Skoltech
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Thesis Changes Log

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PhD Program: Life Sciences

Title of Thesis: Role of breast milk lipid composition in postnatal brain development

Supervisor: Prof. Philipp Khaitovich

The thesis document includes the following changes in answer to the external review process.

I want to thank the reviewers for their valuable comments and discussion. In addition to the corrections mentioned below, I have also added the Conclusions part to my thesis so that it follows the structure as recommended by Skoltech PhD Defence Policy.

Reviewer: Prof. Andrey Mironov

Comment 1. p.12. "Mature lactocytes produce lactose, a unique oligosaccharide found only in milk" – lactose is a disaccharide.

Answer: Thank you for your comment. There are different approaches for carbohydrates classification, in most references carbohydrates consisting of 3 to ten monosaccharide subunits are defined as oligosaccharide (Laurentin, 2013), although in some references carbohydrates consisting of 2 to ten units, including disaccharides and thus lactose, are considered oligosaccharides (Dilworth, 2017). I used the second classification approach in my thesis text.

A. Laurentin, C.A. Edwards, Fiber: Resistant Starch and Oligosaccharides, Encyclopedia of Human Nutrition (Third Edition), Academic Press, 2013, Pages 246-253.

L.L. Dilworth, C.K. Riley, D.K. Stennett, Chapter 5 - Plant Constituents: Carbohydrates, Oils, Resins, Balsams, and Plant Hormones, Pharmacognosy, Academic Press, 2017, Pages 61-80.

Comment 2. P.12 " lactose draws much water under osmotic pressure, diluting milk and thus lowering concentrations of fat and protein" – if lactose binds water the concentration of fat and protein should increase.

Answer: Thank you for the comment. Here I did not want to compare the relative concentrations of lactose versus fat and protein. I wanted to point out that the presence of lactose creates osmotic pressure that attracts much water (National Research Council, 2014), while the amount of fat and protein does not change (Jennes, 1986). Thus milks of some mammals are high in lactose and more diluted, like in humans and great apes, horses, rhinoceroses (Hinde, 2011; Oftedal, 1993a), whereas milks of other mammals are low in lactose and more dense like in elephants, bears, seals (Abbondanza, 2013; Eisert, 2013; Oftedal, 1993b).

Jenness, R. (1986). Lactational performance of various mammalian species. *Journal of Dairy Science*, *69*(3), 869–885.

Hinde, K., & Milligan, L. A. (2011). Primate milk: proximate mechanisms and ultimate perspectives. *Evolutionary Anthropology*, *20*(1), 9–23.

Oftedal, O. T. (1993). The Adaptation of Milk Secretion to the Constraints of Fasting in Bears, Seals, and Baleen Whales. *Journal of Dairy Science*, *76*(10), 3234–3246.

National Research Council (US) Committee on Technological Options to Improve the Nutritional Attributes of Animal Products. (2014). *Designing Foods: Animal Product Options in the Marketplace*. National Academies Press (US).

Abbondanza, F. N., Power, M. L., Dickson, M. A., Brown, J., & Oftedal, O. T. (2013). Variation in the composition of milk of Asian elephants (Elephas maximus) throughout lactation. *Zoo Biology*, *32*(3), 291–298.

Eisert, R., Oftedal, O. T., & Barrell, G. K. (2013). Milk Composition in the Weddell Seal Leptonychotes weddellii: Evidence for a Functional Role of Milk Carbohydrates in Pinnipeds. *Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches*, *86*(2), 159–175.

Oftedal, O. T., Bowen, W. D., & Boness, D. J. (1993). Energy Transfer by Lactating Hooded Seals and Nutrient Deposition in Their Pups during the Four Days from Birth to Weaning. *Physiological Zoology*, *66*(3), 412–436.

Comment 3. p.33 “cows (n= 4)” Domestic cows are a product of selection, including for the fat content of milk. Were the cows of the same breed or different, were the cows of the same age?

Answer: Thank you for the question. In our study we considered Holstein cows both from Moscow and from Shanghai. However, cow’s breed was shown to have little or no effect on the lipid composition of milk (Hanus, 2018). Although previous studies suggest that cows' age has some effect on the milk composition (Khan, 1996), we did not investigate the effect of age in our study because of the lack of meta information.

Hanuš, O., Samková, E., Křížová, L., Hasoňová, L., & Kala, R. (2018). Role of Fatty Acids in Milk Fat and the Influence of Selected Factors on Their Variability-A Review. *Molecules* , *23*(7).

Khan, M. S., & Shook, G. E. (1996). Effects of Age on Milk Yield: Time Trends and Method of Adjustment. *Journal of Dairy Science*, *79*(6), 1057–1064.

Comment 4. Fig 1 – Milk lipidome evolution – incorrect figure title. Evolution is a time process while the figure presents only phylogenetic relation. Panel C – the color notation should be described.

Answer: Thank you for the comment. The title of the figure was changed to "Phylogenetic relationship among studied species". Color notation and description were added to fig.1 (now panel D).

Comment 5. p.35 What is residual variance in MDS (fig.1B)?

Answer: Thank you for the question. The residual variance calculated for the MDS is 12%. I have now added the plot that shows stress decline with increasing dimensionality (fig. 1 panel C).

Comment 6. p.43 PCA (Figures 5,6) shows a significant difference between Moscow and Shanghai populations. Is this difference due to the race difference or nutrition?

Answer: This is a very good question. Maternal nutrition has a great influence on the lipid composition of breast milk, especially in terms of essential LCPUFAs (Martin, 2012; Mulder, 2018; Cimatti, 2018). In this study we did not have an opportunity to record such data, but in our case the two groups represent two different nationalities with different eating habits, so we assume that nutritional factor will be incorporated in the population (or the race) factor. Population is the strongest factor affecting the differences between the two groups of humans, followed by lactation, parity (number of babies a mother has previously had), mode of delivery (natural or Caesarean section), and baby's sex. I have now added the information to part 3.2 Breast milk lipidome analysis in the main cohort of the Chapter 3, Results, p. 44 and added the plot that shows factors that underlie the differences between the two human populations to the Appendices section (supplementary fig. S1 A).

Martin, Melanie A., William D. Lassek, Steven J. C. Gaulin, Rhobert W. Evans, Jessica G. Woo, Sheela R. Geraghty, Barbara S. Davidson, Ardythe L. Morrow, Hillard S. Kaplan, and Michael D. Gurven. 2012. Fatty Acid Composition in the Mature Milk of Bolivian Forager-Horticulturalists: Controlled Comparisons with a US Sample. *Maternal & Child Nutrition* 8 (3): 404–18.

Mulder, Kelly A., Rajavel Elango, and Sheila M. Innis. 2018. Fetal DHA Inadequacy and the Impact on Child Neurodevelopment: A Follow-up of a Randomised Trial of Maternal DHA Supplementation in Pregnancy. *The British Journal of Nutrition* 119 (3): 271–79.

Cimatti, Anna Giulia, Silvia Martini, Alessandra Munarini, Maximilano Zioutas, Francesca Vitali, Arianna Aceti, Vilma Mantovani, Giacomo Faldella, and Luigi Corvaglia. 2018. Maternal Supplementation With Krill Oil During Breastfeeding and Long-Chain Polyunsaturated Fatty Acids (LCPUFAs) Composition of Human Milk: A Feasibility Study. *Frontiers in Pediatrics* 6 (December): 407.

Comment 7. p.53 Clustering on Fig.12A is not obvious.

Answer: Thank you for pointing this out. First panel of figure A is there to show the distribution of the studied fatty acids on the retention time - mass over charge axis, this is a good way to demonstrate the composition of these fatty acids according to their carbon chain length and the number of the double bonds. We use the same coloring for panels A and D although they are not for demonstration of clustering. Cluster analysis was performed on the signal intensities, which are reflected on figures 12 B and 12 C. Another way to show this is on panels C and F, where fatty acids of the same length or the number of double bonds tend to appear in the same clusters.

Comment 8. p.67 I can not understand: "This analysis demonstrated that linoleic (18:2), alphalinolenic (18:3), docosahexaenoic (22:6) and tetracosapentaenoic (24:5) FAs were present at significantly higher levels in human breast milk", but in figure 17 these FA show log fold change about 0, while 28:1, 28:2, 26:1, 27:1 really have a significant fold change in the brain (PFC as well as CB). Moreover, only these FAs define the correlation – without these points, the correlation coefficient became near zero.

Answer: Thank you for the comment and I apologise for confusion. When saying significantly higher in human breast milk, I meant the results of the t-test and the comparison of the concentrations of fatty acids in humans versus macaques. In figure 17 colors represent the results of the t-test: fatty acids colored in purple are significant in milk, fatty acids colored in orange are significant in the brain, fatty acids colored in red are significant in both brain and milk. I have now added the color legend for the figure.

Comment 9. fig.18A – the confidence intervals should be shown

Answer: Thank you for the comment. I have now added confidence intervals at 0.95 confidence level to the plot (fig. 18 A).

Reviewer: Prof. Christoph Borchers

Comment 1. Although this thesis is a fountain of new data, results and insights of the lipids in biological specimens the candidate has just published one publication on this topic, however, I expect more papers will come out of this study.

Answer: Thank you for your comment. We do have a lot of data on the project that is yet to be published. The publication on the comparison of the fatty acid composition in the brain and milk of the seven mammalian species is now in preparation. The data related to the intact lipodome of the brain and the intact lipidome of milk is under analysis.

Reviewer: Prof. Maria do Rosário G.R. Marques Domingues

Comment 1. It would benefit this chapter, namely in section 1.2, if have been added a brief description of the composition of lipid profile (phospholipid and other polar lipids where FA are esterified) of brain in more detail, to complement the very nice description of fatty acid composition.

Answer: Thank you for the comment. I have added the description of lipid profile of the brain to the section 1.2 Role of lipids in the brain development of the Introduction chapter of the thesis (pp. 15-16).

Reviewer: Prof. Yuri Kotelevtsev

Comment 1. The author probably should have taken in account the fact that cows and pigs were the subject of intensive human selection for thousands of years. Particularly cows were selected for milk productivity and contents including fat qualitative and quantitative parameters. I believe thet the work would benefit if the fact of human artificial selection would be taken into account.

Answer: Thank you for the comment. I have now added information on the effect of the artificial selection on the milk composition of domesticated animals to the Chapter 1. Introduction, part 1.1 Evolution of lactation (p. 13).

Reviewer: Prof. Evgeny Nikolaev

Comment 1. On p. 20 of Introduction part 1.3 Mass-spectrometry of lipids, the author states that "the advantage of metabolomics is its lower cost compared to other omics platforms, such as genomics, transcriptomics, and proteomics". Metabolomics is the same cost as proteomics. Please rewrite the phrase.

Answer: Thank you for the comment. I have now modified the phrase.

Comment 2. Speaking about the invention of the two techniques, matrix-assisted laser desorption ionization, MALDI, and electrospray ionization, ESI, the author gives erroneous citation (Han and Gross 1994; Kim 1994) on p.21 of the same chapter. Correct citation has to be included.

Answer: Thank you for the notice. I have changed the reference for MALDI and ESI invention.

Comment 3. Author writes that "double bond configurations cannot be assigned based on tandem mass-spectrometry" p.22, however there are several mass-spectrometry techniques that allow investigation of the double bonds.

Answer: Thank you for the comment. I have now changed the sentence and listed the techniques.

Comment 4. The legend of fig. 4 is not clear, specify how the pattern on the right was obtained.

Answer: Thank you for the comment and I apologise for the confusion. I have now changed the legend to fig. 4.

Comment 5. p-values in fig. 16 p. 69 need to be rounded.

Answer: Thank you for the comment. I rounded p-values and used e notation in fig. 16.

Comment 6. Author uses the identification of lipid species generated by their own annotation method, but it should be taken into account that the method allows to assign particular lipids only with a certain degree of accuracy.

Answer: Thank you for the comment. I have added information on the accuracy for the annotation in Chapter 2. Materials and Methods, part 2.2 Milk hydrolysis, p. 32. I also use different formulations and correct from “we describe” to “we made an attempt to annotate and describe” when writing about the fatty acids analysis in chapter 4. Discussion, p. 81.