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OLIGOSACCHARIDES IN HUMAN MILK, ACHIEVEMENTS IN ANALYSIS: A REVIEW

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Abstract
Human milk oligosaccharides (HMOs) comprise about 20% of the total carbohydrates of human milk. There is currently a growing interest in HMOs as many researchers have recognized the importance of their benefits to infant health. Accumulated evidence suggests that HMOs are anti-adhesive antimicrobials that serve as soluble bait receptors, prevent pathogens from attaching to infant mucous membranes, and reduce the risk of viral, bacterial, and protozoan parasites. It also provides functionality including anti-adhesion and immunomodulators. Even though the composition of human milk in Latvia has been studied in detail, there are no studies on oligosaccharides in human milk. The aim of the study is to find out recent advances in the analysis of HMOs. Semi-systematic method was used to analyze the latest information about the recent advances in the analysis of HMOs by liquid phase separation methods, to investigate any known associations between HMOs composition and maternal nutrition and nutritional factors during lactation and the effect of HMOs on the infant's development and health. The analysis of HMOs is considered very complex because of heterogeneity and different isomeric/anomeric structures of compounds. The proposed methods for analysing HMOs are largely based on liquid chromatography.
Key words: human milk, oligosaccharides, analysis, infant's development.

Introduction

Human milk is recognized as an ideal and unique nutrient base for infants (Ballard & Morrow, 2013). Human milk contains a lot of biologically active compounds such as nucleotides, vitamins, proteins including immunoglobulins, oligosaccharides, and minerals. It was recognized as the first functional food in the life of an infant (Aly *et al*., 2018).

Human milk oligosaccharides (HMOs) comprise about 20% of total carbohydrates, being the third largest solid component presented in human milk at concentrations greater than 20 g L^{-1} (Urashima, Asakuma, & Leo, 2012). A wide variety of oligosaccharides is synthesized in the mammary gland under the influence of specific glycosyltransferases, which sequentially attach N-acetylglucosamine, galactose, fucose, and N-acetylneuramic acid to the main acceptor molecule − lactose (Thurl *et al*., 2017). HMOs consist of a complex mixture of more than 200 non-food and non-digestible carbohydrates (Porfirio *et al*., 2020), varies in size from 3 –22 monosaccharide units, in most cases containing fucose, N-acetylglucosamine (GlcNAc), galactose, sialic acid and glucose. All HMOs have a reducing lactose moiety that can be extended by various numbers of lacto-N-biose (Galβ1,3GlcNAc, LacNAc) or N-acetyllactosamine (Galβ1,4GlcNAc, LacNAc) motifs. Branched HMOs are formed by the action of N-acetyllactosaminide β1,6-N-acetylglucosaminyltransferase (GCNT2), which installs a β1,6-linked N-acetyl-glucosamine (GlcNAc) at an internal galactosyl moiety that can be further extended by structures (Prudden *et al*., 2017). Even though the composition of human milk varies from person to person, HMOs is recognized as one of the most important nutritional components that affects the growth and development of the infant (Thurl *et al*., 2017). In the middle of the twentieth century increased interest in HMOs, the importance of HMOs potential benefits to infant health was recognized (Bode, 2012). HMOs have a wide range of activity, preventing pathogenic organisms from contacting the intestinal epithelium and using them as nutrients in selected beneficial bacteria of the same class (Wu *et al*., 2011). Prebiotic activity of HMOs can be observed in various studies, they reveal that HMOs work in bifidogenic molecules, thereby improving the growth of bifidoflora. In addition, prebiotic contributes functionality including anti-adhesion and immunomodulatory (Euler *et al*., 2005). Research by Smilowitz *et al.* (2014) suggests on how other factors interact with each other with the composition of the HMOs, including mode of delivery parity, environmental factors such as seasonality and geographical location, and maternal factors as dietary quality and age. Nursing mothers' health conditions (obesity, malnutrition or hyperglycemia) have a significant effect on HMOs production and structural diversity (Smilowitz *et al*., 2014).

The aim of the study is to find out recent advances in the analysis of HMOs.

Materials and Methods

The databases of scientific papers Web of Science and Scopus were examined in order to find out data from the latest achievements related to analyses of human milk oligosaccharides; scientific papers in English were used for review, which were published from 2011 to 2021. The search was based on combining two key terms: human milk oligosaccharides and human milk oligosaccharide analysis. In the review, 42 full text articles were collected. When searching for their menologies, the author used a wide range of synonyms to search for the corresponding literature. Semi-systematic method was used in the study.

Results and Discussion

According to statistics, for about 20% of children under six months of age, human milk is the only source of nutrients (Slimību profilakses un …, 2020), its diverse nutrient composition promotes protection against pathogenic bacteria or viruses (El-Hawiet, Kitova, & Klassen, 2015), prevents intestinal inflammation, and constructive modulation of immune system response in infant development (He, Lawlor, & Newburg 2016).

HMOs bioactive factors

During lactation, the composition and amount of HMOs in women varies. It is recognized that the concentration of HMOs in the first lactation stage is higher and gradually begins to decrease during the first trimester of lactation. HMOs are recognized as one of the most important bioactive factors that change the outgrowth of genes in immune cells as microbiota modifiers or immune modulators in the gastrointestinal tract work as prebiotics and counting this affects the immune potential of infants (Ray *et al*., 2019). HMOs are resistant to low stomach pH and pancreatic brush enzymes (Bode, 2012). Therefore, they penetrate the colons, and they are substrates of bacterial metabolism. An important role is played by the defense mechanism, thanks to which HMOs divert gastrointestinal infections, being the bait receptor. The gastrointestinal tract is a functional barrier that diverts pathogenic invasion that provides elements of active lymphoid cell lines, which is the main value for infant immunity (Morozov *et al*., 2018). Derivatives from HMOs work as a binding for cells, for example for Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), respectively, they block reactions between viruses or pathogens as well as their receptors, they work as a bait for receptors. Scientific work has shown that HMOs, or rather their branched chains, block viral infections (Gao *et al*., 2020).

Breastfeeding as a source of early life nutrients and especially non-digestible HMOs among the various bioactive components of human milk are considered critical for healthy microbial colonization of infants, fine-tuning of inflammatory processes, and immune defense and maturation in the early years of life (Ayechu-Muruzabal, Stigt, & Mank, 2018). Many diseases with infectious and immune components in their etiology, including diarrhea, respiratory and urinary tract infections, otitis media, bacteremia, and necrotizing enterocolitis occur less often in breast-than formula-fed infants (Donovan & Comstock, 2016).

Relationship between the mother's diet and the HMOs

Studies show that the mother's breast activation diet alters milk components, they are involved in structuring the gut microbiome, potentially affecting the state of metabolism throughout life (Horta & Mola, 2015). Few scientists have investigated the effects of maternal diet on the bioactive components of HMOs (Maxim *et al*., 2020). HMOs and the milk microbiome are particularly interesting components, because they are associated with changes in the microchip microbiome of newborns and older children (Pannaraj, 2017).

As a result of studies of human milk of different populations during lactation, it turned out that several HMOs are reduced during breastfeeding over time and synthesized oligosaccharides differ in different nursing women (Samuel *et al*., 2019). Studies show significantly different concentrations of HMOs in the milk of exclusively breast infants with excessive weight gain (Larsson *et al*., 2019).

Quin *et al.* (2020) in their work analyzed studies on dietary nutrition during the breastfeeding period, which is insufficient basis, to assess the quality of nutrition. Their work focused only on dietary components, but as a result, it turned out that there was a relationship between diet (total sugar, dietary fiber) and fucose/galactose in HMOs. Their assumption that maternal nutrition is important for HMOs biosynthesis is most likely correct, since the biosynthesis pathway is initiated from activated monosaccharides (Quin *et al.*, 2020). These findings may be pointed out in the most recent work by Seferovic *et al.* (2020), which found that changes in maternal nutrition, mainly, replacement of carbohydrate sources, leads to changes in some major components of HMOs. And all this happens in a short time (Seferovic *et al*., 2020). Kristen *et al.* (2017) found in their work that, the milk microbiome is changing if you follow a high fat and carbohydrates diet. High fat content reduces the concentration of sialylated HMOs compared to galactose, glucose significantly alters the concentration of fucosylated HMOs. Interestingly, as it turned out, the concentration of sialylated HMOs has a relationship with the composition of the microbiome. Presumably, these HMOs play a major role in structuring the milk microbiome (Kristen *et al*., 2017).

Probiotic supplements consumed in the last stages of pregnancy have been found to alter the relative composition of HMOs, probably in the formation of the maternal gut microbiome (Seppo *et al*., 2019). It is likely that this, in turn, forms the milk microbiome (through the entero-mammalian pathway) and successively a common dairy environment of which HMO is a part (Moossavi *et al*., 2019). High fiber diets can also be used, where foods such as fruits and whole grains are consumed. This will be a high fiber diet, which is also a mechanism for the formation of the maternal microbiome. In the works of Azad *et al.* (2018) and Quin *et al.* (2020) there was evidence of a connection between the composition of HMOs and the nutritional composition, and, possibly, the education system is the relationship between the maternal diet, the intestinal microbiome, the milk microbiome and the composition of HMOs in it.

Analytical methods for HMOs analysis

Human milk research began in the early 1900s (Bode, 2012), and various approaches to HMOs analysis have been developed. Elucidating the structures of these oligosaccharides are very important in order to detect their biological functions. Research into methods to separate oligosaccharides from human milk samples is still ongoing. The great heterogeneity and complexity of their monosaccharide compositions and bonds is one reason, which complicates their study. The low concentration of HMOs in the presence of high levels of lactose further exacerbates this problem. In addition, oligosaccharides do not have chromophores or fluorophores, which also prevents their optical detection (Auer, Jarvas, & Guttman, 2021).

Sample Preparation – Methods

Prior to starting the analysis, HMOs need to be isolated from other components that are found in human milk, such as lipid, protein and lactose (Balogh, Jankovics, & Beni, 2015). Currently, methods for preparing HMOs samples comprise similar steps, typically starting with the removal of lipid and protein. The degreasing step is often carried out by centrifugation or solvent extraction. Protein precipitates tend to complement organic solvents such as ethanol, chloroform/methanol, acetone or acetonitrile (Tonon *et al*., 2019). For quantitative characterization of oligosaccharides and their further structural clarification, for example by mass spectrometer (MS), it is very important to choose an effective separation method. This must be the way, which is suitable for separating many polar and branched isomeric structures.

High Performance Liquid Chromatography (HPLC) Analysis of HMOs

At the moment, a large number of HPLC methods have been identified, which are used for the analysis of HMOs. HPLC can be used with label-free detection modes such as charged aerosol, refractive index, pulsed amperometry, evaporative light scattering and MS. Alas, without the release of HMOs, the most used optical recognition techniques cannot be identified due to their insufficient absorption of ultraviolet (UV) radiation and lack of fluorescent characteristics. In any case, several UV-active labels or fluorescent ones can be detected, which contribute to increased detection sensitivity. The most widely used are 2-aminobenzamide (2-AB), 2-aminoacridone (2-AMAC), 2-aminobenzoic acid (2-AA), perbenzoylation and 1-phenyl-3-methyl-5-pyranosolone (PMP) (Leeuwen, 2019).

Other studies compared centrifugation and ultrafiltration methods in 15 acidic and neutral HMOs using ultra-high performance liquid chromatography (UHPLC) and fluorescence detection assay (FLD) for this (Huang *et al*., 2019). The method of obtaining a sample for this analysis required only dilution, ultrafiltration or centrifugation, and the derivatization processes contributed to high sensitivity. At the time when in ultrafiltration the reduction process of disiailacto-N-tetraose (DSLNT) took less than 50 minutes. The greatest drawback of methods that are based on liquid chromatography (LC) is the need to use oligosaccharide standards to identify structural refinement of retention times due to similar public glycan libraries. Despite this, standards are often not presented in commercial access, if they have been purchased for commercial purposes, they are very expensive (Austin *et al*., 2018).

HMOs analysis by capillary electrophoresis

In addition to chromatography methods, capillary electrophoresis (CE) is also often used as a strong glycoanalytic tool due to low sample volume requirements, reduced buffer use, high and fast. CE provides several separation modes that are easy to change during operation. In working with CE, derivatization is needed primarily for uncharged carbohydrates in order to provide them with the necessary electromigration (Sarkozy *et al*., 2020). A team of scientists led by Volpi developed a CE method with conventional 254 nm UV detection to identify HMOs standards and human milk oligosaccharides derived from mothers (Galeotti *et al*., 2014). They applied simple steps of pretreating and derivatizing oligosaccharides with a 2- aminoacridone (AMAC), uncharged and hydrophobic fluorescent label. After removal, the labeled oligosaccharides were separated into a borate buffer containing 20% methanol. This CE approach was able to separate basic neutral and acidic oligosaccharides from human milk in the presence of lactose and other high concentration impurities such as excess fluorophores, proteins and salts (Auer, Jarvas, & Guttman, 2021).

Mass spectrometry (MS) HMOs separation methods (related to liquid phase)

MS is often used in the field of glycan analysis and is also successfully used to analyze milk oligosaccharides for structural characteristics. Electrospray ionization (ESI) and matrix laser desorption/ionization (MALDI) are widely used soft ionization modes for the analysis of sugars based on MS. During MALDI ionization, the sample is mixed or coated with an energy-absorbing standard carbohydrate separation matrix and ionized with a laser beam. This method generates single protonated ions from assays (Lai & Wang, 2017). In contrast, ESI genifies multicharged ions by using an electric field to transform the phase assays of solutions in gas phase ions (Zhong *et al*., 2017). A commonly used method is the permethylation of glycan derivatization, which is used to detect multiple sclerosis, since it contributes to the enhancement and improvement of ionization and makes labile sialic acids stable. During the MS analysis period, reinstallation helps to eliminate fucose overheating and guarantee the diagnosis of fragment ions (Zhou *et al*., 2017). MS associated with chromatographic or electromigratic separation techniques facilitates structural.

The use of biosynthetic routes to generate HMOs has not yet been fully resolved, but complex bioanalytic methods can bring us closer to doing this work (Auer, Jarvas, & Guttman, 2021).

Conclusions

Human milk is the first functional food in an infant life. Human milk oligosaccharides account for about 20% of total carbohydrates, and it is the third largest solid component in human milk. HMOs helps to develop many beneficial properties in the infant body. As prebiotics, they are considered metabolic substrates for beneficial bacteria, which gives them an advantage in growth, when compared with pathogens. As anti-plaque antimicrobials, they act as soluble bait of glycan receptors that help prevent contact with pathogens and the intestinal mucosa.

All the time, the tested data indicate how the HMOs has a beneficial effect on breastfeeding, the formation of the intestinal flora, the modulation of immune responses and the protection of the infant body from infections and diseases. These positive properties directly depend on the structure of the HMOs, which allows us to conclude, that a comprehensive approach to data synthesis is very important for understanding the situation. In turn, the analysis of HMOs is considered very complex that heterogeneity and different isomeric/anomeric structures. The lack of simply chromophore/fluorophore or ionizable oligosaccharide groups that make the analysis difficult to achieve. The proposed methods for analysing HMOs are largely based on liquid chromatography. In addition, a wide range of HPLC methods have been identified, they are often performed to identify the composition of HMOs. HPLC is used in the case of detection, for example as refractive index or light scattering − charged aerosol, pulsed MS and amperometry. Alas, without the release of HMOs, the most common optical detection analyses used are not visible due to insufficient absorption of ultraviolet radiation and lack of fluorescent characteristics.

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