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Changes in Human Milk Lipid Composition and Conformational State During a Single Breastfeed

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Abstract: Fat in human milk forms the main energy source for infants and is the most variable component in terms of concentration and composition. Knowledge on changes in human milk lipid composition and conformational state during a single breastfeed contributes to an in-depth understanding of lipid synthesis in the mammary gland. Therefore, the objective of this study was to evaluate the differences in fatty acid length, degree of unsaturation (lipid composition) and lipid phase (lipid conformational state) of milk released at different stages during a breastfeed (fore-, bulk- and hindmilk). A total of 30 samples from 10 lactating subjects were investigated using confocal Raman spectroscopy. No significant differences in lipid composition were observed between fore-, bulk- and hindmilk samples, which is consistent with literature. A new finding from this study is that the lipid conformational state in human fore-, bulk- and hindmilk was significantly different at room temperature. The lipid phase of foremilk was almost crystalline and the lipid phase of hindmilk was almost liquid. Based on this observation, we hypothesize that lipid synthesis in the mammary gland changes during a single breastfeed.

1. Introduction

Human milk is a complex mixture of nutrients and bioactive factors [1]. The macronutrient fat forms the main energy source for infants and is the most variable component in terms of concentration and composition [2]. Understanding the change in human milk lipid composition and conformational state during a single breastfeed contributes to the understanding of the lipid synthesis in the mammary gland. Human milk lipids are gathered in milk fat globules [3,4]. These globules consist of a core rich in triacylglycerol (TAG) molecules [5] and a membrane rich in phospholipids [4]. TAG molecules make up 95 to 98% of the lipids of milk fat globules and are esters of glycerol with three fatty acids [2,4]. The lipid composition of TAG molecules is determined by the length of the fatty acids, degree of unsaturation of the fatty acids, and the stereospecific position of the fatty acids on the glycerol backbone [6]. Furthermore, the lipid conformational state (i.e. lipid phase) at room temperature is influenced by lipid composition and the stereospecific distribution of fatty acids [7]. Both the lipid composition and the conformational state play an important role in the lipid digestion and its nutritional value [6]. Therefore, the goal of this study is to investigate the change in the lipid composition and conformational state during a single breastfeed.

2. Materials and methods

2.1 Human milk samples

10 healthy, lactating subjects donated three milk sample from a single breastfeed. The three donated samples were (1) the first 2 to 5 mL of a breastfeed, named foremilk, (2) the last 2 to 5 mL of a breastfeed, named hindmilk and (3) 2 mL of milk from the total expressed milk volume during the same breastfeed, named bulk milk. The donated samples were stored at -18 ºC. Ethical approval was obtained from the CMO Arnhem-Nijmegen and all participants gave written informed consent prior to the study. As measurement preparation, each individual sample was thawed, homogenized, and diluted four times with phosphate buffered saline (PBS), within 3,5 months after first storage. Each prepared sample was pipetted into a microscope well glass slide and covered with a borosilicate glass cover slip before measurement with the confocal Raman microscope.

2.2 Confocal Raman spectroscopy measurements

Our home build confocal Raman microscope is elaborately discussed in the reference [8]. In brief, the excitation laser of this microscope had a wavelength of 647.1 nm. A 40x / 0.95 NA objective focused the incident light onto the sample. The power at the sample was 35 mW. The scattered light from the sample was collected by the same objective and passed through a dichroic beam splitter and a long pass filter to reduce the contribution of elastically

scattered light. Afterwards, the Raman scattered light is focused on a pinhole and dispersed by a home build spectrograph. The Raman scattered light is finally detected by a 1600 x 200 pixel CCD camera. Per sample, a total of 8100 Raman spectra were collected in a 3D volume of 88 * 10^3 µm³, by laying a 30 x 30 raster pattern over an area of 56 x 56 µm per layer in a total of 9 layers in depth. All Raman spectra were measured with an integration time of 200 ms at a temperature of 20 ºC. Trapping of milk fat globules was prevented by non-sequential scanning, with a fast motion of the focus over a distance ≥ 28 µm between successive measurement points.

2.3   Data processing and analysis

Each spectrum was pre-processed by (1) removal of cosmic ray events, (2) spectral transmission correction, (3) background correction, and (4) calibration of the wavelength axis. Spectra with a dominant auto-fluorescent contribution were identified using an outlier method based on Hotelling's T2 test of the results of a principal component analysis per sample per layer. All these outlier spectra were removed from the data set. The contribution from water to the Raman signal was removed from each individual spectrum by subtraction of a fitted, pure PBS spectrum from each spectrum. Since the focus of this study was to evaluate the lipid composition and conformational state of human milk, the last data processing step was selection of spectra containing a significant lipid contribution. Only spectra which conform to equation $I_{1745} / (I_{max} - I_{min}) \geq 11$ were used for data analysis, with I_{1745} the intensity of the band located at 1745 cm^{-1} and I_{max} and I_{min} respectively the maximum and minimum value in the region 1800 until 1900 cm^{-1}. Data analysis consisted of band intensity ratios. Each band intensity was calculated by taking the average intensity of the band and subtracting the average baseline close to the band as described in more detail in reference [9].

3. Results and Discussion

3.1   Lipid composition

The lipid composition is defined by the fatty acid length and degree of unsaturation and the stereospecific position of fatty acids [6]. The ratios δ(1440) / ν(1745) and ν(1655) / δ(1440) were used to quantify the fatty acid length and degree of unsaturation respectively [7]. The median values of these ratios for all samples are presented in figure 1a. Fig 1a demonstrates that the lipid composition was different for samples donated by different subjects. Fig 1b shows the median ratio values of the different sample types over all 10 subjects. No significant difference was observed in the fatty acid length and degree of unsaturation between the lipids in fore-, bulk-, and hindmilk. The median value of the fatty acid length and degree of unsaturation are respectively 17.19 ± 0.18 carbon atoms per fatty acid and 0.922 ± 0.023 double bonds per 16 single carbon atoms. This is in agreement with literature [10].
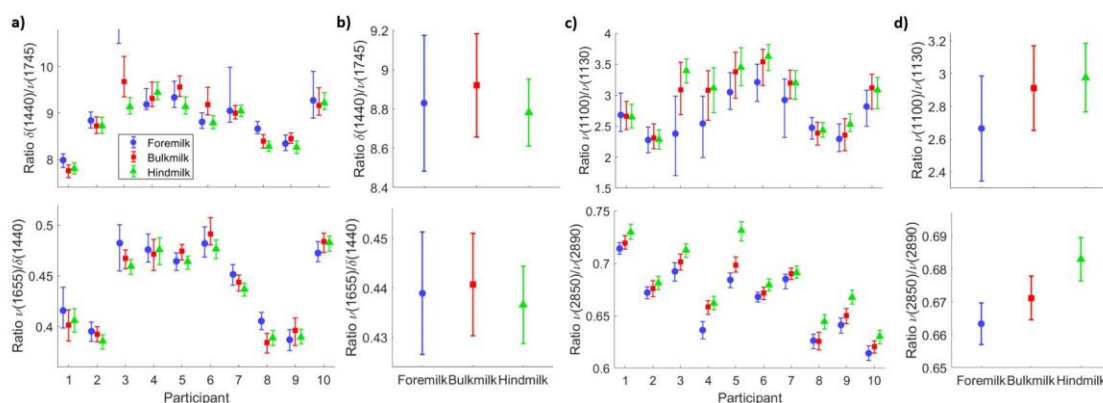

Figure 1. (a) lipid composition ratios for each participant (b) median ratio values of the lipid composition over all 10 participants for each milk type (c) lipid conformational state ratios for each participant (d) median ratio values of the lipid conformational state over all 10 participants for each milk type

3.2   Lipid conformational state

Milk fat at room temperature is in a mixed crystalline/liquid state [7]. The lipid conformational state is related to the lipid phase. The ratio ν(1100) / ν(1130) was used to quantify the ratio gauche to trans conformations of the C-C bonds of the fatty acids. Trans conformation is the out-of-phase conformational state and gauche conformation is the in-phase conformation, these conformations are related to the crystalline and liquid phase respectively [7,11]. The ratio ν(2850) / ν(2890) is sensitive to interchain packing and intrachain conformational disorder of fatty acids, which

is also phase related. The median values of these ratios for all samples are shown in figure 1c. Fig 1c demonstrates that the lipid phase is different for milk donated by different subjects. Fig 1d presents the median ratio values over all 10 participants for the three kind of samples. The ratio $\nu(1100)$ / $\nu(1130)$ indicates a higher presence of gauche conformation in the fatty acid chains of lipids in hindmilk than foremilk. The ratio $\nu(2850)$ / $\nu(2890)$ indicates a higher magnitude of disordering of the fatty acid chains of lipids in hindmilk than foremilk. A higher presence of gauche conformation in fatty acids and the higher magnitude of disordering of the fatty acids correspond to a more fluid state of the lipids in hindmilk than in foremilk at room temperature.

Fig 2 shows results of individual, unaveraged Raman spectra. One spectrum with a high degree of lipids in the crystalline phase (foremilk sample participant 5) and one spectrum with a high degree of lipids in the liquid phase (hindmilk sample participant 5). The difference in the Raman spectra between the crystalline and liquid phase of the lipids is revealed by the difference spectrum in figure 2. The crystalline phase of lipids was identified by sharp high intensity bands located at 1060 and 1130 cm^{-1}, shift of the band around 1300 cm^{-1} from 1302 to 1297 cm^{-1}, a better distinguishable shoulder band located at 1460 cm^{-1}, and the higher intensity of the band located at 2890 cm^{-1}.
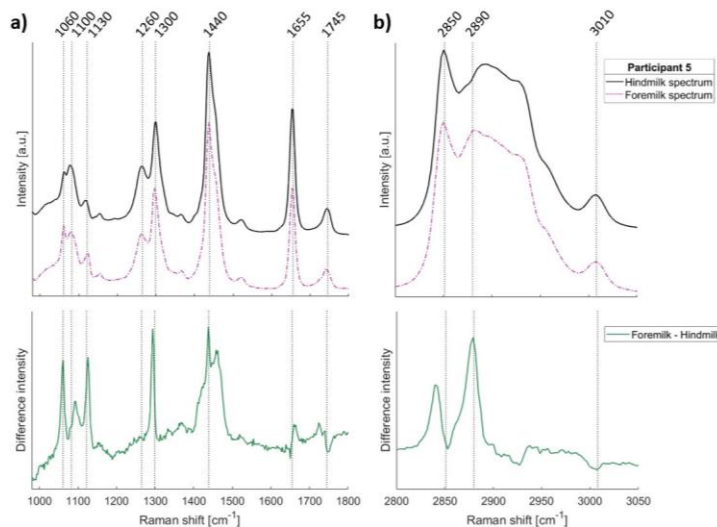

Figure 2: Two typical, individual Raman spectra of fore- and hindmilk samples donated by participant 5 in a) the fingerprint region and b) the high Raman shift region. The foremilk spectrum shows features of lipids in a more crystalline state and the hindmilk spectrum shows features of lipids in a more liquid state. The Raman difference spectrum given in the bottom graph demonstrates the intensity differences between the crystalline and liquid phase lipid spectra.

4. Conclusion

Thus, the intensity ratios and the Raman spectra indicate an increase in the lipid phase during a single breastfeed. Lipid phase is influenced by fatty acid length, degree of unsaturation and stereospecificity of fatty acids [7,11]. Since we did not observe a change in lipid composition, we argue that the change in lipid phase during a single breastfeed is caused by a change in the stereospecific position of fatty acids. The latter has a great influence on the lipid digestion and absorption. The change during a single breastfeed might be explained by a change in the lipid synthesis in the mammary glands during a breastfeed. This study raises important questions about the lipid origin, synthesis and biological function and further research is required.

4. References

[1] Ullah, R., Khan, S., Javaid, S., Ali, H., Bilal, M., and Saleem, M. "Raman spectroscopy combined with a support vector machine for differentiating between feeding male and female infants mother's milk" Biomedical optics express 9.2, 844-851 (2018).
[2] Wei, W., Qingzhe, J., and Xingguo, W. "Human milk fat substitutes: Past achievements and current trends" Progress in lipid research 74: 69-86 (2019).
[3] Yao, Y., Zhao, G , Yan, Y., Mu, H., Jin, Q, Zou, X., and Wang, X. "Milk fat globules by confocal Raman microscopy: Differences in human, bovine and caprine milk" Food Research International, 80:61–69 (2016).
[4] Lopez, C. "Milk fat globules enveloped by their biological membrane: unique colloidal assemblies with a specific composition and structure." Current Opinion in Colloid & Interface Science 16(5): 391-404 (2011).
[5] Argov, N , Wachsmann-Hogiu, S., Freeman, S.L., Huser, T., Lebrilla, C.B., and German, J.B. "Size-dependent lipid content in human milk fat globules" Journal of agricultural and food chemistry, 56(16): 7446–7450 (2008).
[6] Haddad, I, Mozzon, M., and Frega, M.G. "Trends in fatty acids positional distribution in human colostrum, transitional, and mature milk" European Food Research and Technology 235.2: 325-332 (2012).
[7] Gallier, S., Gordon, K.C., Jimenez-Flores, R., and Everett, D.W. "Composition of bovine milk fat globules by confocal Raman microscopy" International Dairy Journal, 21(6):402–412 (2011).
[8] Pully, V.V., Lenferink, A. and Otto, C. "Hybrid Rayleigh, Raman and two-photon excited fluorescence spectral confocal microscopy of living cells". Journal of Raman Spectroscopy 41(6): 599-608 (2010).
[9] Kunstar, A., Otto, C., Karperien, M., van Blitterswijk, C., and van Apeldoorn, A. "Raman microspectroscopy: a noninvasive analysis tool for monitoring of collagen-containing extracellular matrix formation in a medium-throughput culture system". Tissue Engineering Part C: Methods, 17(7): 737-744 (2011).
[10] Yuhas, R., Pramuk, K., and Lien, E.L. "Human milk fatty acid composition from nine countries varies most in DHA" Lipids, 41(9):851–858 (2006).
[11] Dmitriev, A.A. and Surovtsev, N.V. "Temperature-dependent hydrocarbon chain disorder in phosphatidylcholine bilayers studied by Raman spectroscopy" The Journal of Physical Chemistry B, 119(51):15613–15622 (2015).