of industrially produced *TFA*s (Hatem et al., 2024; Precht and Molkentin, 1999).

3.4. Factors effecting the composition of human milk

The composition and concentration of fatty acids in HM can be influenced by a wide range of factors, including maternal health conditions such as obesity and gestational diabetes, maternal diet, geographic location, stage of lactation, infant sex, delivery mode, and heat treatment methods for the milk (Bravi et al., 2021; Hewelt-Belka et al., 2020; Leghi et al., 2020; Peila et al., 2020; Samuel et al., 2022). These variables play a critical role in the composition of the FAs profile of HM.

3.4.1. Maternal health status

3.4.1.1. Obesity

According to the World Health Organization (WHO), overweight and obesity are defined as "abnormal or excessive fat accumulation that may impair health". An adult is considered overweight with a Body Mass Index (BMI) ≥ 25, and obese with a BMI ≥ 30. Recent data indicates that over 2.5 billion adults globally are overweight, of which more than 890 million are obese (WHO, 2024). Projections suggest that by 2030, the number of overweight individuals will exceed 2.16 billion, with 1.12 billion being obese (Kolahi et al., 2018). In Europe, over half of the adult population is overweight or obese, with approximately 40% of women being overweight and over 30% of women during pregnancy being obese (Muc Da Encarnacao and Collaboration, 2017; Simko et al., 2019; WHO, 2022).

During pregnancy, a high BMI increases the risk of complications such as miscarriage, gestational diabetes (GD), stillbirth, and significant postpartum bleeding. It also influences the fatty acid profile in breast milk, potentially impacting infant health and development (Farpour-Lambert et al., 2018; Wang et al., 2017). Research suggests that the breast milk from mothers who were obese during pregnancy may have different fatty acid compositions compared to that of mothers with normal weight. This difference could be related to a higher likelihood of having offspring who are larger for gestational age, potentially leading to subsequent obesity in the child (Amaral et al., 2021; Córdoba-Rodríguez et al., 2022; Ellsworth et al., 2020; Enstad et al., 2021; Fujimori et al., 2015; Lindholm et al., 2013; Yu et al., 2013).

Amaral and colleagues conducted a study involving 107 women, of whom 31.1% had hypertension, and 18.7% had diabetes either before or during pregnancy. In terms of pre-pregnancy nutritional status, 46.3% of the participants were classified as overweight, and 38.3% were considered obese. The study found that being overweight prior to pregnancy was correlated with higher energy and lipid levels in human milk. Conversely, gestational weight gain that fell below the recommended

levels was linked to significant reductions in both energy content and lipid concentration in the milk (Amaral et al., 2021).

Another study reported that in the HM of obese and overweight mothers, the concentration of palmitic acid (C16:0) was 24.34%, and the levels of two LCPUFAs, namely dihomo-gamma-linolenic acid (C20:3 n-6c, 0.54%) and adrenic acid (C22:4 n-6c, 0.14%), were significantly higher compared to the milk of mothers with normal weight (C16:0, 21.96%; C20:3 n-6c, 0.38%; C22:4 n-6c, 0.10%). In contrast, the concentration of CLA significantly decreased from 0.45% in normal-weight mothers to 0.34% in obese and overweight mothers. Additionally, infants born to these obese and overweight mothers were found to have a higher weight-for-length ratio and a greater BMI at birth (Ellsworth et al., 2020).

A study conducted in Sweden examined the FA profile differences in HM between mothers with obesity and those with a normal BMI. The research found that the n-6 to n-3 ratio in HM collected from obese mothers was 40% higher than in milk from mothers with a normal BMI (Lindholm et al., 2013). The authors also reported significant differences in n-3 LCPUFAs, including alpha-linolenic acid (C18:3 n-3c), eicosapentaenoic acid (C20:5 n-3c), and docosahexaenoic acid (C22:6 n-3c) between the studied breast milk samples. In the first three days postpartum, the MUFA ratio was notably higher in the milk of obese mothers, as was the n-6 to n-3 ratio. Additionally, n-3 LCPUFAs were found to be at their lowest levels in the milk of obese mothers. The researchers also highlighted that obese mother generally had lower levels of education, a higher prevalence of smoking before pregnancy, and emphasized that HM composition largely reflects maternal dietary habits. The higher concentrations of n-6 fatty acids relative to n-3 fatty acids in Western diets, particularly in processed and fast foods such as fish sticks, and in animal-derived foods, have been linked to an increased risk of obesity in offspring later in life (Ailhaud et al., 2007). Animal studies have demonstrated that high concentrations of n-3 fatty acids during pregnancy are linked to the development of fewer and smaller adipocytes in the offspring (Korotkova et al., 2005).

Adequate intake of n-3 LCPUFAs during pregnancy is crucial for optimal neuronal development in infants. Lifestyle interventions, such as physical activity, play a significant role not only in enhancing maternal health but also in supporting infant well-being. Research has shown that physical activity can lead to significant changes in FA patterns in HM, even in the absence of dietary modifications (Hull et al., 2011). A study by Baur et al. (1998) indicates that breastfed infants tend to maintain stable blood glucose levels, likely due to the intake of LCPUFAs through breastfeeding. Furthermore, evidence suggests a negative correlation between the frequency and duration of breastfeeding and the risk of diabetes development in children (Pereira et al., 2014).

Several studies have indicated that a higher maternal BMI is linked to an elevated n-6 to n-3 PUFA ratio and increased leptin concentrations in HM. Furthermore, a higher n-6 to n-3 PUFA ratio, combined with the presence of inflammatory cytokines in the milk, has been associated with more rapid weight gain during infancy. These findings highlight the influence of maternal BMI and dietary fatty acid balance on both the composition of breast milk and infant growth patterns (Álvarez et al., 2020; Enstad et al., 2021).

Contemporary research indicates that maternal body composition, dietary habits, and nutritional status before and during pregnancy are crucial determinants of fetal health. Both undernutrition and overnutrition, along with pre-pregnancy and gestational weight issues, are linked to complications in placental, embryonic, and fetal development, affecting fetal growth and leading to perinatal complications. These factors ultimately contribute to suboptimal pregnancy outcomes for both the mother and the infant (Marshall et al., 2022). A study conducted by Giuffrida and colleagues (2022) demonstrated that the fatty acid composition in maternal adipose tissue and plasma is closely correlated with that found in human milk.

The rapid increase in obesity rates is largely attributed to excessive consumption of energy-dense foods combined with insufficient physical activity (Costa et al., 2012). During the periconceptional period and throughout pregnancy, the nutritional quality of a woman's diet is crucial as it significantly affects the course of the pregnancy, the delivery process, and the long-term health of both the mother and child. Besides ensuring the nutritional quality of the diet, maintaining a balanced energy intake is important since excessive weight gain during pregnancy is discouraged (Guelinckx et al., 2008).

Long chain saturated fatty acids, specifically palmitic acid (C16:0), have been implicated in the development of obesity through mechanisms causing hypothalamic inflammation, a critical area for weight regulation. This inflammation undermines insulin's central role in regulating food intake and glucose metabolism, leading to insulin resistance, a pivotal factor connecting obesity with type II diabetes (Chen et al., 2017; Facchini et al., 2001; Thaler et al., 2012; Wang et al., 2017). Moreover, diets high in palmitic acid (C16:0) are associated with leptin resistance, undermining leptin's appetite-suppressing effects and perpetuating a cycle of weight gain (Banks et al., 1999; Caro et al., 1996; El-Haschimi et al., 2000; Knight et al., 2010).

Obesity is linked to elevated levels of proinflammatory cytokines and chemokines in both circulation and tissues. Cytokines, which are protein molecules that facilitate communication between immune cells, play a key role in mediating inflammation. Research on mice fed a high-fat diet showed the development of low-grade hepatitis, which was accompanied by an increase in the production and secretion of various proinflammatory cytokines. This suggests that the inflammatory response may be

triggered either by dietary components or by substances released from increased adipose tissue. Free fatty acids (FFAs) are considered a likely factor in both mechanisms. Elevated plasma FFA levels are associated with insulin resistance in obese individuals and have been implicated in insulin resistance across major insulin target organs, including skeletal muscle, liver, and endothelial cells. Moreover, FFAs have been identified as a major link between obesity, the development of metabolic syndrome, and atherosclerotic vascular disease (Boden, 2008).

3.4.1.2. Gestational diabetes

The American Diabetes Association defines Gestational Diabetes (GD) as diabetes first diagnosed during the mid or late stages of pregnancy. GD is a common pregnancy complication characterized by spontaneous hyperglycemia (ADA, 2018). Unlike other types of diabetes, GD is distinguished by insulin resistance triggered by the release of placental hormones. In GD, the maternal insulin response is insufficient to compensate for this resistance, resulting in elevated blood glucose levels in the mother (McIntyre et al., 2019).

Gestational diabetes affects approximately 14% of pregnancies globally, impacting around 18 million newborns annually. GD is associated with serious maternal complications such as shoulder dystocia and gestational hypertension, and it increases the risk of delivering large babies (macrosomia), which can necessitate cesarean sections. Infants born to mothers with GD are at a higher risk of experiencing hypoglycemia shortly after birth and developing type II diabetes later in life (CDC, 2022; Schaefer-Graf et al., 2018). Approximately 80% of GD cases are marked by β-cell dysfunction amidst chronic insulin resistance, with pregnancy-induced insulin resistance contributing additionally (Buchanan and Xiang, 2005).

A study comparing colostrum and transitional milk between diabetic and healthy mothers highlighted significant differences in fatty acid composition. The milk from diabetic mothers exhibited a reduction in MCFAs such as lauric acid (C12:0), while showing an increased concentration of long chain saturated fatty acids like myristic acid (C14:0), pentadecylic acid (C15:0) and stearic acid (C18:0). Additionally, PUFAs, including linoleic acid (C18:2 n-6c), eicosadienoic acid (C20:2 n-6c), docosapentaenoic acid (C22:5 n-6c), and docosapentaenoic acid (C22:5 n-3c), were present at higher levels. Furthermore, a higher proportion of FFAs was detected in the milk of diabetic mothers, indicating potential impairments in the esterification processes within the mammary gland (Bitman et al., 1989).

Conversely, another study found that the levels of MCFAs in the group of diabetic mothers were similar to those in non-diabetic mothers across all stages of lactation. However, when examining

LCPUFAs, diabetic mothers exhibited lower levels from 14 to 84 days postpartum (Jackson et al., 1994).

Several factors are associated with the development of GD, either directly or indirectly, including obesity, excessive gestational weight gain, a Westernized diet, ethnicity, genetic polymorphisms, advanced maternal age, and family history of GD, among other conditions of insulin resistance (Anghebem-Oliveira et al., 2017; Durnwald, 2015; Levy et al., 2010; Okosun et al., 2004; Zhang et al., 2014). Dietary habits such as high intake of saturated fats, refined sugars, red meat, and processed meats significantly elevate the risk of GD, while diets rich in fiber, micronutrients, and polyunsaturated fats are linked to a reduced risk (Bao et al., 2014; Bowers et al., 2012; Taschereau-Charron et al., 2017; Zhang et al., 2006).

Gestational diabetes has a significant impact on the composition of breast milk. Studies show that women with GD have notably higher concentrations of essential n-6 PUFAs in their breast milk compared to non-GD women, although total PUFA levels are generally negatively correlated with GD (Chertok et al., 2017; Zhong et al., 2022).

In a large-scale lipidomics study conducted in China, Zhong and colleagues (2022) explored the lipid composition of breast milk from mothers with and without GD, seeking relationships between individual lipid components, maternal fasting glucose levels, and infant developmental parameters. The study identified 833 lipid components across 15 categories, with 60 components being significantly associated with elevated maternal fasting glucose levels. Notably, the MUFAs, erucic acid (C22:1) was strongly positively correlated with GD, while the PUFAs, alpha-linolenic acid (C18:3 n-3c) and docosahexaenoic acid (C22:6 n-3c) showed a negative association with GD. These findings indicate that GD induces substantial changes in the breast milk lipid profile. Moreover, the study found that lipid components linked to GD were also associated with key infant growth metrics, such as body weight, length, and head circumference. This underscores the potential protective role of breastfeeding, particularly for infants born to mothers with GD.

Another recent study by a Chinese research team also investigated the lipid composition of human milk in relation to gestational diabetes, mode of delivery, and parity (Yu et al., 2023). The study found that mothers with GD produced breast milk with significantly higher overall lipid content but lower levels of palmitoleic acid (C16:1) and certain triacylglycerols. The researchers noted that higher palmitoleic acid (C16:1) levels in human milk are linked to faster infant growth, while lower levels may result from the metabolic conversion of palmitoleic acid (C16:1) to vaccenic acid (C18:1) or C16:1-OH in GD cases (Yu et al., 2023). Additionally, it was observed that mothers with GD gave birth predominantly to male infants. The association between infant sex and placental hormone

secretion may influence mammary gland development, potentially impacting the lipid content in human milk (Ehrlich, 2021).

3.4.2. Geographical location

Numerous studies have shown that the geographical location of mothers significantly impacts the fatty acid composition of human milk. This is largely due to differences in regional dietary habits, environmental factors, and cultural influences (Bobiński and Bobińska, 2020; Hatem et al., 2024). These variations not only reflect the diversity of global diets but also affect the fatty acid profile of breast milk, which can have implications for infant health outcomes.

In Europe, the levels of SFAs in breast milk tend to be relatively high. For example, pentadecylic acid (C15:0) levels in the milk of mothers from Western countries are elevated, as are the levels of MUFAs like oleic acid (C18:1 n-9c). In contrast, the concentrations of PUFAs, including linoleic acid (C18:2 n-6c), arachidonic acid (C20:4 n-6c), alpha-linolenic acid (C18:3 n-3c), eicosapentaenoic acid (C20:5 n-3c), docosahexaenoic acid (C22:6 n-3c), and total n-6 PUFAs, are relatively low in Western mothers' breast milk (Z. Zhang et al., 2022). The typical Western diet, which is high in n-6 fatty acids from meat and meat products but low in n-3 fatty acids, results in an unbalanced n-6 to n-3 ratio of 15-20:1, far from the ideal 1:1 ratio. This imbalance can trigger pro-inflammatory responses, potentially leading to issues like infections and cellular damage (Medzhitov, 2010; Simopoulos, 2002). Additionally, the Western diet may not provide adequate amounts of DHA, which could result in deficiencies, especially in preterm infants (Granot et al., 2011; Heath et al., 2022).

Furthermore, *trans* fatty acids from baked goods, confectionery, and snack foods are a significant component of the diets of breastfeeding women following both Western and Mediterranean diets (Krešić et al., 2013; Mojska et al., 2007; Samur et al., 2009). These *TFA*s can directly affect the fatty acid composition of breast milk (Mueller et al., 2010). The levels of *TFA*s in HM vary significantly across regions, primarily due to differences in maternal diets (Bahreynian et al., 2020). For instance, European mothers have an average *TFA* level of 1.82% in their breast milk, while Chinese mothers show a lower average of 0.71% (Hatem et al., 2024).

Asian countries exhibit a diverse range of dietary cultures, leading to variations in the fatty acid composition of breast milk by region. In China, the content of SFAs in breast milk is relatively low at around 37.5%, compared to 43.7% in Hungarian mothers. Similarly, Chinese mothers show a MUFA content of 31.8%, compared to 35.8% in Hungarian mothers. However, the proportion of PUFAs in Chinese mothers' milk is 27%, higher than that of many European mothers (Z. Zhang et al., 2022). Even among mothers of the same nationality, regional dietary differences can affect the fatty acid

composition of breast milk. For example, mothers from Hangzhou, China, had the lowest levels of oleic acid (C18:1 n-9c) but the highest levels of docosahexaenoic acid (C22:6 n-3c), whereas breast milk samples from Lanzhou, China, exhibited the lowest levels of docosahexaenoic acid (C22:6 n-3c), total PUFAs, and n-3 PUFAs (Jiang et al., 2016). The high DHA content in Japanese mothers' breast milk is attributed to the traditional Japanese diet, which is rich in seafood (Kasamatsu et al., 2023).

Multiple studies across different countries have consistently demonstrated a positive correlation between a mother's dietary DHA intake and the DHA concentration in her breast milk (Bobiński and Bobińska, 2020). Maintaining a well-balanced diet during breastfeeding is crucial for both maternal health and the optimal development of the breastfed child (Hale and Hartmann, 2017). Consuming foods rich in n-3 fatty acids, such as flaxseed, linseed oils, fish, and seafood, or taking supplements can help achieve a balanced n-6 to n-3 ratio and boost levels of ALA, DHA, and EPA in breast milk (Coniglio et al., 2023; Nelson et al., 2008; Oliver et al., 2020; Petersohn et al., 2024).

To ensure balanced nutrition, dietary guidelines recommend that fats should make up approximately 20% to 35% of total daily calorie intake (EFSA, 2010). However, studies have revealed significant variation in the intake of these fatty acids, particularly DHA, depending on geographical location and access to diets rich in fish and seafood. For instance, women living in inland areas typically consume around 30 mg/day of DHA, while those in coastal regions consume about 180 mg/day (Peng et al., 2009). Supplements can serve as an additional source of EPA and DHA for breastfeeding women. The recommended daily intake for EPA and DHA in adults is 500 mg (Vannice and Rasmussen, 2014).

3.4.3. Lactation period

Human milk adapts dynamically to meet the varying physiological needs of infants at different stages of development. Based on postpartum timing, HM can be categorized into three stages:

Colostrum (0-3 lactation days): Colostrum is produced during the first few days after birth and is characterized by its thick, yellowish appearance. It is rich in proteins, particularly immunoglobulins, which provide essential immune protection to the newborn. Colostrum also contains higher concentrations of certain vitamins and minerals compared to later stages of lactation. While its fat content is relatively low, at approximately 2.2 g/100 mL, it features a unique lipid composition essential for the newborn's early development and immune function. This early milk plays a crucial role in establishing the infant's immune system and provides a concentrated source of nutrients necessary for initial growth and development (Gidrewicz and Fenton, 2014; Giuffrida et al., 2022;

Hoyt et al., 2015; Kristensen-Cabrera et al., 2018). In colostrum, palmitic acid (C16:0) content is around 23.6%, and the LCPUFAs arachidonic acid (C20:4 n-6) and docosahexaenoic acid (C22:6 n-3c) are found at their highest levels, 0.78% and 0.59%, respectively (Z. Zhang et al., 2022).

Transitional milk (15±3 lactation days): Transitional milk represents the phase following colostrum, as the milk composition shifts toward mature milk. During this stage, lactose, fat (3.0 g/100 mL), and caloric content increase to support the infant's rapid growth and metabolic needs. This period is crucial for the infant's early physical and neurological development (Ballard and Morrow, 2013; Gidrewicz and Fenton, 2014; Giuffrida et al., 2022). Studies have indicated that the content of palmitic acid (C16:0) in transitional milk decreases slightly to 21.5% (Z. Zhang et al., 2022).

Mature milk (>15 lactation days): Mature milk has a higher fat concentration, approximately 3.4 g/100 mL, and is influenced by factors such as maternal diet, weight gain during lactation, and geographical location (Gidrewicz and Fenton, 2014; Giuffrida et al., 2022; Martin et al., 2016). The concentrations of SCFAs, such as butyric acid (C4:0), and MCFAs, including caproic acid (C6:0) and caprylic acid (C8:0), increase by approximately 42.18% in mature milk compared to transitional milk (Dai et al., 2020). Additionally, capric acid (C10:0) and lauric acid (C12:0) concentrations rise significantly by over 100% and about 50%, respectively compared to colostrum. Interestingly, palmitic acid (C16:0) remains at similar levels to those in transitional milk. However, the concentrations of linoleic acid (C18:2 n-6c) and alpha-linolenic acid (C18:3 n-3c) increase, while their derivatives, arachidonic acid (C20:4 n-6c) and docosahexaenoic acid (C22:6 n-3c), decrease to approximately 60-65% and 50%, respectively, of the levels seen in transitional milk (Bobiński and Bobińska, 2020).

Despite these shifts, some studies have shown that there are no significant changes in the total content of SFAs, MUFAs, PUFAs, and individual fatty acids like pentadecylic acid (C15:0), heptadecanoic acid (C17:0), oleic acid (C18:1 n-9c), linoleic acid (C18:2 n-6c), alpha-linolenic acid (C18:3 n-3c), or eicosapentaenoic acid (C20:5 n-3c) across different lactation stages (Z. Zhang et al., 2022).

3.4.4. Gender of the infant

Since the 1970s, differences in perinatal outcomes between male and female infants have been referred to as the "male disadvantage." This term originated from observations in the United States, where male infants were found to have a higher risk of neonatal mortality compared to their female counterparts (Naeye et al., 1971). Male newborns have a higher risk of mortality compared to female newborns. Additionally, male infants are more susceptible to respiratory distress syndrome, anemia,

and mineral deficiencies, especially among those with low birth weight (Brothwood et al., 1986; Libster et al., 2009).

Research has shown that male and female offspring respond differently to early-life nutritional stimuli (Galbarczyk, 2011). An animal study found that fetal sex influences mammary gland function during lactation, resulting in different milk compositions for male and female offspring. Notably, milk produced for female offspring shows a 1.3% increase in volume compared to that produced for males. Despite this difference in volume, fat concentration remains relatively consistent, at 3.61% for daughters and 3.62% for sons (Hinde et al., 2014).

Throughout all lactation stages, the total fat content in the breast milk of mothers nursing male infants has generally been observed to be higher than that in mothers nursing female infants. This difference becomes particularly evident in mature milk, where fat concentrations peak, reaching approximately 5.5 g/L for male infants and 4.4 g/L for female infants. Interestingly, while fat content is higher in milk for male infants, levels of carbohydrates and lactose in both colostrum and mature milk tend to be higher in milk for female newborns (Khelouf et al., 2023).

However, a recent human study reported contrasting findings, showing that fat content in the breast milk of mothers nursing female infants was significantly higher than that in mothers nursing male infants (Hosseini et al., 2020).

Clinical studies on human twins have shown that opposite-sex twins receiving the same breast milk may experience different growth and developmental outcomes after birth. On average, breastfed opposite-sex twins tend to be shorter and lighter in weight compared to same-sex twins (Kanazawa and Segal, 2017).

The preterm male infants have a greater need for preterm formula milk with higher energy and micronutrient content compared to preterm female infants to support their growth requirements. When compared to male infants fed standard term formula, these preterm male infants showed improved neurodevelopment over the following 18 months (Lucas et al., 1990).

Understanding whether male and female infants receive different nutrients through breast milk underscores the importance of potentially developing gender-specific nutritional strategies for mothers and their babies (Samuel et al., 2022).

The impact of infant gender on human milk composition remains an area with limited scientific research. While some studies suggest there may be differences in the composition of milk based on the gender of the infant, the specific mechanisms responsible for these variations are still largely unknown. The hormonal and biological factors that may drive sex-specific milk synthesis are not yet fully understood and require further exploration.

3.4.5. Mode of delivery

The rate of cesarean sections (C-sections) as a medical intervention has increased significantly from 12.1% in 2000 to 21.1% in 2015. In Europe, approximately 27% of births are via C-section (Boerma et al., 2018). Compared to vaginal deliveries, C-sections are associated with higher risks of maternal mortality, stillbirth, and preterm birth (Sandall et al., 2018). Postpartum, mothers are more prone to depression and may face challenges with breastfeeding (Clement, 2001). Infants born via C-section are more susceptible to allergies, asthma, and a reduced diversity in their gut microbiota (Sandall et al., 2018). Recent studies also suggest a potential link between C-sections and neurodevelopmental issues in infants (Deoni et al., 2019; Yoshida et al., 2023).

These complications might stem from the reduced transfer of maternal microbiota to the infant, altering immune development. Additionally, C-section deliveries reduce the infant's exposure to the physical stress and hormones associated with vaginal birth, which are critical for the development of the hypothalamic-pituitary-adrenal axis, immune system maturation, organ development, and neurogenesis. Epigenetic modifications in gene expression may also play a role, potentially impacting immune function, metabolic processes, or neurodevelopment pathways critical to the infant's long-term health (Sandall et al., 2018).

In a study by Yu and colleagues (2023), the lipid composition of HM from mothers who had different delivery modes was analyzed. The delivery method is believed to influence the composition of breast milk, as varying hormonal levels and uterine interactions occur during C-section versus vaginal deliveries. These differences may affect milk composition, although the precise mechanisms remain unclear. Yu and colleagues found that mothers who delivered via C-section produced milk with significantly higher fat, protein, and carbohydrate content compared to those who had vaginal deliveries. A Korean study (Hahn et al., 2017), which also reported higher fat content in the milk of mothers who underwent cesarean deliveries. Burianova and colleagues (2019) observed elevated protein levels in the breast milk of cesarean-delivered mothers. However, another study found no significant effect of delivery mode on human milk fat and protein concentration, highlighting inconsistencies in the literature (Zeynali et al., 2022).

Despite these mixed findings, a European longitudinal cohort study provided more insights. The results indicated that breast milk from mothers who delivered vaginally had significantly higher levels of palmitoleic acid (C16:1 n-7c), stearic acid (C18:0), oleic acid (C18:1 n-9c), alpha-linolenic acid (C18:3 n-3c), eicosapentaenoic acid (C20:5 n-3c), and docosahexaenoic acid (C22:6 n-3c). On the

other hand, cesarean-delivered mothers had milk with higher ARA to DHA ratios and n-6 to n-3 PUFA ratios, suggesting a higher n-6 FA content, potentially linked to inflammation due to surgery (Samuel et al., 2022). This discrepancy in fatty acid profiles between delivery modes highlights the complex relationship between maternal factors and breast milk composition.

3.4.6. Holder pasteurization

Human milk is considered the optimal choice for feeding baby, especially the preterm infants (Miller et al., 2018). Research indicates that some mothers face significant challenges in initiating and sustaining lactation during the first six months postpartum (Chapman and Pérez-Escamilla, 1999; Hauck et al., 2011). These difficulties often lead to switch to formula feeding for their infants. The first 2 years of life, encompassing both prenatal and early postnatal periods, are critical for nutritional and hormonal exposures, which have substantial implications for both immediate and long-term health outcomes (Ford et al., 2018). Studies have also indicated that donor milk (DM) is preferred as a substitute for preterm infants over preterm formula (Fang et al., 2021; Poulimeneas et al., 2021). DM obtained from human milk banks is subjected to Holder pasteurization (HoP), conducted at 62.5 °C for 30 minutes, is the internationally endorsed method for this purpose. This pasteurization technique is effective in eliminating potential pathogens while maintaining essential nutrients and immunological properties (Hartmann, 2017; Shenker et al., 2020). Research has indicated that the total fat loss following HoP ranges from 6.2% to 25% (Colaizy, 2021). Additionally, bioactive components such as immunoglobulins and cytokines can be compromised during the process (Wesolowska et al., 2019). The use of DM is associated with a lower incidence of necrotizing enterocolitis in preterm infants compared to formula feeding (Miller et al., 2018). Donor milk is generally preferred over formula due to its closer composition to maternal milk and its associated health benefits, including reduced risk of necrotizing enterocolitis and sepsis in preterm infants (Cañizo Vázquez et al., 2019; Perrin, M.T., 2018).

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Analytical reagents

To ensure high purity and suitability for sensitive fatty acid analysis, the following reagents were sourced from various suppliers:

- Chloroform (stabilized with ethanol for analysis) and Methanol (HPLC and LC-MS grade) were purchased from Carlo Erba Reagents and VWR International, respectively.
- Hydrochloric Acid (37%) was obtained from VWR International.
- Isooctane (>95%) and Disodium Hydrogen Citrate, used in various experimental procedures, were sourced from Fisher Scientific.
- Sodium Sulfate and Sodium Chloride, necessary for drying and preparatory steps, were acquired from REANAL Gyógyszer- és Finomvegyszergyár Zrt and Carl Roth, respectively.
- Tert-Butyl Methyl Ether, used as a solvent, was obtained from VWR International.
- A 37-component FAME Mix (CRM47885), Glyceryl Trinonadecanoate (C19:0 TAG; >99%), Methyl Nonadecanoate (C19:0 ME, analytical standard), Glyceryl Triheptadecanoate (C17:0 TAG), and Heptadecanoic Acid (C17:0 FFA) were purchased from Merck/Sigma-Aldrich.
- Sodium Hydroxide, used for saponification, and n-Hexane, used for extraction and purification, were also obtained from Merck/Sigma-Aldrich.
- High-purity water (>18 MΩcm-1) was prepared using a Millipore Elix Essential 3 UV Water Purification System and used in all experiments.

4.1.2. Human milk samples

Breast milk samples were collected as part of three international research projects conducted in collaboration with the Regional Cooperation in the Fields of Health, Science, and Technology (RECOOP HST) Association and Cedars-Sinai Medical Center. All research followed the guidelines of the Declaration of Helsinki and was approved by the Regional and Local Research Ethics Committee of the University of Pécs, Pécs, Hungary (PTE KK 7072-2018), and the Local Ethics Committee of Lviv City Children's Clinical Hospital, Lviv, Ukraine (16 November 2018 No. 6).

Project 1: Breast milk samples were obtained from 60 mothers in Lviv, Ukraine, and categorized into four groups: (i) mothers with a normal BMI (nBMI), (ii) obese mothers (O), (iii) nBMI mothers

with gestational diabetes (nBMI+GD), and (iv) obese mothers with gestational diabetes (O+GD). Additionally, samples from 9 mothers with a normal BMI were collected from Pécs, Hungary. All samples were collected between the 10^{th} and 12^{th} weeks postpartum.

Project 2: 17 breast milk samples were collected at the Department of Obstetrics and Gynaecology, University of Pécs (Hungary), from mothers between the 10^{th} and 12^{th} postpartum week. Eight mothers were within the normal weight range (BMI: 18.5–25.0) and 9 mothers were classified as overweight or obese (BMI: >25.0).

Project 3: This study involved 56 registered donor mothers with nBMI recruited from the Breast Milk Collection Center at the Unified Health Institution in Pécs, Hungary.

4.2. Methods

4.2.1. Sample preparation for determination of fatty acid composition of human milk

Project 1: The preparation of fatty acid methyl esters (FAME) from the 60 breast milk samples collected in Ukraine and 9 samples from Hungary followed the protocol outlined by Simon Sarkadi et al. (2022). Frozen breast milk samples were first brought to room temperature and homogenized in an ultrasonic bath for 1 min. A 0.2 mL portion of the milk sample was mixed with 1.8 mL of distilled water, followed by the addition of 2 mL of n-hexane. This mixture was vortexed for 2 min and then centrifuged at 2000 rpm for 5 min. 1 mL of the hexane layer was extracted and combined with 4 mL of a 5% methanolic Na-methylate solution, then vortexed at maximum speed for 4 min. The upper hexane layer was carefully removed with a Pasteur pipette and vortexed twice with 1 mL of distilled water for 1 min each time. The samples were dried over Na_2SO_4, and a 0.25 mL aliquot of the clear hexane phase was reserved. All samples were stored frozen at -18 °C until analysis. Fatty acid methyl esters were analyzed using gas chromatography with flame ionization detection (GC-FID) and confirmed by gas chromatography with mass spectrometry (GC-MS).

Project 2: During sample collection, the mothers were instructed to completely empty the breast using a mechanical milk pump into disposable sterile tubes. From the total volume, 2 mL of milk was drawn using a sterile enteral syringe and transferred into microtubes. The human milk samples were immediately stored at -20 °C, then shipped on dry ice to the laboratory at the Department of Food Chemistry and Analysis in Budapest, Hungary, where they were stored at -80 °C until analysis. The preparation of FAME from these samples followed the ISO 16958:2015 standard (ISO/IDF, 2015) with minor modifications (M. Zhang et al., 2022). On the analysis day, frozen breast milk samples were brought to room temperature and thoroughly shaken to ensure even distribution. A 0.5 g portion

of breast milk was transferred into 50 mL centrifuge tubes, and 2.5 mL of tert-butyl methyl ether was added. Next, the samples underwent a transesterification step: 2.5 mL of 5% (m/v) methanolic sodium hydroxide solution was added, and the mixture was vortexed for 10 s using a LABINCO L46 Power Mixer. The tubes were then opened after 180 s, followed by the addition of 1 mL of isooctane. After 30 s, a neutralization solution (5 mL) containing 10% (w/v) disodium hydrogen citrate and 15% (w/v) sodium chloride was added. The samples were shaken carefully once more, and phase separation was achieved by centrifuging at 1750 rpm for 5 min using the Hettich Mikro 22R Refrigerated Centrifuge. Following the modified ISO standard protocol for sample preparation, no dilution was applied, which allowed for the detection of low-proportion FAMEs. The upper phase was carefully transferred to a gas chromatography vial, and the FAMEs were analyzed using GC-FID.

Project 3: Fresh breast milk from each mother was individually collected according to the center's standardized protocol. Each raw milk sample was aliquoted into 3–4 mL portions in microtubes using a sterile pipette, then labeled and immediately stored at -80 °C. To investigate the effect of Holder pasteurization on breast milk composition, our collaborating partners randomly selected and pooled 10 groups from the 56 donors. The pooled samples were subjected to Holder pasteurization (62.5 °C for 30 minutes) at the Unified Health Institution. The pasteurized groups were also divided into 3–4 mL aliquots and stored at -80 °C for further analysis. All procedures involving the 56 raw (unpasteurized) breast milk samples and 10 groups of pooled and pasteurized samples were carried out by our collaborative partners. For the analysis, we implemented the standardized sample preparation methodology described in **Project 2** (Vass et al., 2024) for both the 56 unpasteurized and 10 pasteurized breast milk samples collected from Hungarian mothers. The FAMEs were then analyzed using GC-FID.

4.2.2. Gas chromatography

For **Project 1**, fatty acid methyl ester analysis of human milk samples was conducted using GC-FID and confirmed by GC-MS. Two gas chromatographs were employed: a ThermoFinnigan Trace GC (Milan, Italy) equipped with an AS 2000 sampler, split/splitless injector, FID detector, and a BaseLine N2000 CDS Data system, and an HP 5890 Series II GC with a 7673 autosampler (Palo Alto, CA, USA), split/splitless injector, 5971 MSD, and HP ChemStation software package. Separation was conducted using an SP2340 column (30 m × 0.32 mm ID) with nitrogen gas as the mobile phase at 0.5 mL/min (Simon Sarkadi et al., 2022).

For **Projects 2 and 3**, fatty acid methyl ester analysis was analyzed using an Agilent 6890 GC-FID system (Agilent Technologies, Palo Alto, CA, USA) with an Agilent 7683 autosampler.

Separation was achieved using a Phenomenex Zebron ZB-FAME column (60 m, 0.25 mm, 0.20 μm) with a cyanopropyl stationary phase and hydrogen gas as the mobile phase at 1.2 mL/min (M. Zhang et al., 2022).

FAME identification was performed by comparing relative retention times with authentic FAME standards (FAME MIX c4-c24, Sigma Aldrich 18919-1AMP, and Supelco 37 component FAME Mix; Supelco, Bellefonte, PA, USA). Mass distribution was calculated electronically using peak area quantification with standard normalization. Data are expressed as a percentage of the peak area of total fatty acids. Data acquisition and processing were performed using GC-FID Mass Hunter software (MSD Chemstation F.01.03.2357 (1989–2015 Agilent Technologies Inc.) Palo Alto, CA, USA).

4.2.3. Statistical analysis

The statistical analysis of changes in the fatty acid composition of human milk was performed using IBM SPSS Statistics software (Version 23, IBM, Armonk, NY, USA). Initially, descriptive statistics were applied to the data to summarize the fatty acid profiles. Differences among the groups were assessed using one-way analysis of variance (ANOVA). When significant differences were found ($p < 0.05$), Tukey's post hoc test was employed to identify specific group differences.

To explore the underlying patterns and relationships within the data, Principal Component Analysis (PCA) with Kaiser Normalization was utilized. This analysis helped in visualizing the variation and clustering within the fatty acid compositions across different groups. For model validation, leave-one-out (LOO) cross-validation was implemented to ensure the robustness and reliability of the statistical models.

Furthermore, Quadratic Discriminant Analysis (QDA) was conducted to classify the samples based on their distinct fatty acid profiles. This statistical method was pivotal in identifying and confirming the unique groupings within the data based on the compositional differences.

PCA was performed using The Unscrambler X 10.4 software (CAMO, Oslo, Norway), which provided advanced tools for data visualization and interpretation, facilitating a deeper understanding of complex datasets. Meanwhile, the t-tests, ANOVA, and QDA were executed using SPSS software, offering a comprehensive and robust framework for detailed statistical analysis of the fatty acid data in human milk samples.

5. RESULTS AND DISCUSSION

5.1. Fatty acid composition of human milk samples from mothers with different health status and different geographical locations

The objective of our study was to evaluate the variations in the fatty acid profiles of breast milk among mothers with different health statuses, including those with normal weight, obesity, and/or gestational diabetes. Additionally, we aimed to compare breast milk samples from different geographical locations, specifically Ukraine and Hungary.

5.1.1. Fatty acid composition of human milk: impact of maternal BMI and health condition

In the first project, a total of 69 breast milk samples (60 from Ukrainian and 9 from Hungarian mothers) were analyzed to determine their fatty acid profiles. The samples were categorized into four groups based on maternal BMI and health status (as outlined in Chapter 4.1.2). All participating mothers were Caucasian, aged between 25 and 33 years, and had no chronic illnesses. Additional data, including mode of delivery, gestational age, neonate sex, and maternal education level, were also collected for analysis (Table 3).

Table 3. Data of participants

	Normal BMI	Normal BMI with GD	Obese	Obese with GD
Number of involved mothers	24	15	15	15
Maternal age (years)	27.1 ± 1.2	27.8 ± 1.3	26.3 ± 1.2	30.7 ± 1.8
Gestational age (weeks)	39.5 ± 0.6	38.2 ± 0.2	37.8 ± 0.2	39.6 ± 0.4
Maternal BMI at delivery	23.4 ± 0.3 [a]	23.1 ± 0.4 [a]	31.5 ± 0.6 [b]	32.5 ± 0.5 [c]
Gender of neonate				
Female	11	8	10 [c]	4 [d]
Male	13	7	5 [d]	11 [c]
Delivery				
Natural	16	8	14	11
Caesarean section	8	7	1	4

* BMI: body mass index; GD: gestational diabetes; values marked with different letters are significantly different from each other at $p \leq 0.05$ levels.

After analyzing the data, no significant differences were observed in maternal age and the gestational age across the four groups. However, there was a significant variation in maternal BMI between the groups. Specifically, the BMI of mothers in the obese group (31.5 ± 0.6) was considerably

higher than that of mothers in the normal BMI group (23.4 ± 0.3). This difference persisted both post-delivery and at the time of breast milk sample collection. Notably, among all the samples, obese mothers with GD exhibited the highest BMI (32.5 ± 0.5). Additionally, the data indicated that these mothers had a higher likelihood of giving birth to male infants (Table 3).

The fatty acid profile of 69 breast milk samples was analyzed using GC-FID, and the relative ratios of the compounds in the samples were evaluated. To enhance clarity, the fatty acids were grouped according to their chemical characteristics during data analysis. Our findings showed that the relative ratio of the total SFA content in the breast milk of Ukrainian mothers in the nBMI, nBMI+GD, O, and O+GD groups was 48.38%, 47.53%, 42.82%, and 43.24%, respectively (Table 4). Among the Ukrainian breast milk samples, palmitic acid (C16:0) was the most abundant SFA, followed by myristic acid (C14:0), lauric acid (C12:0), and stearic acid (C18:0).

In addition to the primary fatty acids, trace amounts of caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), arachidic acid (C20:0), and heneicosanoic acid (C21:0) were detected across all four groups. Notably, lignoceric acid (C24:0) was exclusively found in the nBMI and nBMI+GD groups. Furthermore, the lauric acid (C12:0) content in the O+GD group was significantly ($p < 0.05$) lower than in the other groups. Similarly, the myristic acid (C14:0) content in the normal BMI group was higher than in the obese group, and the palmitic acid (C16:0) content in the nBMI group was also lower compared to the other groups.

Table 4. Saturated fatty acid composition of breast milk samples from Ukraine

Fatty acid	Normal BMI (n=15) Mean ± SD (%)	Normal BMI + GD (n=15) Mean ± SD (%)	Obese (n=14) Mean ± SD (%)	Obese + GD (n=14) Mean ± SD (%)
C6:0	1.66 ± 1.71[a]	0.17± 0.42[b]	0.24 ± 0.46[c]	0.11 ± 0.27[d]
C8:0	0.22 ± 0.53	0.04± 0.09	0.01 ± 0.04	0.07 ± 0.11
C10:0	1.05 ± 0.62	0.84± 0.21	0.79 ± 0.22	0.78 ± 0.30
C12:0	5.75 ± 2.20[b]	5.25± 1.99[b]	5.04 ± 1.30[b]	3.57 ± 2.00[a]
C14:0	7.74 ± 2.54[b]	7.14± 3.44[b]	4.13 ± 3.82[a]	4.58 ± 4.70[a]
C16:0	25.80 ± 2.21[a]	28.64± 2.78[b]	27.85 ± 2.75[b]	29.09 ± 3.45[b]
C18:0	5.28 ± 0.55	5.31± 1.70	4.57 ± 1.66	4.86 ± 1.42
C20:0	0.13 ± 0.05	0.08± 0.05	0.17 ± 0.16	0.13 ± 0.09
C21:0	0.41 ± 1.04	0.01± 0.03	0.02 ± 0.03	0.05 ± 0.11
C24:0	0.33 ± 0.69	0.06± 0.13	nd	nd
∑SFA	48.37	47.53	42.82	43.24

(BMI: body mass index; GD: gestational diabetes; caproic acid (6:0); caprylic acid (8:0); capric acid (10:0); lauric acid (12:0); myristic acid (14:0); palmitic acid (16:0); stearic acid (C18:0); arachidic acid (C20:0); eneicosanoic acid (C21:0); lignoceric acid (C24:0); ∑SFA: total saturated fatty acids; values marked with different letters are significantly different from each other at $p \leq 0.05$ levels; n: number of samples; nd: not detected.)

Human milk contains SFAs as the most abundant fatty acid class, accounting for 34% to 47% of the total fatty acid content. Our findings align closely with previous study, which have reported SFA levels in the milk of European women to range between 42% and 47% (Giuffrida et al., 2022). The dominant fatty acids were palmitic acid (C16:0) (approximately 20%), followed by stearic acid (C18:0) (about 6%) and myristic acid (C14:0) (around 4%) (Floris et al., 2020; Giuffrida et al., 2022; Sánchez-Hernández et al., 2019). Significant reduction ($p < 0.05$) in the levels of lauric acid (C12:0) and myristic acid (C14:0) were observed in the milk samples from the O and O+GD groups, while the levels of MCFAs, such as capric acid (C10:0) was higher in the breast milk of the nBMI group. In conclusion, our results align with findings reported in other studies (Lopez-Lopez et al., 2002; Sánchez-Hernández et al., 2019).

A study demonstrated a positive correlation between palmitic acid (C16:0) levels and BMI. In obese individuals, there is an increase in de novo synthesis of palmitic acid (C16:0) as well as heightened lipoprotein lipase activity. This enzyme activity appears to preferentially hydrolyze and store SFA over mono- and polyunsaturated fatty acids in obese individuals. Consequently, the breast milk of obese mothers tends to have higher levels of palmitic acid (C16:0), reflecting these metabolic changes (Garaulet et al., 2011).

High maternal BMI is linked with inflammation and oxidative stress, affecting the composition of immune-related components in breast milk, which may influence the infant's immune system and long-term health (Fischer Fumeaux et al., 2019). Another study also highlighted that women with extremely high BMI had significant concentrations of insulin and leptin in their breast milk, suggesting a direct influence of maternal weight status on hormone levels in breast milk (Fields et al., 2017).

Gestational diabetes is recognized for its potential to delay lactogenesis and alter the chemical makeup of breast milk, impacting the nutritional status and development of the newborn (Hartmann and Cregan, 2001; Peila et al., 2020). It is well-established that the number of insulin receptors in the mammary gland increases during lactation, and insulin plays a crucial role in the physiological regulation of lipogenesis (Vernon, 1989). Insulin sensitivity impacts the activity of enzymes required for the synthesis of long chain fatty acids, such as elongases and desaturases. Saturated and very long chain fatty acids are indicative of biochemical abnormalities like GD, whereas palmitic acid (C16:0) is associated with markers of impaired insulin resistance and an elevated risk of GD.

A Korean study found a positive association between maternal intake of energy, carbohydrates, and cholesterol and milk SFA content (Hyesook Kim et al., 2017). A dietary survey of 61 Latvian women indicated a direct correlation between the consumption of milk and dairy products and the

SFA content in milk (Aumeistere et al., 2019). Interestingly, a moderate but significant positive correlation was noted between maternal meat intake and milk SFA levels (Deng et al., 2018). On the contrary, the intake of coffee and tea was inversely related to milk SFA concentration (Jagodic et al., 2020).

A decrease in medium chain fatty acids, highlighted by Bitman et al. (1989) suggests impaired fatty acid synthesis within the mammary gland, which may be accompanied by an increase in oleic acid (C18:1 n-9) and polyunsaturated fatty acid concentrations, indicative of fatty acid chain elongation processes. Additionally, Nasser et al. (2010) found that high carbohydrate intake in mothers could lead to an increased ratio of lauric acid (C12:0) and myristic acid (C14:0), as carbohydrates are converted into medium chain saturated fatty acids in human milk.

The clinical preference for medium chain fatty acids over long chain fatty acids is due to their rapid metabolism and unique transport mechanisms. They play an essential role in shaping the gut microbiome of newborns, significantly affecting neonatal physiology and immune function (Roopashree et al., 2021). This underscores the importance of maternal balanced dietary and metabolic health in optimizing the fatty acid profile of breast milk to support optimal neonatal development.

The analysis of monounsaturated fatty acids (MUFA) in our study that Ukrainian human milk samples revealed the presence of several key fatty acids: oleic acid (C18:1 n-9) ranged from 3% to 4%, palmitoleic acid (C16:1) from 2% to 3%, and eicosenoic acid (C20:1) was less than 0.4% (Figure 4). Additionally, traces of myristoleic acid (C14:1; <0.1%) were uniquely identified in samples from mothers with normal BMI who had gestational diabetes. The average content of MUFA across all samples was approximately 6%, with no statistically significant differences observed between the various groups.

Across all groups studied, the total MUFA content in breast milk was relatively consistent, with values of 6.39% in the O+GD mothers, 6.31% in obese mothers, 5.88% in nBMI mothers, and 5.87% in the nBMI+GD group with the exception of gondoic acid (C20:1), erucic acid (C22:1), and nervonic (C24:1) acid, which showed a decline over time (Nasser et al., 2010). Despite this, no significant differences in overall MUFA levels were observed among these groups.

Our findings are consistent with those of Sánchez-Hernández et al. (2019), who identified palmitoleic acid (C16:1), oleic acid (C18:1), and eicosenoic acid (C20:1) as the predominant MUFAs in human milk, comprising approximately 3–4% of the total fatty acid content. In our study, we observed similar patterns, with these MUFAs collectively making up a comparable proportion of the total fatty acids.

The MUFA concentrations observed in our study also align closely with recent findings from

European cohorts. Giuffrida et al. (2022) reported that oleic acid (C18:1) was present at approximately 2.4%, palmitoleic acid (C16:1) at 2.3%, and eicosenoic acid (C20:1) at around 0.4%, which are consistent with the values obtained in our analysis.

This uniformity in MUFA content across different maternal health conditions and BMIs suggest that MUFA levels in human milk may be less sensitive to changes in maternal health compared to other fatty acids. However, the observed decrease in specific MUFAs over time might indicate metabolic adjustments or shifts in dietary intake.

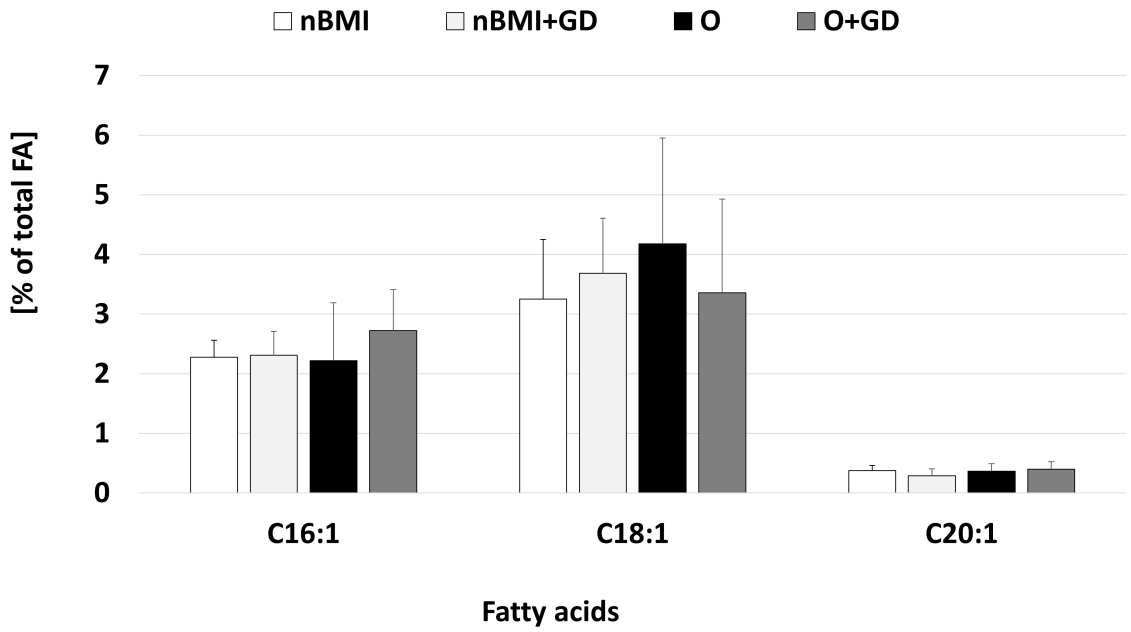

Figure 4. Monounsaturated fatty acids in breast milk samples from Ukrainian mothers
(Total number of samples: 58, 15 per group (nBMI, nBMI+GD), 14 per group (O, O+GD). FA—fatty acid; nBMI—women with normal BMI; nBMI+GD—women with normal BMI and GD; O—obese women; O+GD—obese women with GD; C16:1—palmitoleic acid; C18:1—oleic acid; C20:1—eicosenoic acid.)

Regarding the long-chain polyunsaturated fatty acid (LCPUFA) content of the samples, linoleic acid (LA, C18:2) and alpha-linolenic acid (ALA, C18:3) were the most abundant fatty acids within the LCPUFA group, comprising 23–28% and 15–17% of total fatty acids, respectively (Figure 5). The levels of LA in the milk from mothers with nBMI (23.8%) were significantly lower compared to the nBMI+GD (25.8%), O (26.8%), and O+GD (27.8%) groups.

The total amount of other LCPUFAs (including eicosadienoic acid (C20:2), nervonic acid (C24:2), mead acid (C24:3), arachidonic acid (C20:4), and docosahexaenoic acid (C22:6)) was less than 1.5% in all groups. Interestingly, dihomo-gamma-linolenic acid (DGLA, C20:3 n-6), though present at concentrations below 0.3%, was only detected in the nBMI and nBMI+GD samples.

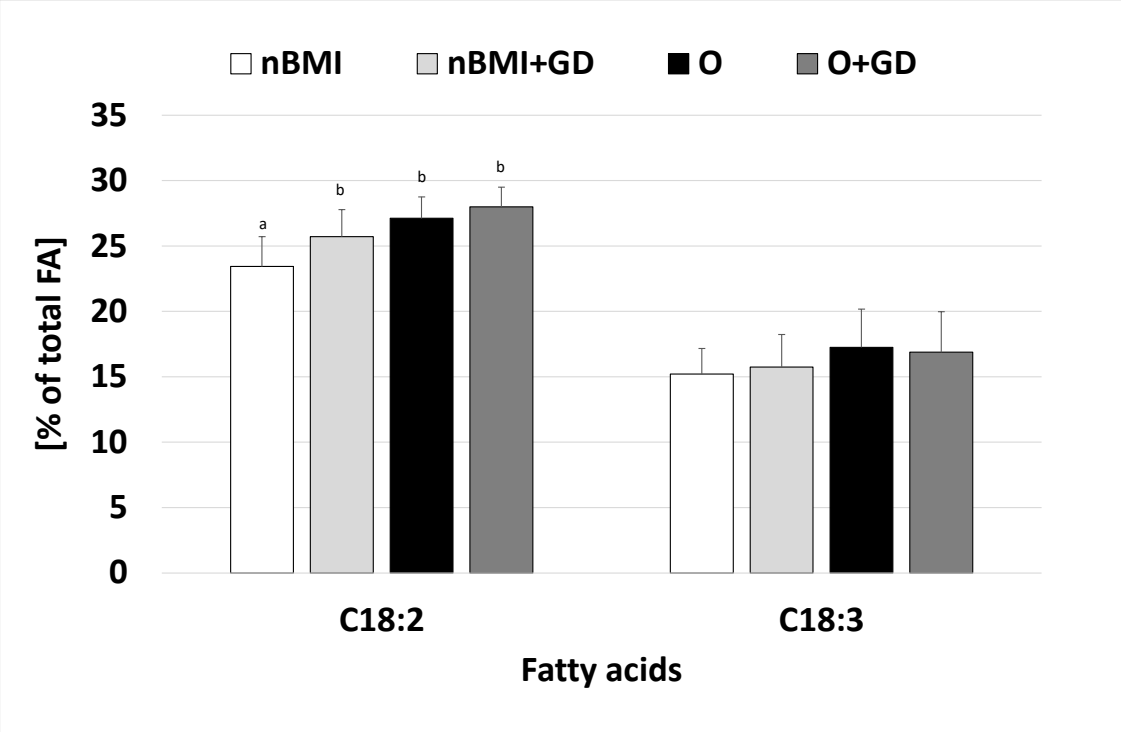

Figure 5. Long chain polyunsaturated fatty acids in breast milk samples from Ukrainian mothers (Total number of samples: 58, 15 per group (nBMI, nBMI+GD), 14 per group (O, O+GD). FA—fatty acid; nBMI—women with normal BMI; nBMI+GD—women with normal BMI and GD; O—obese women; O+GD—obese women with GD; C18:2—linoleic acid; C18:3—alpha-linolenic acid; different letters indicate significant differences at the $p \leq 0.05$ levels.)

Our analysis identified differences in the ratio of unsaturated fatty acids to saturated fatty acids in breast milk samples from mothers with normal BMI compared to those who are classified as obese. Specifically, the unsaturated fatty acids to SFA ratio in breast milk from mothers with a normal BMI was 0.8, whereas in samples from obese mothers, the ratio was 1.1. These findings indicate that obese mothers produce a higher proportion of unsaturated fatty acids in their breast milk.

Long-chain polyunsaturated fatty acid play critical structural and regulatory roles in the body. Notably, n-3 fatty acids are known to promote normal mitochondrial function and mitigate excitotoxicity, which is significant in the context of central nervous system disorders (Bromfield et al., 2008; Garbagnati et al., 2009). DHA and ARA have also been shown to reduce IL-1 induced inflammatory responses in both fetal and adult intestinal epithelial cells, highlighting the potential role of LCPUFAs in maintaining intestinal barrier function and their protective effects against inflammatory bowel diseases (Whiting et al., 2005; Wijendran et al., 2015). Moreover, LCPUFAs are crucial for the development and functioning of the immune system (Harbige, 2003; Laitinen et al., 2006).

In human milk, LCPUFAs such as DHA have been observed to decrease across both preterm and term stages, whereas GLA tends to increase during the transitional and mature phases. LA, EPA and ALA generally remain stable throughout lactation (Floris et al., 2020). Interestingly, some studies have reported elevated levels of LA in obese women compared to non-obese counterparts. LA is converted AA through a series of enzymatic processes, including delta-6 desaturation, elongation, and delta-5 desaturation (Isesele et al., 2022; Sprecher, 2000). Studies have identified a link between increased delta-6 desaturase activity and obesity (Saito et al., 2013; Wolters et al., 2015). This heightened enzymatic activity may lead to a greater conversion of LA to AA, which can influence the composition of fatty acids in the body and may play a role in inflammatory processes. It is noteworthy given that approximately 30% of the LA in breast milk is derived directly from the diet, reflecting dietary intake and plasma levels, while the remainder comes from maternal adipose tissue (Aumeistere et al., 2021; Giuffrida et al., 2022; Isesele et al., 2022).

In our study, the LA content in breast milk samples from mothers with normal BMI (nBMI) was significantly lower at 23.8% compared to 25.8% in nBMI+GD, 26.8% in the O group, and 27.8% in the O+GD group. These findings align with those of Isesele et al. (2022), who also found no significant differences in ARA levels between the normal BMI and obese groups, indicating that maternal obesity does not significantly influence ARA concentrations. However, a minor amount of GLA metabolite (<0.3%) was detected in both nBMI and nBMI+GD breast milk samples.

Dietary contributions account for approximately 65% of the ALA content in breast milk (Demmelmair et al., 2016), with recent reviews highlighting a strong association between maternal fish intake and higher levels of ALA, DHA, and EPA in human milk. Conversely, SFA intake is inversely related to these beneficial fatty acids (Francois et al., 2003; Mazurier et al., 2017; Petersohn et al., 2024). These findings emphasize the importance of dietary influences on the composition of breast milk and underscore the potential for targeted dietary interventions to optimize essential fatty acid profiles for infant health.

In summary, our findings align with previous literature that highlights the variations in human milk FA profiles in relation to maternal health status (Lopez-Lopez et al., 2002; Sánchez-Hernández et al., 2019). The discrepancies observed across studies may be attributed to differences in maternal dietary patterns, sampling conditions, and methodological approaches (de la Garza Puentes et al., 2019).

5.1.2. Statistical evaluation of fatty acid composition of Ukrainian breast milk samples

The objective of our study was to determine whether Ukrainian breast milk samples could be classified according to their fatty acid profiles using statistical methods. To achieve this, we employed supervised discriminant analysis (Figure 6). The analysis demonstrated distinct segregation of samples into two groups based on BMI, but based on presence or absence of GD, clear differentiation was not achievable with this dataset. Our findings indicate that 58.6% of the samples were accurately classified into four categories during cross-validation: women with normal BMI, women with normal BMI and GD, obese women, and obese women with GD. Canonical function 1 predominantly differentiated samples based on maternal BMI, with palmitic acid (C16:0), oleic acid (C18:1) and gamma-linolenic acid (C18:3) making the most significant contributions of the obese group. Conversely, stearic acid (C18:0) and eicosatrienoic acid (C20:3) appeared to influence sample positioning towards the negative range. As seen in Figure 6, Canonical function 2 seemed to facilitate the segregation of nBMI and nBMI+GD mothers, with fatty acids such as caproic acid (C6:0), eicosadienoic acid (C20:2), heneicosanoic acid (C21:0), and lignoceric acid (C24:0) displaying the highest discriminant coefficients.

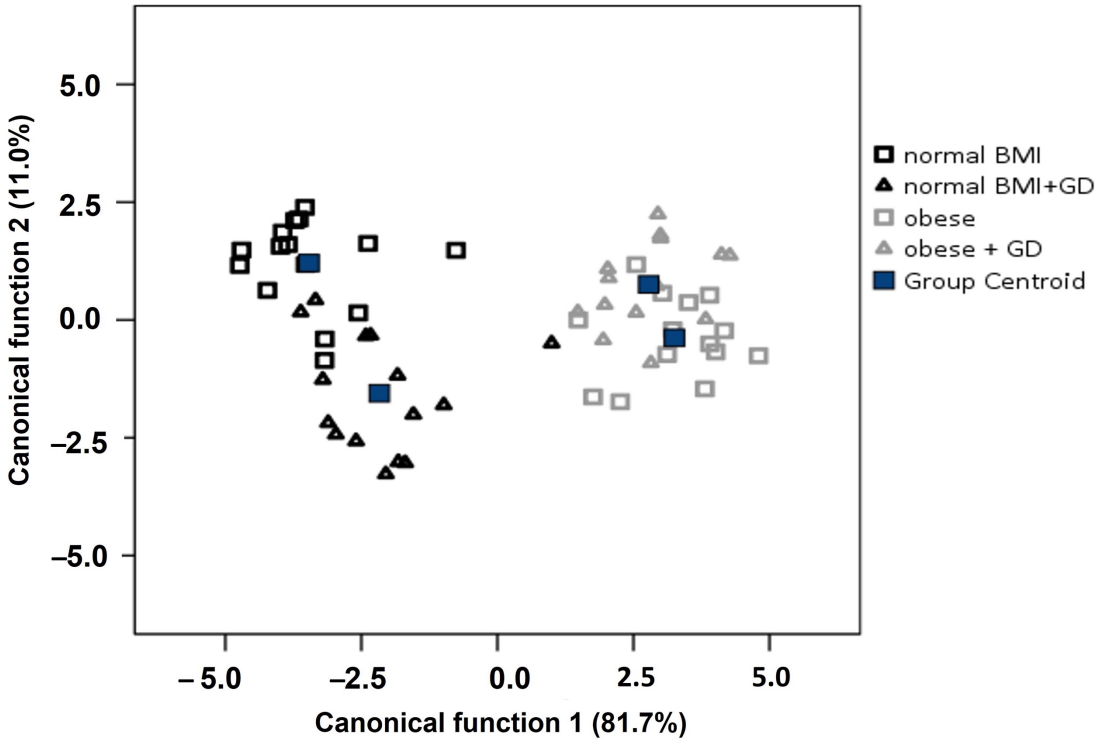

Figure 6. Discriminant analysis of Ukrainian breast milk samples

Based on the results of discriminant analysis conducted on four groups, the analysis was also performed on two groups (Table 5), categorized solely by the mothers' BMI, the model's accuracy post-cross-validation reached 89.8%. Of the misclassified samples, five out of six belonged to the normal BMI group, supporting the efficacy of fatty acid profiles in distinguishing between normal and obese breast milk samples.

Table 5. Results of discriminant analysis of two groups based on the BMI of mothers

			Predicted Group Membership	
			Normal BMI	**Obese**
Original	Count	Normal BMI	29	1
		Obese	0	28
	%	Normal BMI	96.7	3.3
		Obese	0	100
Cross-validated	Count	Normal BMI	25	5
		Obese	1	27
	%	Normal BMI	83.3	16.7
		Obese	3.6	96.4

Original: results of calibration; cross-validated: results after leave-one-out cross-validation.

5.1.3. Variations in fatty acid profiles in human milk based on geographical location

We compared breast milk samples from mothers with normal BMI in Ukraine and Hungary to explore differences in the fatty acid composition based on nationality.

In our study, we found that the average SFA and unsaturated fatty acid percentages in breast milk from normal BMI Ukrainian women (48.37% and 40.30%, respectively) and Hungarian women (53.90% and 38.31%, respectively) were slightly above the typical European range. The composition of HM varies among women of different nationalities and is influenced by the dietary habits specific to each region (Miliku et al., 2019). Extensive literature indicates maternal diet as a primary determinant of breast milk composition (Samuel et al., 2020). The SFA content in breast milk is closely associated with maternal consumption of carbohydrates, fats, total energy, and the mobilization of adipose tissue. High dietary intake of SFAs is typically linked to increased total fat consumption. In Europe, significant sources of SFAs include dairy products, added fats such as butter, lard, and tallow, and meat products, all contributing substantially to the SFA content in breast milk (Harika et al., 2013).

Descriptive statistics revealed significant differences between the Hungarian (H) and Ukrainian (U) samples in the concentrations of several fatty acids. Notably, caproic acid (C6:0) levels were

significantly lower in Hungarian samples (0.02 ± 0.01%) compared to Ukrainian samples (1.66 ± 1.27%). Similarly, palmitoleic acid (C16:1) levels were higher in Hungarian samples (2.91 ± 0.67%) compared to Ukrainian samples (2.26 ± 0.28%), as were palmitic acid (C16:0) (H: 32.44 ± 3.94% vs. U: 25.80 ± 2.35%) and linoleic acid (C18:2) (H: 28.44 ± 2.36% vs. U: 23.43 ± 2.28%). Conversely, alpha-linolenic acid (C18:3) was more prevalent in Ukrainian samples (15.20 ± 1.97%) than in Hungarian samples (9.33 ± 2.43%) (Figure 7).

These differences indicate that Hungarian breast milk samples tend to have higher levels of palmitic acid (C16:0), palmitoleic acid (C16:1), and linoleic acid (C18:2), whereas Ukrainian samples are richer in alpha-linolenic acid (C18:3). In terms of overall fatty acid composition, total levels of SFAs were higher in the Hungarian samples (54 ± 11%) compared to Ukrainian samples (49 ± 11%). Similarly, MUFA levels were slightly higher in Hungarian samples (6.4 ± 1.5%) compared to Ukrainian samples (5.9 ± 1.4%). On the other hand, PUFAs were found to be more prevalent in Ukrainian samples (40 ± 5%) compared to Hungarian samples (38 ± 5%). Interestingly, dihomo-gamma-linolenic acid (C20:3 n-6), a fatty acid with potential anti-inflammatory properties, was detected exclusively in Ukrainian nBMI samples, indicating a possible influence of maternal health status on its presence.

In a related study examining the impact of maternal nationality on breast milk fatty acid profiles, it was found that colostrum from Greek mothers had lower levels of SFA and a lower n-6 to n-3 ratio (Sinanoglou et al., 2017), while containing higher concentrations of oleic acid (C18:1), arachidic acid (C20:0), and MUFA compared to mothers from other nationalities. Specifically, Greek mothers' colostrum contained SFA and MUFA constituting 46.27% and 40.33% of total fatty acids, respectively, which falls within the European range (37.24% to 46.88% for SFA and 39.11% to 45.19% for MUFA) (Fidler and Koletzko, 2000).

Dietary patterns rich in dairy products or non-hydrogenated fats tend to increase palmitic acid (C16:0) levels in human milk, while a higher PUFAs to SFAs ratio in meals correlates with reduced palmitic acid (C16:0) concentrations (Bravi et al., 2016). Protein intake is positively associated with palmitic acid (C16:0) levels in milk, yet it demonstrates a negative correlation with carbohydrate intake (Deng et al., 2018). Notably, the linoleic acid (C18:2) content in Hungarian maternal milk is higher (28.44%) compared to Ukrainian milk (23.43%), possibly due to the prevalent use of sunflower oil in Hungary, which is rich in linoleic acid (C18:2) (Premnath et al., 2016).

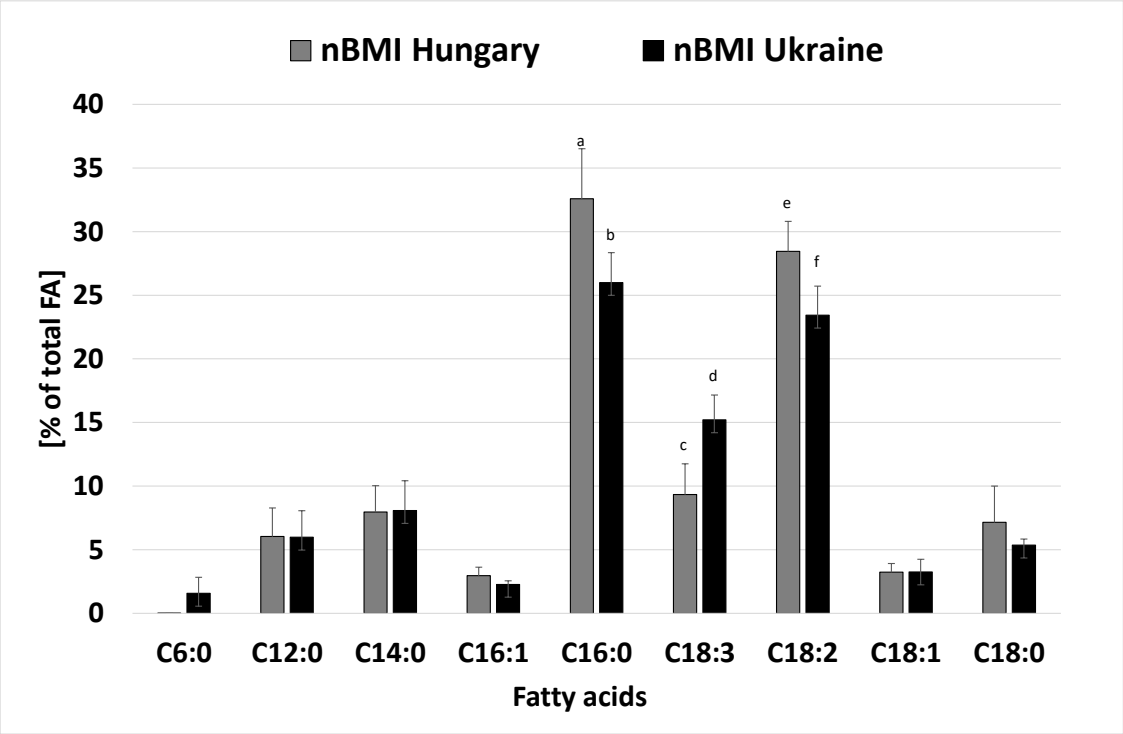

Figure 7. Main fatty acids in breast milk of nBMI women from Ukraine and Hungary
(FA—fatty acid; nBMI—women with normal BMI; C6:0—caproic acid; C12:0—lauric acid; C14:0—myristic acid; C16:0—palmitic acid; C18:0—stearic acid; C16:1—palmitoleic acid; C18:1—oleic acid; C18:2—linoleic acid; C18:3—alpha-linolenic acid; different letters indicate significant differences at the $p \leq 0.05$ levels.)

The levels of DHA in breast milk are predominantly influenced by maternal dietary intake, as endogenous production of DHA within the mammary gland is limited. Consumption of fish and DHA-rich supplements significantly increases DHA levels in breast milk (Sherry et al., 2015). Higher levels of DHA in Ukrainian breast milk samples compared to Hungarian samples may be due to more accessible and frequent fish consumption in Ukraine, as fish directly provides DHA and EPA rather than relying on endogenous conversion from alpha-linolenic acid (C18:3) (Burhaz and Matviienko, 2023; Liu et al., 2016).

In Hungary, fatty fish intake is limited, primarily occurring during Christmas, and mainly consists of freshwater species low in n-3 LCPUFA. Furthermore, the prevalence of DHA supplementation among lactating mothers is low, which may contribute to the lower DHA levels observed in Hungarian breast milk. Studies have shown that EPA and DHA levels in breast milk can vary significantly depending on dietary fish oil supplementation during pregnancy and lactation. Mothers who did not supplement displayed lower EPA and DHA concentrations compared to those who did supplement (0.07% and 0.16% versus 0.10% and 0.23% during pregnancy; 0.13% and 0.27% during lactation) (Mihályi et al., 2015; Miliku et al., 2019).

Our analysis revealed that there were notable variations in the fatty acid profiles between the two countries, which may be attributed to different dietary habits of mothers from different countries or regions.

5.1.4. Statistical analysis of breast milk samples from Ukraine and Hungary

The fatty acid profiles of nBMI breast milk samples from Ukraine and Hungary were statistically analyzed using Principal Component Analysis (PCA). PCA can extract and visualize the most informative features from large datasets, it can easily identify trends, patterns, or outliers while preserving the most relevant information from the initial dataset.

The analysis explained 79% of the dataset's total variance with PC1 (59%) and PC2 (20%) (Figure 8A) where PC1 was primarily responsible for differentiating samples based on geographic origin. Significant differences in the levels of palmitic acid (C16:0), linoleic acid (C18:2), and alpha-linolenic acid (C18:3) between the two groups were confirmed by t-tests, highlighting their influence on classification. The roles of eicosadienoic acid (C20:2) and eicosatrienoic acid (C20:3) were particularly notable in Ukrainian samples. According to the loading plot (Figure 8B), the distribution of samples along PC2 was most influenced by the levels of capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), and linoleic acid (C18:2).

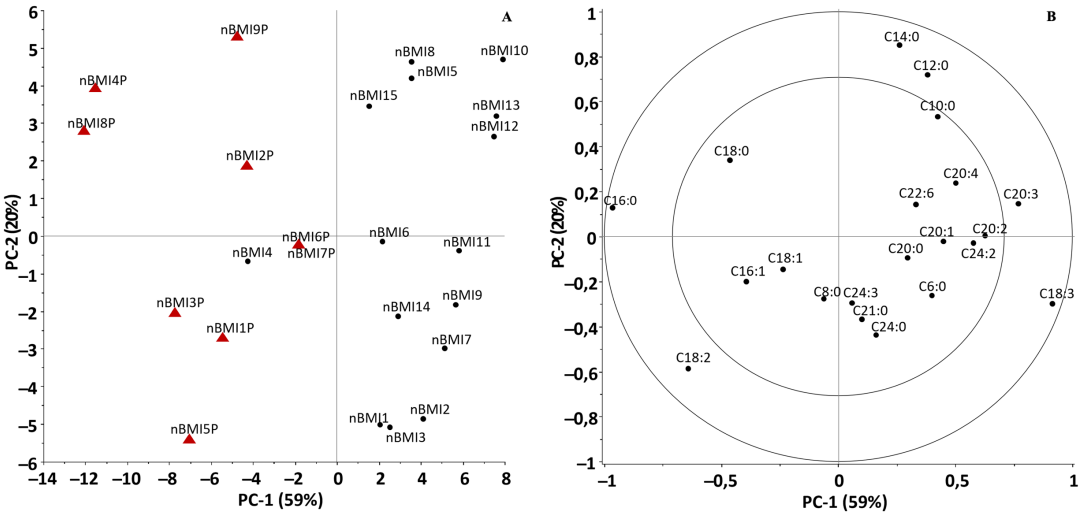

Figure 8. (A) Scores plot of principal component analysis, (B) Principal component loading of the fatty acid profile of breast milk samples from women with normal BMI in Hungary and Ukraine
(Red triangles: Hungary; black dots: Ukraine. C6:0, caproic acid; C8:0, caprylic acid; C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C20:0, arachidic acid; C21:0, heneicosylic acid; C24:0, lignoceric acid; C16:1, palmitoleic acid; C18:1, oleic acid; C20:1, eicosenoic acid; C18:2, linoleic acid; C20:2, eicosadienoic acid; C18:3, alpha-linolenic acid; C20:3, dihomo-gamma-linolenic acid; C20:4, arachidonic acid; C22:6, docosahexaenoic acid. Total number of samples was 24.)

One of the primary limitations of our study was the lack of comprehensive data on the dietary practices of the pregnant and lactating women who participated. This information is critical, as recent studies have demonstrated the substantial influence of maternal nutrition during pregnancy on fetal metabolism and overall neonatal health. Understanding the specific dietary intakes of these mothers would have provided valuable context for interpreting the observed variations in breast milk composition. Without detailed dietary information, our ability to fully explain the differences in nutrient profiles among the breast milk samples was limited. Future studies should aim to collect more thorough dietary data from participants to enable a deeper understanding of how maternal diet impacts breast milk composition.

5.2. Fatty acid composition of human milk in Hungarian mothers with varying BMI levels

In this study, we conducted an in-depth analysis of the fatty acid composition of HM among Hungarian mothers with different BMI levels. The objective was to investigate how maternal BMI influences the nutritional profile of HM, with a particular focus on the types and proportions of fatty acids present.

5.2.1. Fatty acid composition of human milk: impact of maternal BMI

In this study, 17 breast milk samples were collected from mothers at the Department of Obstetrics and Gynaecology of the University of Pécs (Hungary) during the 10^{th} to 12^{th} week postpartum. The samples were categorized into two groups based on the mothers' BMI: normal weight (n=8, BMI: 18.5–25.0) and overweight or obese (n=9, BMI: >25.0) (refer to Section 4.1.2).

Following sample preparation, the fatty acid content was analyzed using GC-FID. A total of 20 distinct fatty acids were identified in the breast milk samples. Our analysis confirmed the presence of all key fatty acids, including palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2), across all samples (Figure 9).

Figure 9 presents typical GC-FID chromatograms: Figure 9A illustrates the fatty acid profile of a breast milk sample from a mother with a normal BMI, while Figure 9B shows the profile from a mother with obesity. The retention times observed in both chromatograms are consistent, indicating reliable measurement conditions. Additionally, the peak shapes across both profiles demonstrate high quality, thereby confirming the reproducibility of the results and the robustness of the analysis method.

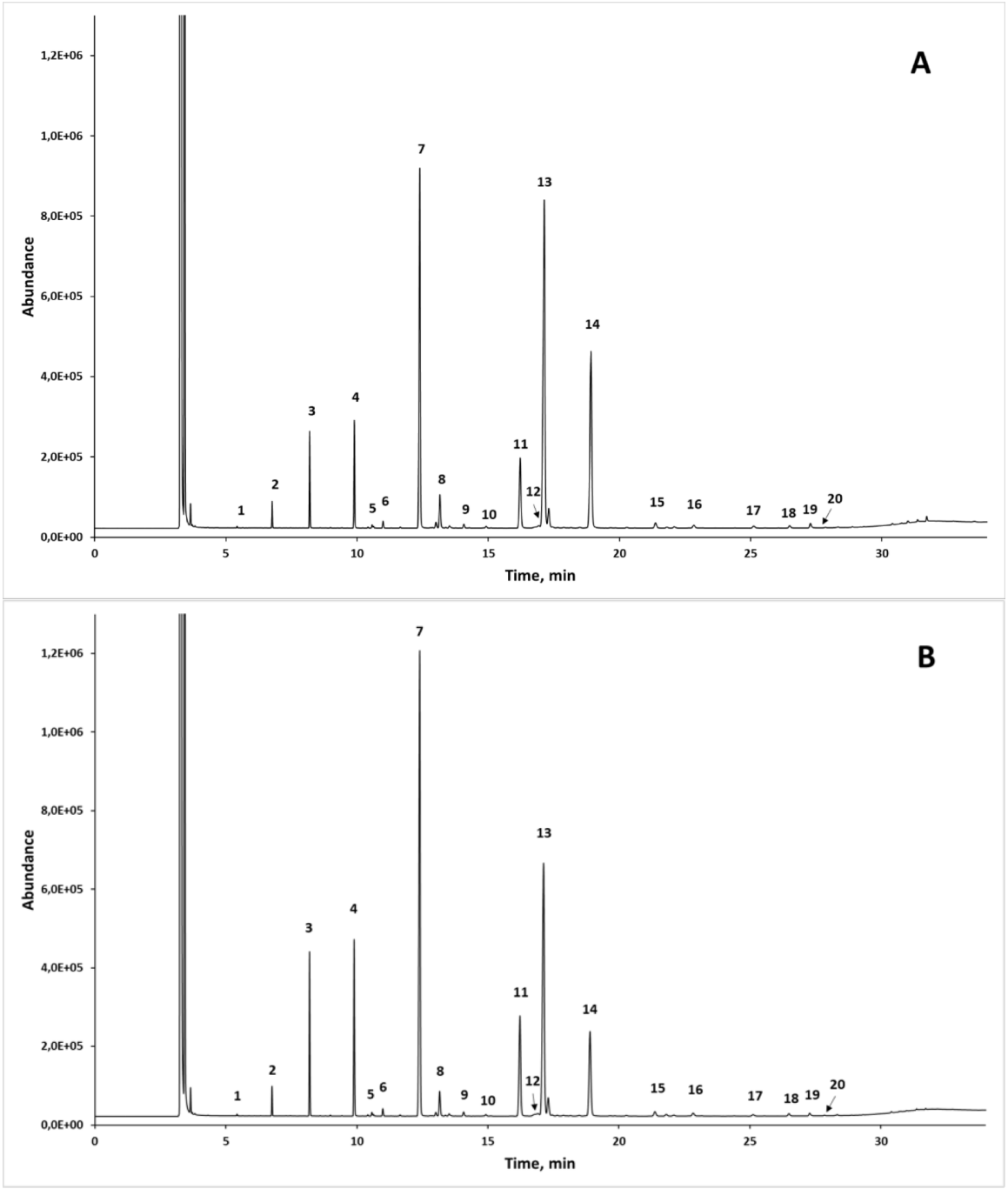

Figure 9. GC-FID chromatograms of the fatty acid composition of the selected human milk sample from a mother with normal BMI (A) and a mother with obesity (B)

(1: C8:0 – caprylic acid; 2: C10:0 – capric acid; 3: C12:0 – lauric acid; 4: C14:0 – myristic acid; 5: C14:1 – myristoleic acid; 6: C15:0 – pentadecanoic acid; 7: C16:0 – palmitic acid; 8: C16:1 – palmitoleic acid; 9: C17:0 – heptadecanoic acid; 10: C17:1 – heptadecenoic acid; 11: C18:0 – stearic acid; 12: C18:1 tr (n9) – oleic acid; 13: C18:1 cis (n9) – oleic acid; 14: C18:2 – linoleic acid; 15: C18:3 – alpha-linolenic acid; 16: C20:1- eicosenoic acid; 17: C20:2 – eicosadienoic acid; 18: C20:3 – dihomo-gamma-linolenic acid; 19: C20:4 – arachidonic acid; 20: C22:0 – behenic acid.)

As previously noted, the adjustment to the ISO standard sample preparation allowed for the detection of minor fatty acid compounds (Figure 10). Although these compounds are present in concentrations of less than 1%, they play a critical role in the subsequent discriminant analysis.

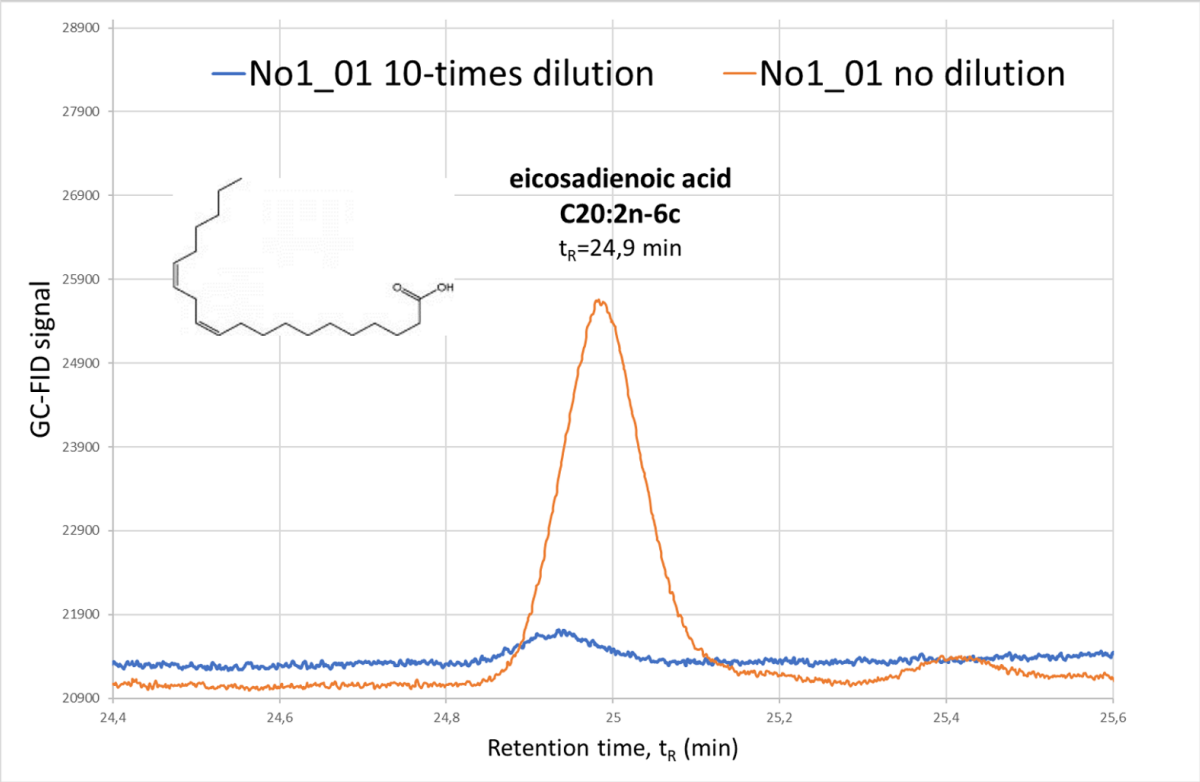

Figure 10. Comparison of chromatographic peaks of eicosadienoic acid between 10-fold dilution and non-dilution methods
(No1_01 code represents the sample name)

Saturated fatty acids were the most prevalent, with higher concentrations observed in the milk from obese mothers (54.21%) compared to those with normal BMI (48.33%). Monounsaturated fatty acids followed, with similar levels in both the normal BMI (33.35%) and obese groups (33.52%). Polyunsaturated fatty acids were less abundant, with the normal BMI group having 15.76% and the overweight or obese group showing a lower percentage at 11.50%. It was observed that the most abundant fatty acids, including oleic acid (C18:1 n-9), palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0), and lauric acid (C12:0) were higher in the obese samples, except for linoleic acid (C18:2 n-6), which showed a lower concentration (Table 6).

Our findings indicated that the milk from overweight and obese mothers contains higher levels of SFAs and lower levels of PUFAs compared to the milk of mothers with a normal BMI. These results are consistent with those of Barreiro et al. (2018), who reported similar differences in fatty acid composition linked to maternal obesity. We observed a relationship between high maternal BMI and lower proportions of n-3 PUFAs, such as α-linolenic acid (C18:3 n-3), in the breast milk of overweight and obese mothers. Additionally, these mothers exhibited lower levels of arachidonic acid (C20:4 n-6) and a higher n-6 to n-3 PUFA ratio. These findings are consistent with those reported by

Hua et al. (2024) and will be discussed in more detail in the next chapter.

Table 6. Fatty acid composition of Hungarian breast milk samples from mothers with different health statuses

		Normal BMI (n=8)		Overweight and obese (n=9)	
		Mean (%)	SD	Mean (%)	SD
Fatty acid		23.32[a]	1.8	27.34[b]	3.63
C8:0	Caprylic acid	0.03	0.07	0.02	0.04
C10:0	Capric acid	0.96[a]	0.42	1.13[b]	0.21
C12:0	Lauric acid	4.57[a]	1.35	5.59[a]	1.33
C14:0	Myristic acid	6.55[a]	2.22	7.75[a]	2.15
C14:1n-5c	Myristoleic acid	0.11	0.08	0.08	0.1
C15:0	Pentadecanoic acid	0.35	0.09	0.33	0.23
C16:0	Palmitic acid	28.48[a]	4.27	31.06 [a]	3.73
C16:1n-7c	Palmitoleic acid	2.25[a]	0.37	1.98[a]	0.55
C17:0	Margaric acid	0.31	0.05	0.19	0.19
C17:1n-7c	Heptadecenoic acid	0.08	0.11	0.02	0.06
C18:0	Stearic acid	7.06[a]	0.63	8.14[a]	1.47
C18:1n-9t	Elaidic acid	n.d.	n.d.	0.14	0.29
C18:1n-9c	Oleic acid	30.64[a]	3.93	31.21[a]	4.8
C18:2n-6c	Linoleic acid	14.59[a]	4.06	11.13[a]	2.46
C18:3n-3c	α-Linolenic acid	0.45[a]	0.33	0.13[b]	0.22
C20:1n-9c	Eicosenoic acid	0.28	0.18	0.09	0.14
C20:2n-6c	Eicosadienoic acid	0.15	0.16	n.d.	n.d.
C20:3n-6c	Dihomo-gamma-linolenic acid	0.25	0.12	0.06	0.11
C20:4n-6c	Arachidonic acid	0.33	0.2	0.18	0.19
C22:0	Behenic acid	0.02	0.04	n.d.	n.d.
∑SFA	Sum of saturated fatty acids	48.33[a]	6.92	54.21[a]	5.51
∑MUFA	Sum of monounsaturated fatty acids	33.35[a]	3.92	33.52[a]	4.77
∑PUFA	Sum of polyunsaturated fatty acids	15.76[a]	4.59	11.50[a]	2.29

SD: standard deviation; BMI: body mass index; n: number of samples; n.d.: not detected; values marked with different letters are significantly different from each other at $p \leq 0.05$ levels.

Evidence suggests that obese women tend to consume more foods rich in n-6 PUFAs and fewer sources of n-3 PUFAs. The typical Western diet, which often has a high n-6 to n-3 PUFA ratio, is known to exacerbate inflammation (Patterson et al., 2012). This imbalance in fatty acids in human milk may contribute to accelerated weight gain in infants (Enstad et al., 2021). In contrast, diets like the Mediterranean, which contain higher amounts of n-3 PUFAs, are known for their anti-inflammatory properties (Scoditti et al., 2014).

The fatty acid composition of breast milk from mothers with nBMI in our study exhibited a relatively high SFA content (48.34%) compared to recent European cohorts, which reported ranges of 41.09% to 41.93%. However, this SFA content was similar to that observed in Korean mothers (48.1%). The MUFA content (33.35%) in our study closely matched that of Greek women (35%), with oleic acid (C18:1 n-9) being the predominant MUFA, consistent with findings from other European populations (34–36%). The PUFA content (15.76%) was lower than that observed in other European countries (21.5% and 18.6%) but was comparable to European averages (16.30%–16.53%) (Giuffrida et al., 2022; Hyesook Kim et al., 2017).

Notably, the SFA levels in the breast milk of overweight and obese Hungarian mothers (54.21%) were higher than those reported for obese mothers in Sweden (47.50%) and Spain (27.80%) (de la Garza Puentes et al., 2019; Lindholm et al., 2013).

The primary fatty acids identified in the milk samples were oleic acid (C18:1 n-9; nBMI: 30.64%, O: 31.21%) and palmitic acid (C16:0; nBMI: 28.48%, O: 31.06%). These findings are consistent with prior research, which also identifies oleic acid (C18:1 n-9) and palmitic acid (C16:0) as the predominant fatty acids in human milk (Giuffrida et al., 2022, 2016; Samuel et al., 2019; Thakkar et al., 2013; Wu et al., 2010).

The column diagram (Figure 11) illustrates the distribution of FAs that were significantly different in the FAME profiles between samples from normal-weight and obese mothers. Dark bars represent the nBMI group (normal BMI), grey bars correspond to the O group (obese). Despite the presence of standard deviations, statistically significant differences were observed for the FAs. The results indicate that most fatty acids with significant differences exhibited higher percentage ratios in the O group, except for palmitoleic acid (C16:1) and linoleic acid (C18:2), which were higher in the nBMI group. Although alpha-linolenic acid (C18:3) is not displayed in the figure due to its low percentage, it was also observed to be higher in the nBMI group.

The levels of C16:0 (31.06%) and C18:0 (8.14%) in the breast milk of overweight and obese Hungarian mothers were slightly higher than those observed in obese mothers from Sweden (C16:0: 25.90%; C18:0: 5.90%) and Spain (C16:0: 21.20%; C18:0: 6.03%) (de la Garza Puentes et al., 2019; Lindholm et al., 2013). The elevated palmitic acid (C16:0) levels may reflect higher overall dietary intake of SFAs, which are associated with weight gain in mothers (Jenkins et al., 2015; Panagos et al., 2016; Petersen et al., 2019; Pfeuffer and Jaudszus, 2016; Vidakovic et al., 2016). The increased palmitic acid (C16:0) in Hungarian mothers' milk could also be linked to their consumption of animal products, fast food, processed foods, and high-fat dairy products, which are significant sources of SFAs (Lindholm et al., 2013; Mihályi et al., 2015).

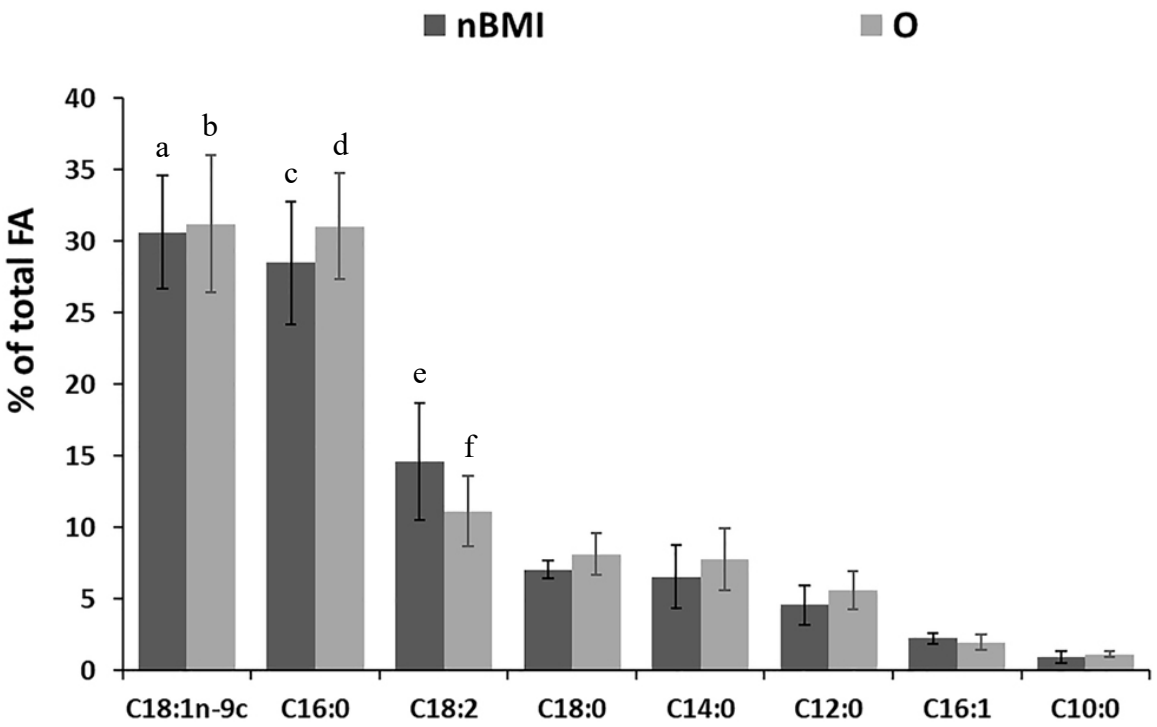

Figure 11. Distribution of significantly different fatty acids in breast milk samples from mothers with different health statuses

(FA: fatty acid; nBMI: women with normal body mass index (BMI); O: overweight and obese women; different letters indicate significant differences at the $p \leq 0.05$ levels.)

5.2.1.1. Level of n-3 and n-6 fatty acid in human milk from mothers with different BMI

In our detailed analysis of the measurement results, we focused on the composition of PUFAs, specifically within the n-3 and n-6 families. The following n-3 PUFAs were identified: alpha-linolenic acid (C18:3 n-3c), eicosatrienoic acid (C20:3 n-3c), eicosapentaenoic acid (C20:5 n-3c), and docosahexaenoic acid (C22:6 n-3c). The n-6 PUFA group included linoleic acid (C18:2 n-6t), gamma-linolenic acid (C18:3 n-6c), eicosadienoic acid (C20:2 n-6c), dihomo-gamma-linolenic acid (C20:3 n-6c), arachidonic acid (C20:4 n-6c), and docosadienoic acid (C22:2 n-6c).

When comparing the PUFA profiles of breast milk from mothers with normal BMI to those who were overweight and obese, we found that the n-6 to n-3 ratio was 53% higher in milk from the O group (Figure 12). Furthermore, the n-3 PUFA content in milk from O group mothers was lower, with alpha-linolenic acid (C18:3 n-3) reduced to 0.1%, compared to 0.4% in the nBMI group. This elevated n-6 to n-3 ratio aligns with findings from similar studies in Sweden (Lindholm et al., 2013) and Spain (de la Garza Puentes et al., 2019), both of which reported lower levels of n-3 PUFAs in breast milk from overweight or obese mothers. These results are consistent with those reported by Chamorro et al., (2022), highlighting the impact of maternal BMI on breast milk fatty acid composition, potentially

influencing the nutritional quality available to the infant.

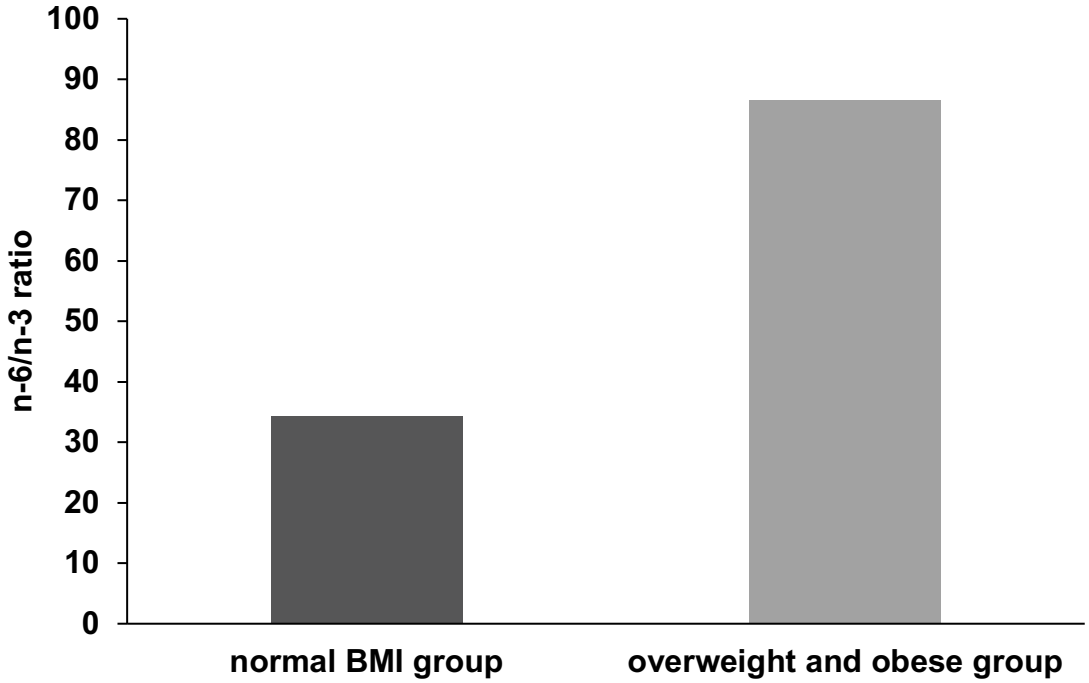

Figure 12. n-6/n-3 ratio in breast milk from mothers with different health statuses

In our results, the n-6 to n-3 ratio observed in the O group was significantly higher than the levels reported in other studies (Miliku et al., 2019; Thakkar et al., 2019). Previous literature has suggested that a higher maternal BMI is associated not only with an increased n-6 to n-3 PUFA ratio but also with elevated levels of leptin and insulin in breast milk (Hashemi Javaheri et al., 2024). Obesity is widely recognized as a condition characterized by chronic, low-grade inflammation, heavily influenced by dietary habits and nutritional intake (Shivappa et al., 2014). This imbalance in the n-6 to n-3 PUFA ratio, combined with a pro-inflammatory lipid profile, may contribute to greater adipose tissue deposition in infants breastfed by obese mothers, potentially affecting early metabolic programming (Sinanoglou et al., 2017).

The prevalence of high n-6 fatty acids in Western diets is often attributed to modern agricultural practices, the expansion of agribusiness, increased availability of processed foods, and widespread production of hydrogenated and refined vegetable oils as well as animal fats. Collectively, these factors influence dietary patterns that impact breast milk composition (Bhardwaj et al., 2016; Chaves et al., 2019; Simopoulos, 2016, 2011).

These findings emphasize the significant impact of maternal health and nutritional intake on breast milk composition and highlight the potential long-term effects on infant health. This

underscores the necessity for dietary guidelines aimed at optimizing fatty acid intake in expectant and nursing mothers to improve health outcomes for both mothers and their offspring.

5.2.2. Statistical evaluation of fatty acid composition in human milk from mothers with different BMI

The fatty acid composition of breast milk samples was statistically analyzed using PCA to identify hidden patterns within the data. The dataset was mean-centered, and full cross-validation was utilized to assess the model's parameters (Figure 13). The analysis revealed that the first three principal components accounted for a significant portion (95%) of the total variance: PC1 explained 69%, PC2 accounted for 14%, and PC3 for 12%. Residual variances for PC1 through PC3 during calibration and validation phases were recorded as 0.85/1.14, 0.46/0.99, and 0.14/0.29, respectively.

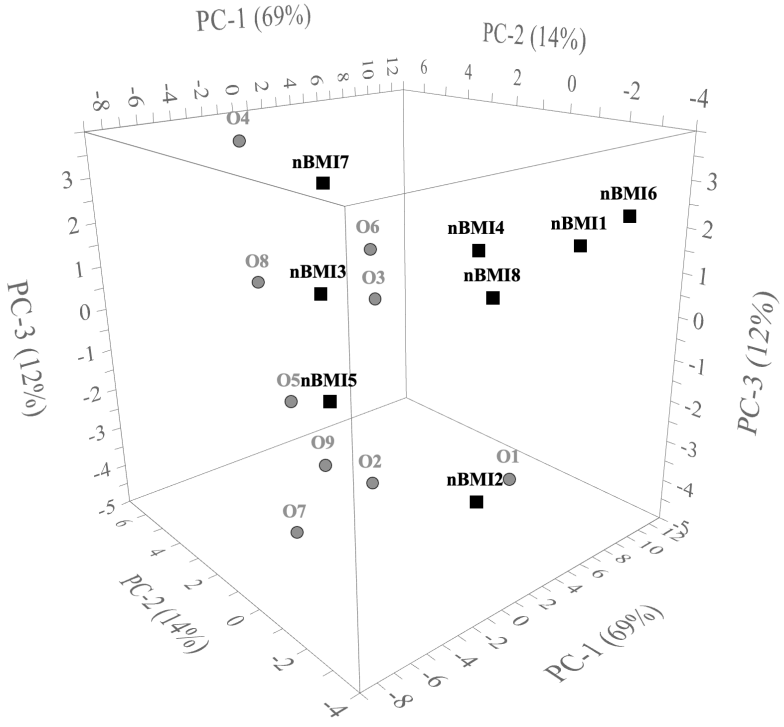

Figure 13. The scores plot of principal component analysis of the fatty acids in human milk samples (squares: normal BMI; dots: overweight and obese samples)

Despite the application of PCA, no distinct separation between the normal nBMI and O groups was observed based on the fatty acid profiles alone. However, PC1 demonstrated the most substantial influence, positioning most samples from the overweight and obese groups towards the negative end of the scale, in contrast to the normal group.

The PCA biplot, as illustrated in (Figure 14), highlights the fatty acids that significantly impacted the positioning of the samples within the PCA plot. The greater the distance of a component from the center of separation, the more influential it is. The biplot of sample scores and loadings indicated that fatty acids, mainly SFAs, such as myristic acid (C14:0), palmitic acid (C16:0), as well as unsaturated fatty acids like oleic acid (C18:1) and linoleic acid (C18:2), were key contributors due to their variability in percentage among the samples. These findings are consistent with our observation that SFA compounds are more prevalent in the breast milk of obese mothers.

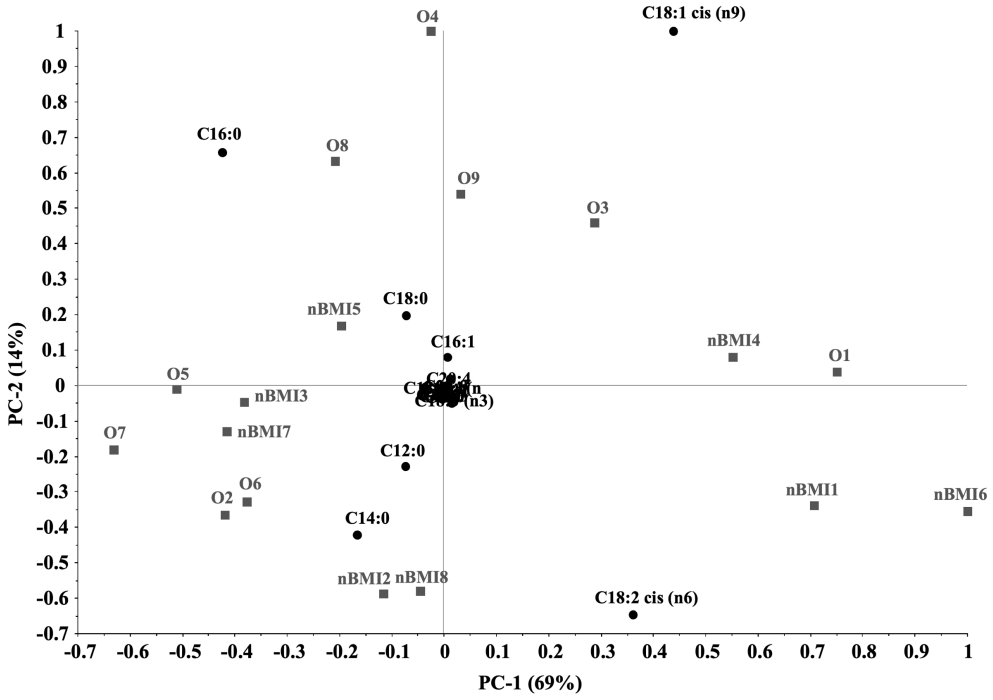

Figure 14. Bi-plot of PCA loadings and scores of the fatty acids of human milk samples
(nBMI: normal body mass index; O: overweight and obese samples)

In addition to PCA, quadratic discriminant analysis (QDA) was applied to further classify the samples based on the relative percentages of fatty acids to the total fatty acid's ratio, as depicted in (Figure 15). The QDA model achieved a classification accuracy of 88.24%. Two samples, specifically O6 and nBMI3, were misclassified using this approach, however, the model maintained a high degree of accuracy in predicting and classifying external samples. The discriminant analysis effectively differentiated samples belonging to the normal BMI group from those in the obese group based on their distinct fatty acid profiles.

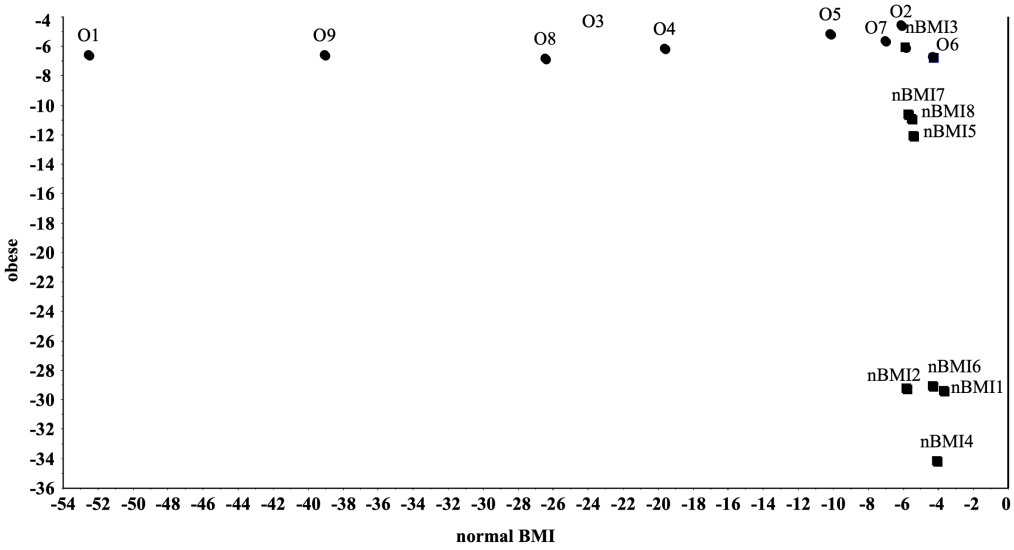

Figure 15. Quadratic discriminant analysis (QDA) of human milk samples
(squares: normal BMI; dots: overweight and obese samples)

By taking advantage of statistical evaluation methods, we were able to support the results showing that there is a qualitative difference in the fatty acid profile between breast milk from obese mothers and breast milk from normal weight mothers.

The majority of studies have shown that obese women tend to have higher fat concentrations in their breast milk (Leghi et al., 2020), leading to alterations in the fatty acid profile and concentrations of specific fatty acid metabolites (Walker et al., 2022). Typically, mothers with high BMI experience metabolic dysregulation or consume poor-quality fats in their diets (Fujimori et al., 2015; Hyesook Kim et al., 2017). Regardless of the underlying factors contributing to obesity, the fatty acid composition of HM significantly influences the properties of milk TAGs, which in turn affects energy intake and infant development. This may contribute to an increased risk of obesity later in life (Meng et al., 2023; Prentice et al., 2016).

5.3. Fatty acid composition of human milk based on infant gender, delivery mode, and pasteurization

In the third project, we aimed to analyze the fatty acid composition of human milk in relation to infant gender, delivery method, and the effects of pasteurization. To achieve this, 56 breast milk samples were collected from donor mothers at the Breast Milk Collection Center of the Unified Health Institution in Pécs, Hungary. The donors had an average age of 30.53 ± 5.44 years and an average BMI of 24.45 ± 3.38. The infants were born between the 38th and 42nd weeks of gestation, with an

average gestational age of 39.06 ± 2.71 weeks.

The breast milk samples were individually collected, labeled, and stored. Subsequently, the donated milk samples were randomly pooled and subjected to Holder pasteurization (62.5 °C for 30 min) at the Unified Health Institution. The fatty acid profiles of both raw (unpasteurized) and pasteurized milk samples were analyzed. The samples were prepared according to the ISO standard protocol with minor modifications (sections 4.2.1, 4.2.2).

Thirty-two FAME compounds were quantified using the GC-FID technique, and the fatty acid composition of the raw breast milk samples is presented in Table 7.

Table 7. Fatty acid composition of the raw breast milk samples

Fatty acids	Common name	Mean ± SD (%)	Fatty acids	Common name	Mean ± SD (%)
C6:0	Caproic acid	0.01±0.01	**C18:2n-6t**	Linolelaidic acid	<0.01
C8:0	Caprylic acid	0.06±0.03	**C18:2n-6c**	Linoleic acid (LA)	15.13±5.02
C10:0	Capric acid	0.98±0.20	**C18:3n-6c**	γ-Linolenic acid (GLA)	0.10±0.06
C11:0	Undecanoic acid	<0.01	**C18:3n-3c**	α-Linolenic acid (ALA)	0.54±0.18
C12:0	Lauric acid	5.46±1.92	**C20:0**	Arachidic acid	0.18±0.08
C13:0	Tridecylic acid	0.03±0.02	**C20:1n-9c**	Eicosenoic acid	0.40±0.11
C14:0	Myristic acid	7.02±1.99	**C20:2n-6c**	Eicosadienoic acid	0.30±0.09
C14:1n-5c	Myristoleic acid	0.14±0.06	**C20:3n-6c**	Dihomo-gamma-linolenic acid	0.35±0.08
C15:0	Pentadecylic acid	0.31±0.15	**C20:4n-6c**	Arachidonic acid (ARA)	0.40±0.08
C16:0	Palmitic acid	26.01±4.38	**C22:0**	Behenic acid	0.01±0.03
C16:1n-7c	Palmitoleic acid	1.90±0.44	**C22:1n-9c**	Erucic acid	0.02±0.03
C17:1n-7c	Heptadecenoic acid	0.12±0.10	**C20:5n-3c**	Eicosapentaenoic acid (EPA)	<0.01
C17:0	Margaric acid	0.27±0.09	**C22:2n-6c**	Docosadienoic acid	0.01±0.02
C18:0	Stearic acid	7.73±1.87	**C24:0**	Lignoceric acid	0.02±0.05
C18:1n-9t	Elaidic acid	0.12±0.27	**C24:1n-9c**	Nervonic acid	0.02±0.08
C18:1n-9c	Oleic acid	32.29±4.06	**C22:6n-3c**	Docosahexaenoic acid (DHA)	0.07±0.16

The overall FAME profile of the raw milk samples is consistent with our earlier findings (see Table 6). Additional analyses were performed on the dataset to investigate factors beyond maternal health, such as infant gender, delivery mode, and pasteurization, that may influence the quality of human breast milk.

5.3.1. Effect of infant gender on human milk fatty acid composition

The impact of infant gender on human milk composition remains an underexplored area with

limited scientific research. Evidence from animal models suggests that infant sex may be a predictive determinant of maternal milk composition. Some studies indicate that male and female infants exhibit sexually dimorphic responses to their early nutritional environment, suggesting sex-specific nutritional requirements during the early stages of life for optimal growth and development (Galante et al., 2018; Khelouf et al., 2023).

The aim of our study was to investigate whether differences in FA composition exist in the breast milk of mothers who delivered infants of different sexes (male or female).

Among the recruited mothers, 40.6% had male infants, while 59.4% had female infants.

To assess the normality of the data, the Kolmogorov-Smirnov test was applied. The results indicated that the fatty acid data in the analyzed samples did not follow a normal distribution in all cases. Consequently, the Kruskal-Wallis test was performed to compare the groups based on the sex of the infant (male or female). Differences were found in breast milk composition between the two groups. Notably, the concentration of eicosadienoic acid (C20:2 n-6c) was significantly higher in the breast milk produced for female infants (Table 8).

Table 8. Fatty acid composition of HM influenced by the sex of infants

Fatty acid	Female Mean ± SD (%)	S	Male Mean ± SD (%)	Fatty acid	Female Mean ± SD (%)	S	Male Mean ± SD (%)
C6:0	<0.01		0.02±0.01	**C18:2n-6t**	<0.01		<0.01
C8:0	0.06±0.03		0.06±0.01	**C18:2n-6c**	16.66±4.72		12.01±2.68
C10:0	1.00±0.23		0.97±0.15	**C18:3n-6c**	0.10±0.07		0.10±0.04
C11:0	<0.01		<0.01	**C18:3n-3c**	0.55±0.18		0.54±0.17
C12:0	5.35±2.32		5.30±1.28	**C20:0**	0.17±0.09		0.21±0.04
C13:0	0.02±0.02		0.04±0.01	**C20:1n-9c**	0.41±0.10		0.39±0.10
C14:0	6.51±2.11		7.50±1.81	**C20:2n-6c**	0.34±0.07	*	0.25±0.04
C14:1n-5c	0.12±0.06		0.17±0.05	**C20:3n-6c**	0.38±0.08		0.30±0.04
C15:0	0.29±0.15		0.34±0.11	**C20:4n-6c**	0.42±0.09		0.36±0.05
C16:0	25.89±4.55		27.18±3.36	**C22:0**	0.02±0.03		0.01±0.02
C16:1n-7c	1.83±0.41		2.12±0.43	**C22:1n-9c**	0.02±0.04		0.01±0.03
C17:0	0.08±0.09		0.19±0.05	**C20:5n-3c**	<0.01		<0.01
C17:1n-7c	0.26±0.10		0.31±0.04	**C22:2n-6c**	0.01±0.02		<0.01
C18:0	7.55±1.93		8.18±1.26	**C24:0**	0.02±0.05		0.02±0.07
C18:1n-9t	0.15±0.28		0.03±0.13	**C24:1n-9c**	0.02±0.05		0.03±0.12
C18:1n-9c	31.68±3.75		33.35±3.89	**C22:6n-3c**	<0.01		<0.01

S= significance * p<0.05

The average percentage ratios were calculated as 0.25 ± 0.04 for boys and 0.34 ± 0.07 for girls. The average of eicosadienoic acid (C20:2 n-6c) values in breast milk for boys and girls are illustrated in Figure 16.

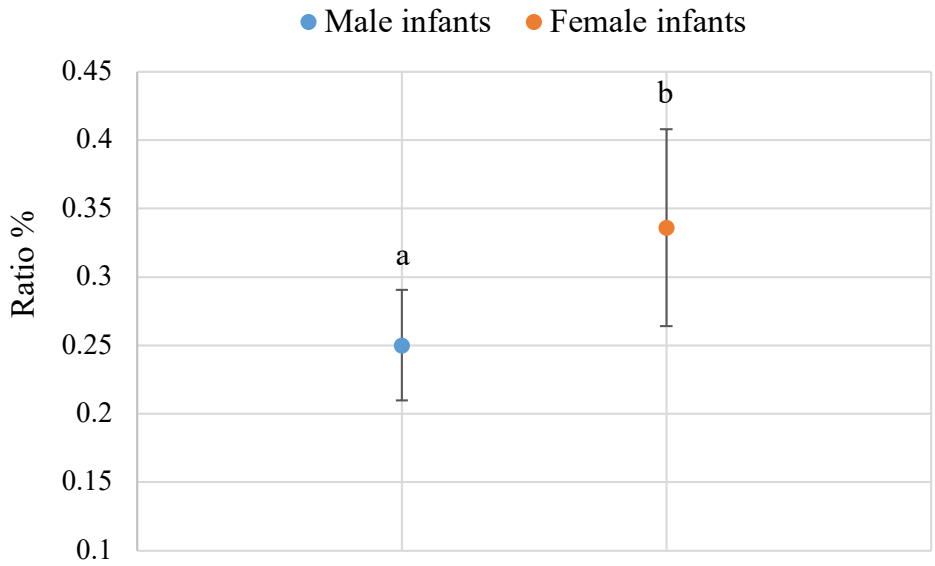

Figure 16. Average values of eicosadienoic acid (C20:2 n-6c) in different groups of human milk samples

(Orange dots represent human milk for girls, blue dots represent human milk for boys; different letters show significant differences at $p \leq 0.05$ levels.)

Our research revealed that HM produced for female infants contained significantly higher concentrations of eicosadienoic acid (C20:2 n-6c), aligning with the findings of Thakkar et al. (2013). Eicosadienoic acid (C20:2n-6c) is a fatty acid associated with anti-inflammatory properties and improved growth velocity in preterm infants (Ahmed et al., 2023). This sex-specific difference may be attributed to the varying nutritional and developmental needs of male and female infants.

Principal component analysis (PCA) was performed for data visualization and pattern recognition. PCA on human milk samples identified that the first three PCs explained 98% of the total variance within our dataset, with PC1 accounting for 60%, PC2 for 28%, and PC3 for 10%. No outliers were observed based on the Hotelling T2 values and F-residuals. The PCA revealed that the positioning of the samples on the first two principal components was primarily influenced by palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), oleic acid (C18:1 n-9c), and linoleic acid (C18:2 n-6c). Other significant contributors included lauric acid (C12:0), pentadecanoic acid (C15:0), and dihomo-gamma-linolenic acid (C20:3 n-6c), although these did not facilitate clear group separations, as visualized in Figure 17.

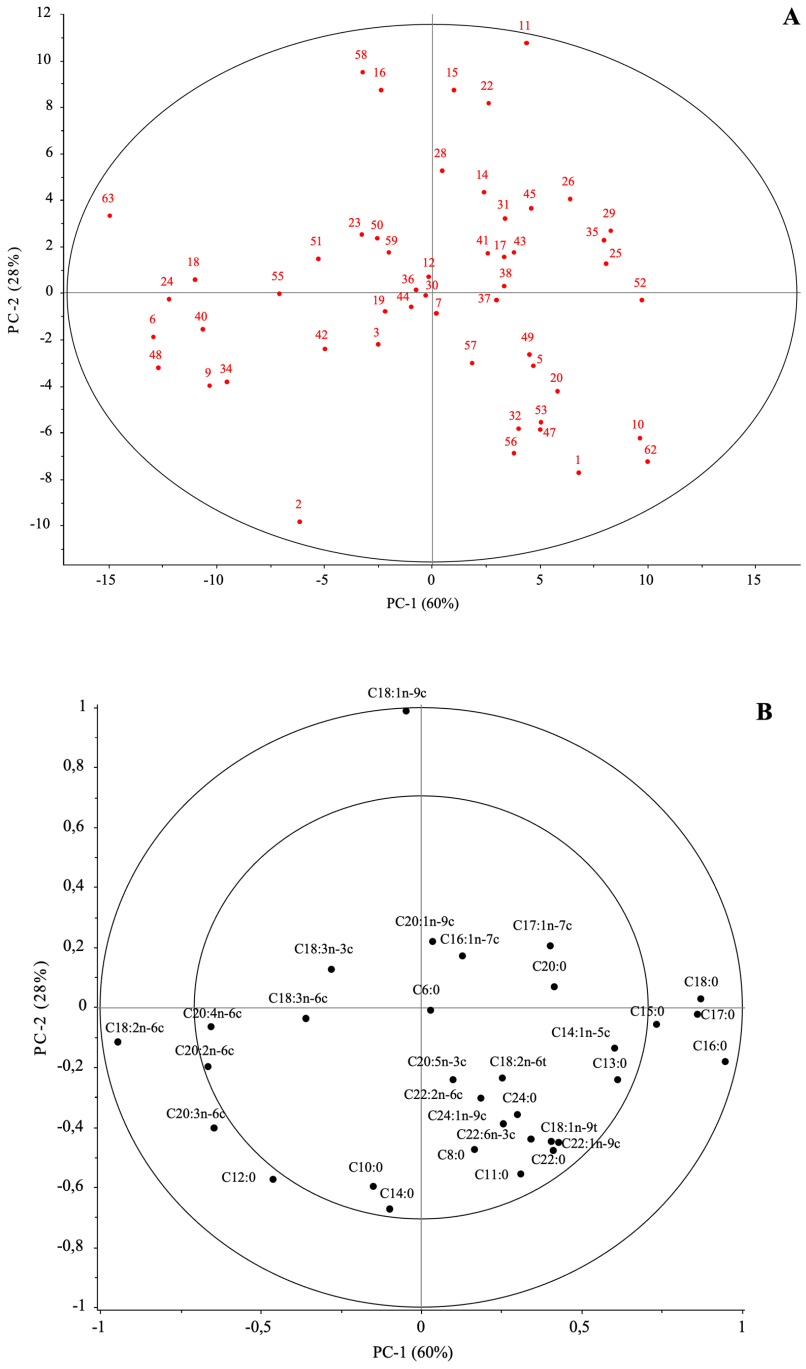

Figure 17. Scores (A) and correlation loadings (B) of the human milk samples

However, when considering the third principal component (PC3), the samples showed distinct separation based on the sex of the infants. Clear differences between the samples were visible for the second and third principal components (PC2 and PC3). For PC2, the concentration of oleic acid (C18:1 n-9c) fatty acid significantly influenced the positioning; for PC3, the contents of myristic acid (C14:0), lauric acid (C12:0), and capric acid (C10:0) substantially impacted the positioning of samples,

facilitating the observed separation. Positions in the negative range on PC2 could be explained by higher contents of linoleic acid (C18:2 n-6c), while samples in the positive range were influenced by margaric acid (C17:0). According to the corresponding loadings (Figure 18), the latter had a lesser impact.

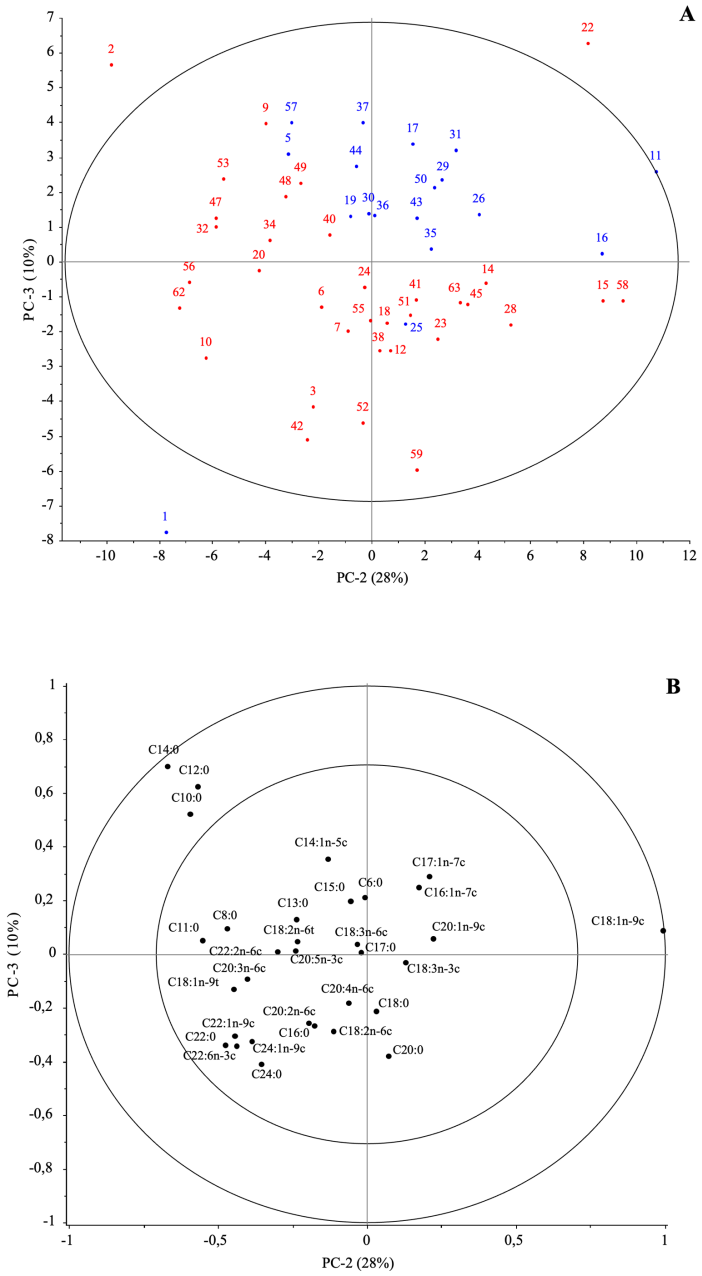

Figure 18. Scores (A) and correlation loadings (B) of the samples (PC2-PC3)
(The samples were marked with colors according to the sex of the infant: girl-red; boy-blue.)

Our results showed that myristic acid (C14:0) levels in HM differed based on the infant's sex, consistent with the findings of Meng et al. (2023). Additionally, they reported that milk provided to female infants contained higher concentrations of caprylic acid (C8:0), myristic acid (C14:0), myristoleic acid (C14:1), nervonic acid (C24:1), alpha-linolenic acid (C18:3 n-3), and n-3 LCPUFAs in mature milk. Furthermore, a recent study by Thakkar et al. (2013) found that the concentrations of linoleic acid (C18:2 n-6), eicosadienoic acid (C20:2 n-6), and total PUFAs were also higher in milk provided to female infants compared to male infants.

As supported by Galbarczyk (2011), the sex of the infant significantly alters the fatty acid composition of human milk. Animal studies using rhesus monkeys (Macaca mulatta) as models have shown that lactating female monkeys with male offspring produce less milk with higher energy content, while those with female offspring produce more milk with lower energy per unit, resulting in similar total energy content across milk samples, regardless of the offspring's sex (Hinde, 2009). Other studies have similarly reported higher energy content in milk from mothers of male infants (Hahn et al., 2017; Powe et al., 2010).

Studies suggest that mothers of male infants typically have higher fat content in their breast milk (Fischer Fumeaux et al., 2019) and pregnant women carrying male fetuses generally consume about 10% more energy than those carrying female fetuses (Tamimi et al., 2003). Bennett et al.(2018) suggested that this gender-related energy intake difference could stem from variations in physiological composition. Further research has confirmed that due to different growth rates between male and female infants, male infants have a relatively higher demand for energy for growth and development (Thakkar et al., 2013).

Our findings also revealed no significant differences in postpartum maternal BMI effects, although most mothers fell within the normal BMI range. However, Khelouf et al. (2023) found that mothers of male infants typically have higher pre-pregnancy and pregnancy weights and BMIs than those with female infants, suggesting a potential link between maternal weight, BMI, and the sex of the offspring, which might influence the nutritional components of human milk.

To date, the mechanisms responsible for the specific synthesis of breast milk components tailored to different infant genders remain unclear, and data on this topic are quite limited. However, the necessity of addressing the varying nutritional requirements associated with different infant genders calls for further research to elucidate this phenomenon, in order to optimize the distinct needs for their growth.

5.3.2. Impact of different neonatal delivery modes on human milk fatty acid composition

Nearly 57.6% of the participating mothers delivered their babies by caesarean section, while 42.4% delivered vaginally. Regarding the mode of delivery, our study found that breast milk from mothers who delivered via C-section contained lower concentrations of specific fatty acids, including lauric acid (C12:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), and *trans*-oleic acid (*trans*-C18:1 n-9) compared to those who delivered vaginally. In contrast, levels of linoleic acid (C18:2 n-6c), behenic acid (C22:0), lignoceric acid (C24:0), and docosahexaenoic acid (C22:6 n-3c) were significantly higher in breast milk from mothers who delivered by C-section (Table 9).

Table 9. Fatty acid composition of HM in mothers delivered spontaneously or by C-section

Fatty acid	Spontanously Mean ± SD (%)	S	C-section Mean ± SD (%)	Fatty acid	Spontanously Mean ± SD (%)	S	C-section Mean ± SD (%)
C6:0	0.01±0.01		0.01±0.01	**C18:2n-6t**	<0.01		<0.01
C8:0	0.06±0.03		0.06±0.03	**C18:2n-6c**	14.59±4.84	*	15.25±4.74
C10:0	1.01±0.19		0.95±0.21	**C18:3n-6c**	0.08±0.06		0.11±0.06
C11:0	<0.01		<0.01	**C18:3n-3c**	0.53±0.16		0.54±0.20
C12:0	5.60±1.90	*	5.19±1.98	**C20:0**	0.17±0.10		0.20±0.06
C13:0	0.03±0.02		0.03±0.02	**C20:1n-9c**	0.40±0.11		0.40±0.11
C14:0	7.19±1.80		6.83±2.22	**C20:2n-6c**	0.29±0.11		0.30±0.08
C14:1n-5c	0.14±0.06		0.14±0.06	**C20:3n-6c**	0.34±0.08		0.36±0.08
C15:0	0.31±0.16	*	0.29±0.14	**C20:4n-6c**	0.38±0.07		0.42±0.08
C16:0	26.57±4.53	*	26.19±4.37	**C22:0**	0.01±0.03	*	0.02±0.03
C16:1n-7c	1.88±0.37		1.92±0.51	**C22:1n-9c**	0.02±0.03	*	0.02±0.04
C17:0	0.29±0.09	*	0.26±0.08	**C20:5n-3c**	0.01±0.03		<0.01
C17:1n-7c	0.10±0.10		0.14±0.09	**C22:2n-6c**	<0.01		0.01±0.02
C18:0	8.15±1.96	*	7.45±1.57	**C24:0**	0.02±0.06	*	0.03±0.07
C18:1n-9t	0.14±0.31	*	0.11±0.22	**C24:1n-9c**	0.03±0.10		0.03±0.11
C18:1n-9c	31.58±4.33		32.66±4.03	**C22:6n-3c**	0.07±0.17	*	0.09±0.18

S= significance * p<0.05

Our research demonstrated that the mode of delivery influences the fatty acid composition in HM. Specifically, we found that breast milk from mothers who had natural deliveries contained higher levels of stearic acid (C18:0) and eicosapentaenoic acid (C20:5 n-3c), both known for their anti-inflammatory properties. Interestingly, our results also revealed elevated levels of DHA in the breast milk of mothers who underwent C-sections, consistent with previous finding (Samuel et al., 2022).

Furthermore, we observed that linoleic acid (C18:2 n-6c), eicosadienoic acid (C20:2 n-6c), and arachidonic acid (C20:4 n-6c), all n-6 PUFAs, were higher in the milk of C-section mothers. These n-6 PUFAs are known for their pro-inflammatory properties (Şimşek et al., 2015), contrasting with the higher levels of anti-inflammatory n-3 PUFAs observed in the milk of mothers who delivered naturally.

The n-6 to n-3 fatty acid ratios were calculated accordingly. As shown in Figure 19, this higher rate can be attributed to the fact that both ARA and DHA levels are elevated in mothers who have undergone a caesarean section, with the increase in ARA being more pronounced.

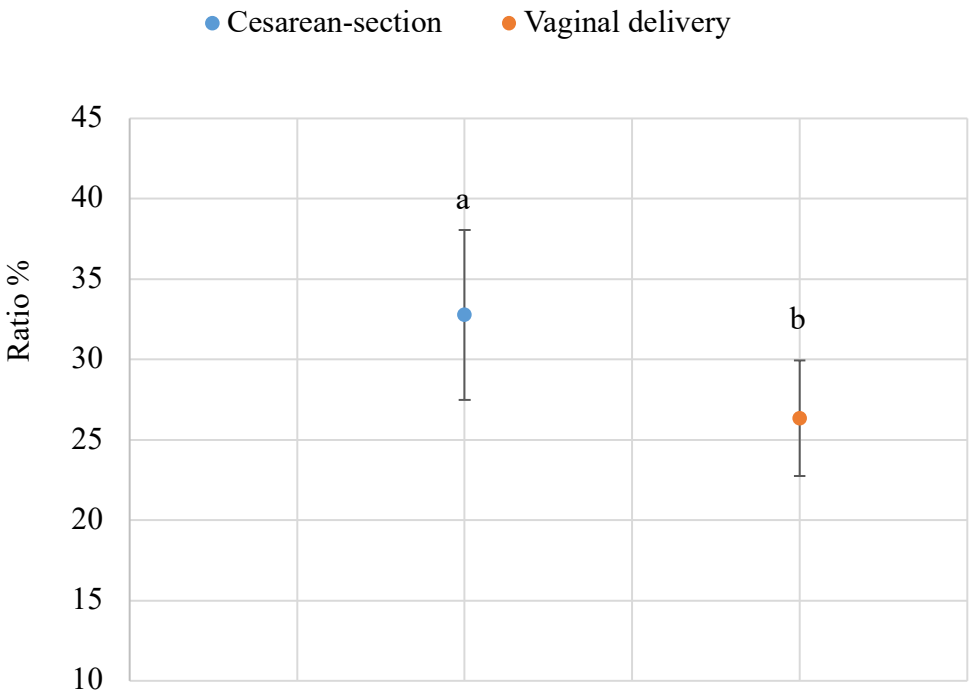

Figure 19. Comparison of inflammatory n-6 to n-3 fatty acid average ratios between groups of C-section and vaginal delivery
(Orange dots represent vaginal delivery, blue dots represent C-section; different letters show significant differences at $p \leq 0.05$ levels.)

These findings were further supported by PCA statistical analysis. When the samples were categorized by mode of delivery in the PCA plot (Figure 20), a clear separation between the groups emerged based on their fatty acid profiles. In the case of PC1, the variance was primarily influenced by the levels of both saturated and unsaturated fatty acids. Mothers who delivered via caesarean section had higher levels of linoleic acid (C18:2 n-6c) in their breast milk compared to those who delivered vaginally. Additionally, higher levels of arachidonic acid (C20:4 n-6c) and eicosadienoic acid (C20:2 n-6c) were also observed in the caesarean section group.

For the second principal component, sample positioning was largely influenced by the levels of dihomo-gamma-linolenic acid (C20:3 n-6c), lauric acid (C12:0), and oleic acid (C18:1 n-9c). Samples with elevated concentrations of these fatty acids were positioned in the negative region of PC1. Conversely, samples located in the positive region of PC1 exhibited higher percentages of palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), and pentadecanoic acid (C15:0).

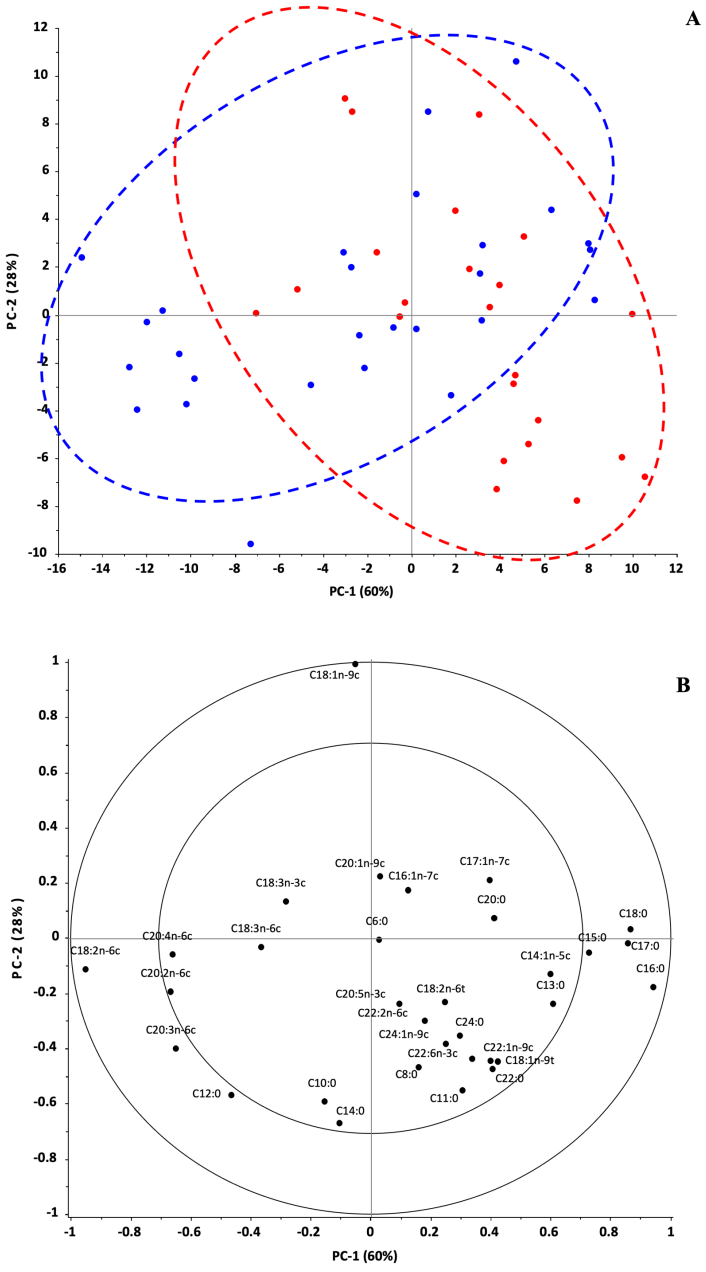

Figure 20. PCA results of fatty acids in breast milk from caesarean and vaginal deliveries
(Blue dots represent caesarean section samples, and red dots represent vaginal delivery samples. (A) Scores and (B) correlation loadings of the samples (PC1-PC2).)

Previous research has suggested that the mode of delivery influences fatty acid composition in breast milk, with C-section linked to increased fat content (Hahn et al., 2017). Significant differences in infant weight between cesarean and vaginal deliveries, İsik et al. (2016) have indicated that cesarean delivery may cause a delay in milk production. With cesarean-born infants generally weighing less, indicate that delivery mode may impact early growth and development. Moreover, cesarean delivery may hinder the effective acquisition of maternal microbiota by infants, potentially affecting their immune system development. This insufficiency may lead to changes in the immunological development of cesarean-born infants, thereby influencing their disease resistance (Sandall et al., 2018). Studies have also linked cesarean delivery to increased oxidative stress in colostrum, which may alter the nutritional components of human milk, particularly by increasing levels of n-6 PUFA, thus promoting an inflammatory state (Şimşek et al., 2015). This discrepancy suggests a complex interaction between delivery mode and milk composition that merits further exploration.

5.3.3. Holder pasteurization's impact on the fatty acid composition of donated human milk

Donor milk (DM) is often recommended by researchers as a preferred substitute for preterm infants compared to preterm formula (Poulimeneas et al., 2021). Donor milk obtained from human milk banks undergoes Holder pasteurization (HoP), a process conducted at 62.5 °C for 30 minutes. This method effectively eliminates potential pathogens while preserving essential nutrients and immunological properties.

Our findings indicate significant changes in the concentrations of 14 fatty acids following Holder pasteurization, as shown in Table 10. Specifically, the concentrations of caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), myristoleic acid (C14:1 n-5c), oleic acid (C18:1 n-9c), gamma-linolenic acid (C18:3 n-6c), alpha-linolenic acid (C18:3 n-3c), and arachidonic acid (C20:4 n-6c) decreased after HoP treatment. In contrast, concentrations of myristic acid (C14:0), palmitic acid (C16:0), *trans*-oleic acid (*trans*-C18:1 n-9), lignoceric acid (C24:0), nervonic acid (C24:1 n-9c), and docosahexaenoic acid (C22:6 n-3c) increased post-treatment.

PCA was employed to analyze the fatty acid profiles of both pasteurized and unpasteurized (raw) samples, with the results summarized in (Figure 21). The first two principal components accounted for 93% of the variance in the data. The results clearly indicated that pasteurization significantly affected the fatty acid profiles of the samples, showing good separation along PC1 and PC2 for all samples except for sample p10. Both principal components had a significant impact on the positioning

of the samples. Except for sample p1, the pasteurized samples were predominantly positioned in the negative region of PC1, which was primarily associated with a higher percentage of oleic acid (C18:1 n-9c). This significance is supported by the Kruskal-Wallis test, which identified statistically significant differences ($p<0.05$). The positive region of PC1 was mainly explained by a higher proportion of palmitic acid (C16:0), more common in the unpasteurized samples (Figure 21).

Table 10. Effect of Holder pasteurization on breast milk

Fatty acid	Raw Mean ± SD (%)	S	Pasteurized Mean ± SD (%)	Fatty acid	Raw Mean ± SD (%)	S	Pasteurized Mean ± SD (%)
C6:0	0.07±0.03	*	0.01±0.00	**C18:2n-6t**	<0.01		<0.01
C8:0	0.19±0.04	*	0.06±0.02	**C18:2n-6c**	17.10±2.47		15.21±1.63
C10:0	1.26±0.14	*	1.00±0.07	**C18:3n-6c**	0.15±0.03	*	0.10±0.03
C11:0	<0.01		<0.01	**C18:3n-3c**	0.65±0.12	*	0.54±0.07
C12:0	5.88±0.83		5.55±0.65	**C20:0**	0.19±0.02		0.18±0.02
C13:0	<0.01		0.03±0.01	**C20:1n-9c**	0.38±0.05		0.39±0.06
C14:0	6.05±0.89	*	7.03±0.68	**C20:2n-6c**	0.31±0.05		0.30±0.05
C14:1n-5c	0.16±0.02	*	0.14±0.02	**C20:3n-6c**	0.39±0.07		0.36±0.05
C15:0	0.25±0.04		0.29±0.06	**C20:4n-6c**	0.51±0.06	*	0.40±0.05
C16:0	22.98±2.06	*	26.10±1.16	**C22:0**	<0.01	*	0.014±0.015
C16:1n-7c	1.94±0.18		1.90±0.18	**C22:1n-9c**	<0.01	*	0.02±0.02
C17:0	0.24±0.02		0.27±0.03	**C20:5n-3c**	<0.01		<0.01
C17:1n-7c	0.08±0.09		0.11±0.03	**C22:2n-6c**	<0.01		<0.01
C18:0	6.98±0.75		7.71±0.61	**C24:0**	0.01±0.03	*	0.02±0.03
C18:1n-9t	0.01±0.03	*	0.11±0.13	**C24:1n-9c**	0.02±0.05	*	0.03±0.05
C18:1n-9c	34.16±3.59	*	32.05±2.46	**C22:6n-3c**	0.02±0.07	*	0.07±0.09

S= significance * $p<0.05$

Additionally, classification methods were applied to evaluate the effects of pasteurization. The cubic support vector machine provided the best results, achieving complete separation of the samples. The accuracy of the model was 100% (Figure 21), demonstrating the distinct impact of pasteurization on the fatty acid composition in human milk.

Consistent with findings from over 55 publications, our study confirmed that linoleic acid (C18:2 n-6c) remains stable and unaffected by the pasteurization process (Delgado et al., 2014; Floris et al., 2020; Valentine et al., 2010). We also observed a significant increase in palmitic acid (C16:0) levels and a significant decrease in oleic acid (C18:1 n-9c) levels following Holder pasteurization. Only minor alterations were noted in the relative composition of MCFAs, which is consistent with previous studies (Ewaschuk et al., 2011). Additionally, in our study, docosahexaenoic acid (C22:6 n-3c) was initially detected at low concentrations in raw milk samples but showed a significant increase after

pasteurization (Table 10). This suggests that Holder pasteurization may enhance the availability of certain fatty acids, potentially improving their nutritional value (Peila et al., 2016).

Recent studies underscore that while Holder Pasteurization effectively reduces microbial and viral contaminants, it preserves significant biological activity in human milk, retaining critical components such as anti-infective properties, immune-modulating factors, and growth factors, albeit with some loss of functionality (Parra-Llorca et al., 2018; Peila et al., 2016; Schlotterer and Perrin, 2018).

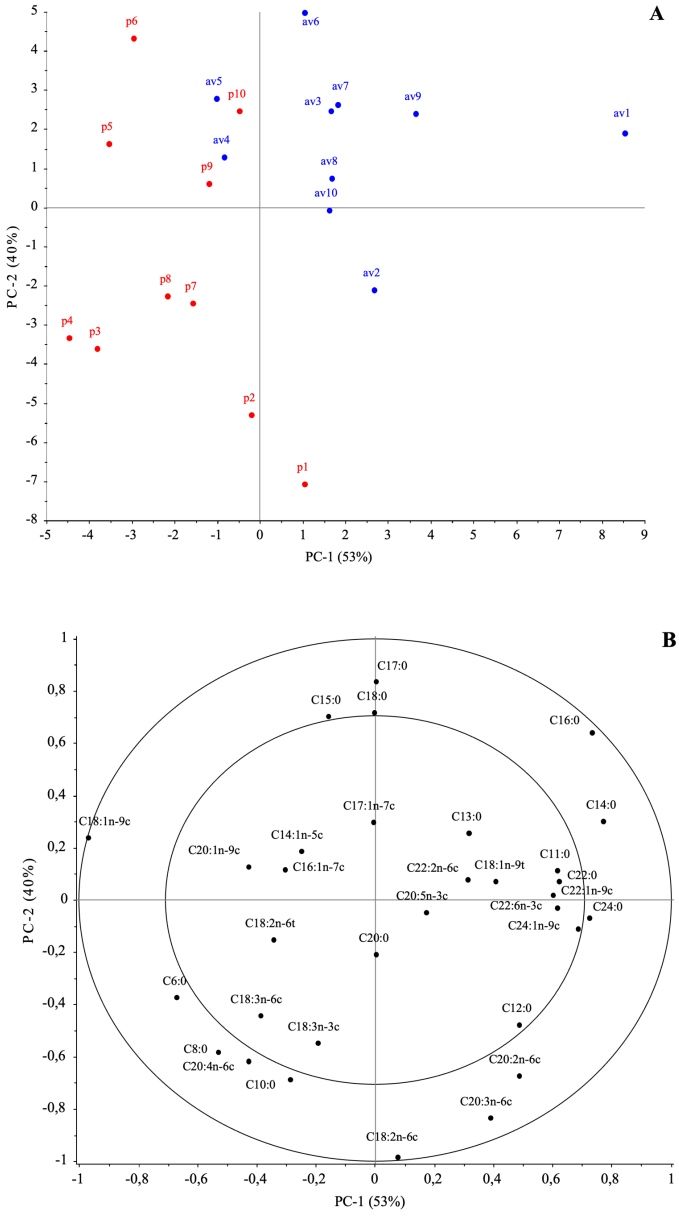

Figure 21. PCA results of raw and holder pasteurized human milk samples
(Scores plot (A); Correlation loadings (B). Holder-pasteurized samples are indicated in red, and raw samples are indicated in blue.)

6. CONCLUSIONS AND RECOMMENDATIONS

Our research investigated the influence of maternal health conditions (normal body mass index (BMI), obesity, and gestational diabetes), geographical location, infant characteristics (sex and delivery mode), and Holder pasteurization (HoP) on the fatty acid composition of human milk. The findings provide valuable insights into how these factors affect the nutritional profile of breast milk, with potential implications for infant development and long-term health outcomes.

One key conclusion is that maternal health status significantly impacts the fatty acid composition of human milk. Specifically, the levels of saturated fatty acids (SFAs) varied significantly among mothers with different health statuses. Mothers with normal BMI exhibited lower levels of linoleic acid (C18:2 n-6) compared to those with obesity and gestational diabetes (GD). Additionally, the lauric acid (C12:0) content was significantly lower in the breast milk of mothers with obesity and GD compared to other groups. These differences in the nutritional quality of breast milk may be related to infant development and have potential long-term health implications.

Geographical differences were also found to play a notable role in the fatty acid composition of human milk. Hungarian mothers with a normal BMI exhibited higher levels of saturated fatty acids (SFAs), such as palmitic acid (C16:0), and n-6 polyunsaturated fatty acids (PUFAs), including linoleic acid (C18:2 n-6). In contrast, Ukrainian mothers demonstrated higher concentrations of alpha-linolenic acid (C18:3 n-3). These variations are likely influenced by differing dietary habits and cultural factors, suggesting that region-specific nutritional guidelines for breastfeeding mothers could be beneficial.

Additionally, infant sex, delivery mode, and Holder pasteurization were found to influence the fatty acid composition of breast milk. For example, milk provided to female infants contained higher concentrations of specific fatty acids, such as eicosadienoic acid (C20:2 n-6). Cesarean deliveries were associated with a higher ratio of n-6 fatty acids. HoP significantly reduced the concentrations of certain SFAs, including caproic acid (C6:0), caprylic acid (C8:0), and capric acid (C10:0), as well as MUFAs like myristoleic acid (C14:1 n-5c) and oleic acid (C18:1 n-9), and PUFAs such as alpha-linolenic acid (C18:3 n-3), gamma-linolenic acid (C18:3 n-6), and arachidonic acid (C20:4 n-6). However, it increased the concentration of docosahexaenoic acid (C22:6 n-3c).

Based on these findings, several recommendations can be made:

1. **Maternal Health Considerations**: Healthcare providers should take maternal health status into account when offering nutritional counseling to breastfeeding mothers. Special attention should be given to obese mothers and those with gestational diabetes, as their milk may require dietary modifications to optimize infant nutrition.
2. **Geographical-Specific Interventions**: Region-specific dietary interventions should be promoted to address the geographical disparities observed in milk composition. These tailored interventions can help ensure that maternal diet supports optimal fatty acid profiles in breast milk.
3. **Infant Sex, Delivery Mode, and Holder pasteurization**: Other factors, such as infant sex, delivery mode, and the heat treatment of human milk, also influence the fatty acid profile. Healthcare providers should consider these variables when advising mothers on breastfeeding practices.
4. **Future Research**: Further research is needed to explore the long-term effects of variations in fatty acid profiles in human milk on infant growth and development. Investigating how these altered profiles influence infant health outcomes over time would provide deeper insights and help refine breastfeeding recommendations.

7. NEW SCIENTIFIC RESULTS

Based on my research findings, I have demonstrated that maternal health status significantly influences the fatty acid composition of human milk. The following are my new scientific results:

1. I found significant differences in the levels of saturated fatty acids (SFAs) among Ukrainian mothers with different health statuses. Specifically, short-chain SFAs, such as caproic acid (C6:0), medium-chain SFAs like capric acid (C10:0) and lauric acid (C12:0), as well as long-chain SFAs, including myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0), varied notably among the groups. Obese mothers exhibited significantly higher levels of oleic acid (C18:1 n-9), whereas mothers with a normal BMI showed significantly lower concentrations of linoleic acid (C18:2 n-6).
2. My research revealed that overweight and obese Hungarian mothers have significantly higher levels of SFAs, including capric acid (C10:0), lauric acid (C12:0), and myristic acid (C14:0). They also exhibited elevated levels of oleic acid (C18:1 n-9), but no significant difference was observed in the total MUFA content between the groups. The obese mothers had lower ratios of polyunsaturated fatty acids (PUFAs), such as linoleic acid (C18:2 n-6) and alpha-linolenic acid (C18:3 n-3), compared to normal BMI mothers. We found that the n-6 to n-3 ratio was about 50% higher in milk from the obese group compared to the normal BMI mothers.
3. I identified significant differences in the fatty acid composition of human milk based on geographical location. Hungarian mothers with normal BMI showed higher levels of palmitic acid (C16:0) and linoleic acid (C18:2 n-6) in their milk, while Ukrainian mothers exhibited higher levels of PUFAs, particularly alpha-linolenic acid (C18:3 n-3).
4. I discovered that the sex of the infant influences the fatty acid profile of human milk. Milk provided to female infants contained significantly higher levels of eicosadienoic acid (C20:2 n-6) compared to milk provided to male infants.
5. I demonstrated that Holder pasteurization significantly alters the fatty acid profile of human milk. Pasteurization resulted in reduced levels of SFAs (caproic acid (C6:0) to capric acid (C10:0)), MUFAs (oleic acid (C18:1 n-9)), and PUFAs (alpha-linolenic acid (C18:3 n-3), gamma-linolenic acid (C18:3 n-6) arachidonic acid (C20:4 n-6)), while increasing the levels of long-chain SFAs (palmitic acid (C16:0)) and MUFAs (nervonic acid (C24:1 n-9)). I found that considering the fatty acid profile of the samples, it is possible to classify the raw and pasteurized samples with up to 100% accuracy using the cubic support vector machine classification model.

8. SUMMARY

The aim of our research was to investigate the factors influencing the fatty acid composition of human milk, with a particular focus on maternal health conditions (such as normal body mass index (BMI), obesity, and gestational diabetes), geographical differences, infant characteristics (such as sex and delivery mode), and post-processing techniques like Holder pasteurization (HoP). The research was structured around three distinct projects:

Project 1 examined the effects of maternal health conditions, including normal BMI, obesity, and gestational diabetes, on the fatty acid profile of breast milk in Ukraine, while also comparing the fatty acid profiles of normal BMI mothers from Ukraine and Hungary. The results revealed significant variations in saturated fatty acids (SFAs) based on maternal health status. Obese mothers exhibited higher levels of monounsaturated fatty acids (MUFAs), particularly oleic acid (C18:1 n-9), whereas normal BMI mothers had lower levels of polyunsaturated fatty acids (PUFAs), especially linoleic acid (C18:2 n-6). Furthermore, Hungarian mothers with normal BMI had significantly higher levels of palmitic acid (C16:0) and linoleic acid (C18:2 n-6) compared to Ukrainian mothers, who showed elevated concentrations of alpha-linolenic acid (C18:3 n-3). These findings underscore the influence of both maternal health status and nationality on the fatty acid composition of human milk.

Project 2 focused on the fatty acid composition of human milk from Hungarian mothers with varying health statuses. Our study found that overweight and obese mothers had significantly higher levels of SFAs, particularly palmitic acid (C16:0) and stearic acid (C18:0), which together comprised 39.2% of the total fatty acids. Additionally, these mothers exhibited elevated levels of MUFAs, such as oleic acid (C18:1 n-9). In contrast, normal-weight mothers had higher levels of PUFAs, such as linoleic acid (C18:2 n-6). These results suggest that maternal BMI has a notable impact on the fatty acid composition of breast milk, with obese mothers producing milk richer in SFAs and lower in PUFAs.

Project 3 explored additional maternal factors influencing the fatty acid composition of breast milk, including infant sex, delivery mode, and the impact of Holder pasteurization. Our study found that infant sex influenced the fatty acid profile, with higher concentrations of eicosadienoic acid (C20:2 n-6) detected in the milk provided to female infants. Moreover, the mode of delivery (cesarean section vs. vaginal birth) significantly affected the fatty acid composition, with cesarean deliveries associated with a higher n-6 to n-3 fatty acid ratio. The study also assessed the effects of HoP on the fatty acid composition of human milk. Results showed that HoP significantly reduced the

concentrations of short- and medium-chain fatty acids, such as caproic acid (C6:0) and capric acid (C10:0), as well as MUFAs like oleic acid (C18:1 n-9) and PUFAs, including alpha-linolenic acid (C18:3 n-3), gamma-linolenic acid (C18:3 n-6), and arachidonic acid (C20:4 n-6). In contrast, long-chain SFAs, such as palmitic acid (C16:0), increased post-pasteurization. Although the core fatty acids remained largely intact, the process altered the nutritional composition of the milk, potentially enhancing certain fatty acids that may benefit preterm infant development.

In conclusion, our research highlights the intricate interplay between maternal health conditions, neonatal factors (such as sex and delivery mode), and post-processing techniques in determining the fatty acid composition of human milk. These findings provide valuable insights for healthcare professionals, emphasizing the need for tailored nutritional guidance and personalized breastfeeding strategies based on maternal and infant characteristics. Furthermore, the research underscores the significance of geographical and cultural dietary differences, providing a foundation for region-specific recommendations aimed at optimizing both maternal and infant health outcomes.

APPENDICES

A1: References

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PUBLICATIONS

Publication–Journal

1. **Zhang, M**., Simon Sarkadi, L., Üveges, M., Tormási, J., Benes, E., Vass, R.A. and Vari, S.G., 2022. Gas chromatographic determination of fatty acid composition in breast milk of mothers with different health conditions. *Acta Alimentaria*, 51(4), pp.625–635, [Available from: https://doi.org/10.1556/066.2022.00120].
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1. **Zhang, M**., Üveges, M., Muránszky, G., Simon Sarkadi, L., Matsyura, O., Ertl, T., Vass, R., Vari, S.G. (2021): Fatty Acid Composition of Mother Milk. XXI EuroFoodChem, 22-24 November 2021, online conference. Book of Abstract (ISBN 978-989-8124-34-0), pp.170.
2. Simon Sarkadi, L., Üveges, M., **Zhang, M**., Tormási, J., Benes, E., Vass, R., Vari, S.G. (2022): Fatty acid composition of human milk of pregnant women with obesity and Gestational Diabetes. RECOOP 17th Bridges in Life Sciences Conference, 6-9 April, 2022, Prague, Czech Republic. Book of Abstract (ISBN 978-615-6006-03-5), pp. 88.
3. Simon Sarkadi, L., **Zhang, M**., Muránszky, G., Vass, R.A., Matsyura, O., Vari, S.G. (2022): Impact of pregnant women's obesity and Gestational Diabetes on the fatty acid composition of human milk. RECOOP 17th Bridges in Life Sciences Conference, 6-9 April, 2022, Prague, Czech Republic. Book of Abstract (ISBN 978-615-6006-03-5), pp. 89.
4. **Zhang M**., Simon Sarkadi L., Üveges M., Tormási J., Benes E., Vass R., Vari SG. (2023): Gas chromatographic determination of fatty acid composition in breast milk of mothers at different lactation periods. RECOOP 5th International Student Conference, 20-21 April, 2023, Budapest, Hungary. Book of Abstract (ISBN 978-615-6006-04-2), pp. 66.

5. Üveges M., **Zhang M**., Simon Sarkadi L., Tormási J., Benes E., Vass R., Vari SG. (2023): Fatty acid profile of human milk from healthy mothers at various lactation periods. RECOOP 18th Bridges in Life Sciences Conference, 20-21 April, 2023, Budapest, Hungary. Book of Abstract (ISBN 978-615-6006-04-2), pp. 117.
6. **Zhang M**., Simon Sarkadi L., Üveges M., Benes E., Vass R., Matsyura O., Vári S. (2023): Comparison of fatty acid composition of human milk of pregnant women with obesity and gestational diabetes. NUTRITION SCIENCE RESEARCH XI. PhD Conference, 5 May, 2023, Budapest, Hungary. Book of Abstract (ISBN 978-615-5606-14-4), pp. 37.
7. **Zhang M**., Üveges M., Tormási J., Benes E., Simon Sarkadi L., Vass R., Vári S. (2023): Determination of fatty acid composition in breast milk. 4th Young Researchers' International Conference on Chemistry and Chemical Engineering (YRICCCE IV), 1-3 June, 2023, Debrecen, Hungary. Book of Abstract (ISBN 978-615-6018-16-8), pp. 82–83.
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10. **Zhang M**., Simon Sarkadi L., Üveges M., Tormási J., Kolobarić N., Drenjančević I., Vari SG. (2024): Analysis of Fatty Acid Composition in Human Aortic Endothelial Cells Using Gas Chromatography-Flame Ionization Detection. RECOOP 19th Bridges in Life Sciences Conference, 11-12 April, 2024, Bratislava, Slovakia. Book of Abstract (ISBN 978-615-6006-05-9), pp. 129.
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Oral presentation

1. Simon Sarkadi, L., Üveges, M., **Zhang, M**., Tormási, J., Benes, E., Vass, R., Vari, S.G. (2022): Fatty acid composition of human milk of pregnant women with obesity and Gestational Diabetes. RECOOP 17th Bridges in Life Sciences Conference, 6-9 April, 2022,

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Poster presentation

1. **Zhang, M**., Üveges, M., Muránszky, G., Simon Sarkadi, L., Matsyura, O., Ertl, T., Vass, R., Vari, S.G. (2021): Fatty Acid Composition of Mother Milk, XXI EuroFoodChem, 22-24 November, 2021, online, Budapest, Hungary.
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