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# Relationship between Nutritional Composition of Donor Human Milk and Eating Habits and Body Composition of Human Milk Donors: A cross-sectional pilot study conducted by the Human Milk Bank in Portugal's Northern Region

# *Relação entre a Composição Nutricional do Leite Humano de Dadora e os Hábitos Alimentares e a Composição Corporal de Dadoras de Leite Humano: estudo piloto transversal conduzido no Banco de Leite Humano da Região Norte de Portugal*


Sara Lopes Salgueiro da Silva

**ORIENTADO POR: PROFª DOUTORA DIANA MARIA VELOSO E SILVA**
**COORIENTADO POR: MESTRE BEATRIZ DE OLIVEIRA TEIXEIRA**



TRABALHO DE INVESTIGAÇÃO
1.º CICLO EM CIÊNCIAS DA NUTRIÇÃO | UNIDADE CURRICULAR ESTÁGIO
FACULDADE DE CIÊNCIAS DA NUTRIÇÃO E ALIMENTAÇÃO DA UNIVERSIDADE DO PORTO

**TC**

**PORTO, 2023**

**Abstract**

**Introduction**: Donor Human Milk (DHM) is recommended in Neonatal Intensive Care Units (NICUs) when maternal breast milk is not available.

**Aim:** To explore the association between eating habits, body mass index (BMI) and body composition of Human Milk Donors (HMD) recruited by the Human Milk Bank in the Northern Region of Portugal with the nutritional composition of their donor milk.

**Methodology:** A cross-sectional observational study involved 25 HMD aged from 26.0 to 39.0 years. Questionnaires were administered through interviews to collect sociodemographic, lifestyle, and clinical data. Height, weight and fat mass (FM) were measured and BMI calculated. Eating habits were assessed using a Food Frequency Questionnaire (FFQ). DHM samples were analysed before and after pasteurization using the Miris Human Milk Analyzer™. Associations were estimated using Spearman's correlations.

**Results:** Eleven (44%) HMD were overweight with a median fat mass of 27.5%. No significant associations were found between BMI, FM, demographic or lifestyle data of HMD and the nutritional composition of DHM. However, protein content in DHM showed positive correlations with daily energy intake before pasteurization ($r=0.440$, $p=0.028$) and with carbohydrate intake before ($r=0.611$, $p=0.001$) and after pasteurization ($r=0.499$, $p=0.011$). Fish and seafood consumption were negatively correlated with energy ($r=-0.473$, $p=0.017$) and fat ($r=-0.480$, $p=0.015$) content in DHM. Rice and pasta intake were positively associated with carbohydrate content in DHM before and after pasteurization ($r=0.659$, $p<0.001$; $r=0.495$, $p=0.012$).

**Conclusion:** These results highlight the importance of HMD' nutritional monitoring to optimize their nutritional status and the composition of their donor milk.

**Keywords:** body mass index, donor human milk, eating habits, human milk donors, nutritional composition.

**Resumo**

**Introdução:** O Leite Humano de Dadora (LHD) é recomendado nas Unidades de Cuidados Intensivos Neonatais (UCIN) quando o leite materno está indisponível.

**Objetivo:** Explorar a associação entre hábitos alimentares, índice de massa corporal (IMC) e composição corporal de Doadoras de Leite Humano (DLH) recrutadas pelo Banco de Leite Humano da Região Norte de Portugal com a composição nutricional de leite doado.

**Metodologia:** Estudo transversal com 25 DLH entre os 26,0 e os 39,0 anos. Obtiveram-se dados sociodemográficos/clínicos/estilo de vida por aplicação de questionários. Mediu-se a altura, peso e massa gorda (MG) e calculou-se o IMC. Os hábitos alimentares foram avaliados usando um Questionário de Frequência Alimentar. Amostras de LHD antes e após a pasteurização foram analisadas com o Miris Human Milk Analyzer™. As associações foram estimadas através de correlações de Spearman.

**Resultados:** Onze (44%) DLH apresentavam excesso de peso e uma mediana de MG de 27,5%. Não foram encontradas associações significativas entre IMC, MG, dados demográficos/estilo de vida das DLH com a composição nutricional do LHD. Antes da pasteurização, o teor proteico do LHD associou-se positivamente à ingestão energética diária (r=0,440; p=0,028) e à de hidratos de carbono (HC) antes (r=0,611; p=0,001) e após a pasteurização (r=0,499; p=0,011). O consumo de pescado associou-se negativamente à energia (r=-0,473; p=0,017) e gordura (r=-0,480; p=0,015) do LHD. A ingestão de arroz e massa associou-se positivamente aos HC do LHD antes e após a pasteurização (r=0,659; p<0,001; r=0,495; p=0,012).

**Conclusão:** O acompanhamento nutricional das DLH é crucial para otimizar o seu estado nutricional e a composição nutricional do leite doado.

**Palavras-Chave:** composição nutricional, dadoras de leite humano, hábitos alimentares, índice de massa corporal, leite humano de dadora.

**Abbreviations**

BMI – Body Mass Index

CHUSJ – Centro Hospitalar Universitário de São João

cm – centimetres

DHA - Docosahexaenoic acid

DHM – Donor Human Milk

FFQ – Food Frequency Questionnaire

FM – Fat Mass

g – grams

HM – Human Milk

HMD – Human Milk Donors

kcal – kilocalories

kg – kilograms

ml – millilitres

MOM - Mother's Own Milk

NICU – Neonatal Intensive Care Unit

p25 – percentile 25

p75 – percentile 75

SPSS - Statistical Package for the Social Sciences

WHO – World Health Organization

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**Introduction**

Maternal breast milk is a natural and adequate food that helps to meet the energy and nutritional needs of any newborn. Currently, the beneficial effects of breastfeeding are widely recognized on infants' and mothers' health[1, 2]. Literature has been associating breastfeeding with a lower risk of morbidity and mortality, as well as improvements in infants' neurodevelopment. Also, breastfeeding has been associated with a lower incidence of breast and ovarian cancer in women[1, 3].

The World Health Organization (WHO) currently recommends that infants should be breastfeed exclusively during the first 6 months of life and should continue breastfeeding for 2 years alongside with food diversification[4]. However, there may be circumstances where a mother's own milk (MOM) is not available or is insufficient to meet infants' needs, which is a common challenge in Neonatal Intensive Care Units (NICUs)[5]. In these cases, the American Academy of Pediatrics suggests donor human milk (DHM) as a viable alternative due to the supremacy of human milk (HM) characteristics compared to commercial formulas[6, 7]. Using DHM from Human Milk Banks is particularly important for preterm or newborns with low birth weight, when MOM cannot be used[6].

According to previously randomized clinical trials[7], feeding infants with DHM instead of commercial formulas has several advantages such as the higher protection conferred against necrotizing enterocolitis, bronchopulmonary dysplasia and different types of infections[8]. However, although HM has the necessary nutrients for the healthy development of infants up to 6 months of age, literature suggest that several factors such as mother's diet and maternal body composition, can cause variations in the concentration of certain nutrients[3]. Actually, changes in fat and protein content of HM, but not in lactose, are being associated with lactating women' diet in literature[9]. Also, it has been increasingly suggested that maternal overweight may be associated with nutritional composition changes of HM, particularly in terms of fat and lactose levels[10].

As already mentioned, although DHM is a highly beneficial choice for preterm infants or newborns with low birth weight, it is crucial to further our understanding about the factors associated with its nutritional composition, in order to promote its optimization[2]. In Portugal, there is a rising interest in personalized fortification of DHM. Studies have already assessed the influence of DHM's nutritional composition on the growth of recipient infants[11], however the potential impact of Human Milk Donors (HMD)' diet on DHM's composition has not yet been investigated. Also, given the high prevalence of Portuguese women in a reproductive age with obesity[12], it is urgent to understand the possible implications of their eating habits, body mass index (BMI) and body composition on the DHM's nutritional composition.

**Aim**

The present work aimed to study the association between the eating habits, BMI and body composition of HMD recruited by the Human Milk Bank in the Northern Region of Portugal, specifically at the Centro Hospitalar Universitário de São João (CHUSJ) with the nutritional composition of their donor milk.

**Methodology**

1. Study design and sampling

A cross-sectional observational study was conducted between March and May 2023 at CHUSJ in HMD. To became a HMD, women have to follow specific pre-established criteria such as (a) being a healthy lactating woman who, despite exclusively breastfeeding her own child, has to have excess milk, (b) be the mother of an infant with less than 6 months of life, (c) do not smoke or consume alcoholic beverages with a high intake of caffeine content and (d) not having a positive test for HIV 1 or 2, Hepatitis B or C, Human T-cell Lymphotropic Virus I or II or Syphilis. The complete description of the inclusion criteria is described else-where(13). Besides this, all participants must have ≥18 years and cannot have any special dietary needs. At the start of the study, 26 HMD were active and 5 were included, resulting in a total of 31 HMD. Of these, 25 were included in this study (participation proportion = 80.6%).

2. Ethics

The present research was approved by the Ethics Committee of the CHUSJ / FMUP (report number 78/2023) and all ethical principles of Declaration of Helsinki, the Portuguese Law and the Good Clinical Practice Guidelines were followed. Informed consents were obtained from each participant, authorizing their participation in the study, after presenting all the necessary information and the respective rights regarding their collaboration in this research.

3. Data collection

In order to achieve the study aim, a questionnaire with sociodemographic, lifestyle, and clinical data was created and applied thought an interview during a clinical appointment at the Pediatrics/Obstetrics Service of CHUSJ. Nutritional composition of milk samples of each participant was analysed and recorded at the Human Milk Bank in the Northern Region of Portugal.

Sociodemographic data included participants' age, in years, as well as their educational level, according to 11 items listed from "without formal education" to "doctorate". We further categorized this into two groups: secondary level (12 years of schooling) and having a superior degree (>12 years of schooling), once there was not anyone with a less educational level. Profession was asked as an open answer and subsequently categorized as either healthcare or non-healthcare professional.

Lifestyle data related to smoking and physical activity were collected with dichotomous questions (yes/no). The participants answered 'yes' if they had ever smoked in their life, and regularly do physical activity. Regarding to sleeping habits, their daily hours of sleep during week and weekends were asked. Then, the average of both periods was used. Eating habits of the previous year were assessed, by interview, thought a semiquantitative Food Frequency Questionnaire (FFQ) validated for Portuguese adult population[14, 15]. Food frequency was indicated by the participants according to 9 categories from “never or less than once per month” to “more than six times a day”. For each food item, the amount ingested was registered according to the comparison with pre-established average portions. The consumption of each food, in grams, was calculated by multiplying the frequency of consumption by the portion size reported by the participants and, if appropriable, by a seasonal variation factor. The Food Processor Plus software (ESHA Research, Salem, Oregon) was used to convert FFQ items into nutrient intakes expressed in grams[14, 15].

Anthropometric measures were collected according to standard procedures[16]. Height was measured in centimeters (with 0,1 cm of resolution) using a SECA 287 ultrasonic measuring station or, if not possible, it was obtained from the reported height on the citizen card. Body weight, in kilograms (kg), and fat mass (FM), in percentage (%), were determined using the TANITA® TBF-300 Bioimpedance scale. BMI was calculated and categorized according to WHO criteria: underweight (<18.5 $kg/m^2$), normal weight (18.5-24.9 $kg/m^2$); pre-obesity (25.0-29.9 $kg/m^2$) and obesity (≥30.0 $kg/m^2$)[17].

HMD' obstetric history was evaluated. The number of pregnancies and the type of delivery were asked, as well as the birth weight and gestational age of their infant which had resulted from their last pregnancy. Infants were classified as preterm if their gestational age was less than 37 weeks[18].

DHM samples' nutritional composition was analysed, before and after pasteurization, using the Miris Human Milk Analyzer™ (Miris AB, Uppsala, Sweden), an analytical innovative technique based on mid-infrared transmission spectroscopy principles[19]. The samples of raw DHM were collected by HMD in their own homes after they were instructed on good practices for extracting and cold storing milk. When the raw DHM arrives at the Human Milk Bank, samples' conformity parameters were checked (for example organoleptic characteristics, bottles condition and the range of storage temperature of the raw DHM). The Miris Human Milk Analyzer™ measured milk samples' true protein, crude protein, fat and carbohydrate contents, in grams (g) per 100 millilitres (ml), and its energy, in kilocalories (kcal) per 100 ml, using 3 ml sample of DHM. Prior to analysis, the device was calibrated by the calibration solution Miris Calibration

Control Kit™ (Miris AB, Uppsala, Sweden) and the samples were homogenized using the Miris Ultrasonic Processor™ (Miris AB, Uppsala, Sweden) and warmed according to the respective protocol[(19)]. Finally, DHM samples were injected into this device and analysed before and after pasteurization.

4. Statistical analysis

The collected data was compiled in a database using the IBM SPSS® program (Statistical Package for Social Sciences), version 28.0, for the respective statistical analysis. The normality of each quantitative variable was tested using the Shapiro-Wilk test. To streamline the analysis, the median and 25th percentile (p25) and 75th percentile (p75) were used, as certain variables did not follow a normal distribution. Categorical variables were expressed as absolute and relative frequencies.

Comparison between DHM's nutritional composition before and after pasteurization was conducted using the Wilcoxon Test. The percentual impact (Delta %) of each DHM's component analysed was calculated by the ratio, in percentage, between the difference of each component before and after pasteurization and the respective value before pasteurization. The energy and macronutrients contents of DHM before/after pasteurization was compared with the *gold standard* of mature HM [(20)] using a One-Sample Wilcoxon Signed Rank Test.

Correlations between DHM's nutritional composition before/after pasteurization and age, sleep hours, BMI and FM of HMD and birth weight and gestational age of their infants were determined using Spearman's correlation coefficients. An identical approach was used to identify the correlations between the HMD' dietary habits and respective energy and macronutrient intake with the nutritional composition of DHM before and after pasteurization. An alpha level of <0.05 was set for all analyses.

## Results

The median age of this sample was 34.0, ranging from 26.0 to 39.0 years old. Twenty-two (88%) had a superior degree, 32.0 % (n=8) were pregnant for the first time, 48.0 % (n=12) had a caesarean section, 44 % (n=11) practiced regular physical activity and slept a median of 7.3 hours per day (Table 1). Also, 44% (n=11) were overweight with a median FM value of 27.5% (Table 2). In Attachment A it presents the descriptive characteristic of the HMD´ infants.

**Table 1- Human Milk Donors' characteristics (n=25)**

Legend – p25: 25th percentile; p75: 75th percentile; h: hours

| | |
|---|---|
| **Age, years, median (p25,p75)** | 34.0 (30.5, 36.5) |
| **School level, n (%)** | |
| *Secondary level* | *3 (12.0)* |
| *Superior degree* | *22 (88.0)* |
| **Health professional, n (%)** | 8 (32.0) |
| **First pregnancy, n (%)** | 8 (32.0) |
| **Cesarean delivery, n (%)** | 12 (48.0) |
| **Smoking (at least once during life), n (%)** | 8 (32.0) |
| **Regular physical activity, n (%)** | 11(44.0) |
| **Sleep hours, h, median (p25,p75)** | 7.3 (6.0, 8.0) |

**Table 2 - Human Milk Donors' nutritional status and body composition (n=25)**

Legend – p25: 25th percentile; p75: 75th percentile; BMI: body mass index; $kg/m^2$: kilogram per square meter; %: percentage
*According to WHO classification: World Health Organization. Obesity: preventions and managing the global epidemic. Report on a consulive meeting. Geneva, Switzerland: Author; 1997.;
**Body fat percentage. [website]. Tanita; 2012 - 2022. [cited on: 23 Jun 18]. Availiable at: https://tanita.eu/understanding-your-measurements/body-fat-percentage

| | |
|---|---|
| **BMI, $kg/m^2$*, median (p25,p75)** | 21.2 (20.1, 29.2) |
| ***With pre-obesity (25.0-29.9 $kg/m^2$), n (%)*** | *6 (24.0)* |
| ***With obesity (≥30.0 $kg/m^2$), n (%)*** | *5 (20.0)* |
| **Fat mass**, in %, median (p25, p75), n=17** | 27.5 (20.5, 38.7) |

Attachment B characterizes the energy and nutritional profile of the HMD' diet, indicating the following median intake values: 2429.0 kcal/day, 117.9 g/day, 93.6 g/day and 282.6 g/day of energy, protein, fat and carbohydrates intake, respectively. Attachment C describes their eating habits by food group, during the last year. A higher consumption of meat was observed compared to fish and seafood (214.0 vs. 74.5 g/day). Daily fruit intake was higher than vegetable consumption (396.7 vs. 191.6 g/day). Rice and pasta were considered the primary sources of carbohydrates, while bread and potatoes were consumed in smaller quantities (85.7 vs. 55.8 and 68.5 g/day, respectively). Additionally, there was a higher intake of unsaturated fats compared to saturated fats (12.5 vs. 2.0 g/day, respectively).

The median volume of DHM analysed from each HMD was 885.0 ml (min: 85.0, max: 5055.0 ml). Table 3 represents the impact of pasteurization on DHM's nutritional composition. Pasteurization significantly affected the energy and all macronutrients of DHM ($p<0.05$). There was a superior percentage impact on the energy, crude protein and fat content of DHM (Delta %: -4.7, -7.7 and -8.0, respectively). In Table 4 statistically significant differences were observed in the DHM's carbohydrate content before pasteurization when compared to the g*old standard* value[20] for mature human milk (8.0 vs. 7 g/100ml). Additionally, significant differences were noted in both fat (3.2 vs. 3.8 g/100ml) and carbohydrate contents (8.0 vs. 7.0 g/100ml) after pasteurization.

**Table 3. Impact of pasteurization on Donor Human Milk's nutritional composition (n=25)**

Legend – p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; g: grams; ml: mililiters
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)
**Wilcoxon test
***The percentage reduction (Delta %) in energy and macronutrients for DHM was calculated by the ratio between the difference of energy and macronutrients before and after pasteurization and the value of energy and macronutrients before pasteurization.
The statistically significant values (with p<0.05) are highlighted in bold

| Variables | Before Pasteurization* | After Pasteurization* | Difference (After-Before) | p-value** | Delta%*** |
|---|---|---|---|---|---|
| | | | Median (p25, p75) | | |
| **Energy, kcal/100 ml** | 69.0 (61.0, 76.9) | 66.8 (59.7, 71,4) | -3.0 (-5.3, -1.5) | **<0.001** | -4.7 (-8.3, -2.2) |
| **True protein, g/100 ml** | 0.9 (0.8, 1.0) | 0.8 (0.7, 1.0) | 0.0 (-0.1, 0.0) | **0.010** | 0.0 (-12.5, 0.0) |
| **Crude protein, g/100 ml** | 1.1 (1.0, 1.3) | 1.0 (0.8, 1.2) | -0.1 (-0.2, 0.0) | **0.002** | -7.7 (-12.7, 0.0) |
| **Fat, g/100 ml** | 3.6 (2.8, 4.4) | 3.2 (2.7, 4.1) | -0.3 (-0.5, -0.1) | **<0.001** | -8.0 (-12.3, -3.5) |
| **Carbohydrate, g/100 ml** | 8.0 (7.9, 8.1) | 8.0 (7.9, 8.0) | -0.1 (-0.1, 0.0) | **0.008** | -1.2 (-1.3, 0.0) |

**Table 4. Comparison between g*old standard* of mature Human Milk and Donor Human Milk composition before and after pasteurization (n=25)**

Legend - HM: Human Milk; p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; g: grams; ml: millilitres
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)
**One-Sample Wilcoxon Signed Rank Test
[1] According to: Rêgo C, Lopes C, Durão C, Pinto E, Mansilha H, Pereira-da-Silva L, et al. Alimentação Saudável dos 0 aos 6 anos – Linhas De Orientação Para Profissionais E Educadores. PNPAS; 2019.
The statistically significant values (with p<0.05) are highlighted in bold

| | ***Gold standard* HM[1]** | **Before Pasteurization*** | **p-value**** | **After Pasteurization*** | **p-value**** |
|---|---|---|---|---|---|
| | | Median (p25, p75) | | Median (p25, p75) | |
| **Energy, kcal/100 ml** | *66* | 69.0 (61.0, 76.9) | 0.142 | 66.8 (59.7, 71,4) | 0.647 |
| **Crude protein, g/100 ml** | *1,0* | 1.1 (1.0, 1.3) | 0.091 | 1.0 (0.8, 1.2) | 0.869 |
| **Fat, g/100 ml** | *3,8* | 3.6 (2.8, 4.4) | 0.230 | 3.2 (2.7, 4.1) | **0.007** |
| **Carbohydrate, g/100 ml** | *7,0* | 8.0 (7.9, 8.1) | **<0.001** | 8.0 (7.9, 8.0) | **<0.001** |

Any demographic, lifestyle and anthropometric data of HMD or their infants was associated with the nutritional composition of the DHM before or after pasteurization, as indicated in Attachments D and E.

By analysing DHM's nutritional composition true and crude protein of DHM was positively correlated with daily energy intake, in kcal (r=0.440, p=0.028 and r=0.431, p=0.031, respectively), before pasteurization and with carbohydrate intake before (r=0.611, p=0.001; r=0.600, p=0.002, respectively) and after pasteurization (r=0.499, p=0.011 and r=0.590, p=0.002, respectively), as shown in Attachments F and G.

Regarding the correlations between dietary habits of HMD and the nutritional composition of DHM before pasteurization, detailed in Attachment H, eggs consumption showed a negative association with contents of true and crude protein (r=-0.497, p=0.012 and r=-0.479, p=0.015, respectively) and a positive association with DHM's carbohydrate content (r=0.507, p=0.010). Fish and sea food intake were negatively correlated with energy (r=-0.473, p=0.017) and fat (r=-0.480, p=0.015) contents of DHM. On the other hand, meat consumption showed a strong positive correlation with DHM's fat content (r=0.788, p=0.057), but it was not statistically significant. Considering, carbohydrate sources, rice and pasta intake were positively associated with the carbohydrate content in DHM (r=0.659, p<0.001). Potatoes consumption showed a positive correlation with both true and crude protein (r=0.484, p=0.014 and r=0.466, p=0.019, respectively).

After pasteurization, similar associations were observed for fish and seafood intake as well as for rice and pasta, as present in Attachment I. Potatoes intake had a positive correlation with crude protein DHM content (r=0.408, p=0.043), whereas the group of meat, fish, and eggs consumption showed a negative correlation with this macronutrient (r=-0.427, p=0.033). Saturated fats were positively associated with carbohydrate content of DHM (r=0.487, p=0.014).

**Discussion**

In this cross-sectional study, it was found that Holder pasteurization have a slight impact on the nutritional composition of DHM. Significant differences were observed, particularly in the energy and fat content of DHM, with a higher percentage reduction in energy, fat and true protein, which aligns with previous research findings(21). The percentage impact on these macronutrients differs from the findings of *Adhisivam et al.* (energy: -4.7% vs -8.1%; fat: -8.0% vs -14.9%; true protein: -7.7% vs -8,9, respectively)(21). However, it is evident that fat is consistently the macronutrient that is most significantly affected in both studies. These results have practical implications in clinical settings, especially for the individualized fortification of DHM provided to premature or very low birth weight infants, who are the primary recipients of DHM(11). Considering the influence of processing DHM, it was observed that the macronutrient most deviating from the *gold standard* for mature HM was carbohydrate, which was minimally affected by pasteurization. We also observed a slight statistically significant difference in fat content of DHM after pasteurization, which can be attributed to the impact of DHM's processing on its fat content(21). It is crucial to know that the DHM undergoes pasteurization process before to its distribution to the recipient infants. Therefore, when interpreting the relationship between the eating habits of HMD and the composition of DHM, it is essential to consider the nutritional implications of this processing step.

In this sample, 44% of participants were overweight, which is greater than a prevalence observed in a recent study with Portuguese women at reproductive age (35%) and lower than the prevalence found in Portuguese women according to the *National Food, Nutrition and Physical Activity Survey 2015-2016* (55%)[12, 22]. Furthermore, the median FM value (27.5 %) of our sample fell within the recommended range for adult women aged 20 to 39 years (21.0 - 33.0%)[23]. Generally, no associations were found between DHM nutritional composition and HMD' body composition in this study, but only with HMD' eating habits.

Factors such as age and sleep hours of HMD, as well as their infants' gestational age and birth weight, also do not seem to affect DHM's nutritional composition in this work. *Dritsakou et al.* revealed a positive relationship between maternal age and HM fat content, while birth weight of their infants exhibited a negative association with energy and all macronutrients of HM[24]. A possible explanation for these differences may be the small sample of this work and the fact that it only includes mature DHM samples (>2 weeks postpartum) while *Dritsakou et al.* included HM samples from different stages of lactation[24]. Gestational age was not found to be correlated with DHM composition in this study. It is noteworthy that the majority of infants were born with more than 37 weeks and, according to previous research conducted by *Gidrewicz et al.*, there were no significant differences observed in the nutritional composition of preterm and term HM after 3 months postpartum[25] making reasonable to think that infants' gestational age do not seem to have a long-term impact on the composition of DHM. However, more studies are needed to understand the role of these variables in DHM's nutritional composition.

Among the characteristics of HMD, BMI and FM exhibited the most robust positive Spearman correlations with DHM fat content, although without statistical significance. The literature suggested that lactating women who are overweight or have a higher percentage of FM may produce milk with a higher fat content[10]. So, further research is needed to validate our findings, considering the limited number of participants in this study.

Our specific results revealed that increased energy and carbohydrate intake of HMD were associated with higher true and crude protein content of raw DHM. The impact of energy intake on true and crude protein lost statistical significance after DHM pasteurization, likely due to its susceptibility to the pasteurization process. However, positive correlations between energy intake and protein content were still observed. It is important consider that the association between dietary intake and DHM's protein content may hold relevant implications for the nutrition of preterm infants, who require twice the amount of protein compared to full-term infants[18]. These findings are supported by *Binder et al.*, that also observed that higher dietary macronutrients intake was reflected in higher levels of respective macronutrients in DHM[18], which contrasts with our findings. *Binder et al.* used a 24-hour recall method to assess participants' eating habits, whereas our study utilized a FFQ, which limits the validity of direct comparisons between the two studies.

In a study conducted on Portuguese pregnant women[22], a lower consumption of fish compared to meat was also observed, while vegetable intake exceeded that of fruit, which is contrary to our findings. It is worth noting that this previous study did not distinguish between carbohydrate sources or different types of fats. Thus, our current study can contribute to advancing knowledge in this specific field.

This work indicates an expected positive association between the consumption of carbohydrate sources and carbohydrate content in DHM.

Although statistical significance was not detected, the consumption of these foods seems to be also positively correlated with the energy value of DHM. Surprisingly, a higher intake of potatoes appears to be associated with higher protein content in DHM, which may explain the observed relationship between dietary carbohydrate and DHM protein value. Also, in the present study, we found that the consumption of protein sources (meat, fish and eggs) were inversely associated with the protein content of DHM, with statistical significance observed only for eggs. A previous study conducted on lactating women in China found a negative correlation between protein content in colostrum and food sources of animal protein intake[26]. Considering fish intake, literature is particularly interested in its relation to specific fatty acids in HM, as its consumption has been associated with higher levels of Docosahexaenoic acid (DHA) in HM[27]. Consequently, it would be interesting to explore its potential negative association with the total fat content of DHM, as observed in our study. There is a lack of evidence regarding the relationship between HMD' food intake and the nutritional composition of DHM, making further studies in this area necessary to better understand all of these results.

This same study of lactating women in China[26] affirmed that, in mature milk, a positive association was observed between the intake of food sources of animal protein and carbohydrate concentration of DHM. Interestingly, our results also revealed a positive association between eggs consumption and the carbohydrate content of DHM. However more studies are needed to provide a comprehensive understanding of this topic.

Another unexpected result was the positive correlation between saturated fat consumption and the carbohydrate content in DHM. However, it is essential to note that the only saturated fat considered in our work was butter, which is commonly consumed alongside carbohydrate food sources.

There are some limitations in this study that should be noted. The first limitation is the small sample size, which may have impacted the ability to detect significant associations. Additionally, being a cross-sectional study, makes it not possible to establish a causal relationship. On the other hand in this study, it was used a validated FFQ for the Portuguese adult population and this was the first study conducted in the Human Milk Bank in the Northern Region of Portugal. Our findings reinforce the importance of nutritional monitoring during the lactation period, particularly in the context of HM donation. It can also contributes to the development of public policies aimed at improving the dietary habits of HMD, thereby adjusting the nutritional composition of DHM to meet the specific nutritional needs of recipient infants. Based on the high percentage of overweight HMD and the potential associations between diet and the nutritional composition of DHM demonstrated in this study, we recommend that HMD receive nutritional guidance and food education. Additionally, we propose increasing awareness about the importance of donating HM to premature infants and those with high-risk conditions.

**Conclusion / Final Considerations**

No relationship was found between BMI or FM of HDM and the nutritional composition of their DHM. Energy and carbohydrate intake were positively associated with DHM protein content. Further research needed to confirm findings, considering major covariates and larger sample sizes.

**Acknowledgements**

This work would not be possible to do without the invaluable contribution of the multidisciplinary team of Human Milk Bank in the Northern Region of Portugal, including Doctors Susana Pissarra, Susana Fraga, Rita Moita, as well as nurses Carmen Prucha, Cristina Martins, Catarina Sousa, Joana Morais and Raquel Marcolino. We sincerely thank to all HMD who participate in this study.

Many thanks are extended to Prof. Doctor Cláudia Camila Dias for her indispensable assistance with statistical analysis and to Doctor Sofia Costa for her support in conversion of FFQ items into nutrient intakes.

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**Attachments Index**

## Attachment A - Characteristics of Human Milk Donors’ infants (n=25)

Legend - p25: 25th percentile; p75: 75th percentile; g: grams

| | |
|---|---|
| **Male sex, n (%)** | 11 (44.0) |
| **Preterm infant (<37 weeks), n (%)** | 2 (8.0) |
| **Gestacional age, weeks, median (p25,p75)** | 39.0 (38.0, 40.0) |
| **Birth weight, g, median (p25,p75)** | 3295.0 (2867.5, 3662.5) |

## Attachment B - Human Milk Donors’ energy and macronutrient intake (n=25)

Legend – p25: 25th percentile; p75: 75th percentile; kcal/day – kilocalories per day; g/day – grams per day

| | **median (p25, p75)** |
|---|---|
| **Energy, kcal/day** | 2429.0 (2053.8, 2772.7) |
| **Protein, g/day** | 117.9 (89.2, 134.6) |
| **Fat, g/day** | 93.6 (81.7, 107.7) |
| **Carbohydrate** | 282.6 (227.6, 326.0) |

## Attachment C - Human Milk Donors' eating habits (n=25)

Legend – p25: 25th percentile; p75: 75th percentile; kcal/day – kilocalories per day; g/day – grams per day
[1] Whole milk, semi-skimmed milk, skimmed milk, yogurt and cheese
[2] Chicken, turkey, rabbit meat, beef, pork and lamb
[4] Fatty, lean and canned fish, cod fish, squid, octopus and shellfish
[5]Apple, pear, orange, tangerine, banana, kiwi, strawberries, cherries, peaches, plum, melon, watermelon, persimmon, figs, loquats and grapes
[6] White or savoy cabbage, bunch, kale, broccoli, cauliflower, brussels sprouts, turnip greens, spinach, green beans, lettuce, watercress, onion, carrot, turnip, tomato, pepper, cucumber
[7] Almonds, hazelnuts, walnuts, pistachios and peanuts
[8] White or wholemeal bread, toast, cornbread
[9] Rice and pasta
[10]Fried, boiled, roasted, stewed and mashed potatoes
[11]Butter
[12] Olive oil, oil, margarine

| | **median (p25, p75)** |
|---|---|
| **Dairy[1], g/day** | 162.4 (46.5, 377.7) |
| **Meat, fish and eggs, g/day** | 214.0 (171.3, 259.8) |
| ***Eggs g/day*** | *22.2 (22.2, 44.4)* |
| ***Meat[2], g/day*** | *120.0 (68.0, 156.4)* |
| ***Fish and seafood[4], g/day*** | *74.5 (33.3, 92.1)* |
| **Fruits[5], g/day** | 396.7 (248.6, 558,8) |
| **Vegetables[6] , g/day** | 191.6 (119.3, 314.2) |
| **Nuts[7], g/day** | 30.5 (4.9, 45.7) |
| **Bread, rice, pasta and potatoes, g/day** | 218.4 (146.6, 297.0) |
| ***Bread[8], g/day*** | *55.8 (38.6, 95.7)* |
| ***Rice and pasta[9], g/day*** | *85.7 (58.9, 157.1)* |
| ***Potatoes[10], g/day*** | *68.6 (23.8, 83.9)* |
| **Fats, g/day** | 13.8 (9.1, 18.6) |
| ***Saturated fats[11] , g/day*** | *2.0 (0.0, 4.7)* |
| ***Unsaturared fats[12], g/day*** | *12.5 (5.8, 15.5)* |

## Attachment D - Spearman's correlation between Human Milk Donors' and their infants' characteristics and Donor Human Milk's nutritional composition before pasteurization (n=25)

Legend: HMD – Human Milk Donors; BMI - body mass index ; kcal – kilocalories; g – grams; ml – millilitres; h – hours; kg/m2 – kilogram per square meter; % - percentage
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)

| | Donor Human Milk composition before pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **HMD' charateristics** | | | | | | | | | | |
| **Age, years** n = 25 | 0.175 | 0.401 | -0.151 | 0.472 | -0.112 | 0.593 | 0.133 | 0.528 | 0.181 | 0.387 |
| **Sleep hours, h** n = 25 | 0.046 | 0.829 | 0.118 | 0.573 | 0.120 | 0.567 | 0.058 | 0.784 | -0.113 | 0.590 |
| **BMI, $kg/m^2$** n = 25 | 0.172 | 0.411 | -0.099 | 0.637 | -0.076 | 0.719 | 0.236 | 0.257 | 0.157 | 0.454 |
| **Fat mass, %** n = 17 | 0.183 | 0.483 | 0.091 | 0.728 | 0.118 | 0.652 | 0.292 | 0.255 | 0.361 | 0.155 |
| **Infants' charateristics** | | | | | | | | | | |
| **Gestacional age, weeks** n = 25 | -0.231 | 0.266 | 0.276 | 0.182 | 0.286 | 0.165 | -0.240 | 0.248 | -0.019 | 0.928 |
| **Birth weight, g** n = 25 | 0.207 | 0.322 | 0.202 | 0.333 | 0.174 | 0.406 | 0.223 | 0.283 | -0.037 | 0.859 |

## Attachment E - Spearman's correlation between Human Milk Donors' and their infants' characteristics and Donor Human Milk's nutritional composition after pasteurization (n=25)

Legend: HMD – Human Milk Donors; BMI - body mass index ; kcal – kilocalories; g – grams; ml – millilitres; h – hours; $kg/m^2$ – kilogram per square meter; % - percentage
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)

| | Donor Human Milk composition after pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **HMD' charateristics** | | | | | | | | | | |
| **Age, years** n = 25 | 0.071 | 0.737 | -0.033 | 0.876 | -0.089 | 0.671 | 0.117 | 0.577 | -0.016 | 0.941 |
| **Sleep hours, h** n = 25 | 0.035 | 0.870 | 0.289 | 0.161 | 0.238 | 0.252 | 0.072 | 0.731 | -0.032 | 0.878 |
| **BMI, $kg/m^2$** n = 25 | 0.151 | 0.472 | -0.170 | 0.416 | -0.256 | 0.217 | 0.200 | 0.337 | 0.077 | 0.715 |
| **Fat mass, %** n = 17 | 0.146 | 0.576 | 0.041 | 0.875 | -0.009 | 0.973 | 0.282 | 0.274 | 0.075 | 0.776 |
| **Infants' charateristics** | | | | | | | | | | |
| **Gestacional age, weeks** n = 25 | -0.178 | 0.395 | 0.325 | 0.113 | 0.317 | 0.122 | -0.225 | 0.279 | -0.070 | 0.741 |
| **Birth weight, g** n = 25 | 0.204 | 0.328 | 0.067 | 0.749 | 0.142 | 0.498 | 0.201 | 0.335 | -0.038 | 0.856 |

## Attachment F - Spearman's correlation between Human Milk Donors' energy and macronutrient intake and Donor Human Milk's nutritional composition before pasteurization (n=25)

Legend: p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; Kcal/ day: kilocalories per day; g: grams; g/day: grams per day; ml: millilitres
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)
The values in bold represent statistically significant correlations, with p<0.05

| | Donor Human Milk composition before pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **Energy and macronutrient intake** | | | | | | | | | | |
| **Energy, kcal/day** | 0.295 | 0.153 | **0.440** | **0.028** | **0.431** | **0.031** | 0.281 | 0.174 | 0.195 | 0.351 |
| **Protein, g/day** | 0.258 | 0.213 | 0.224 | 0.281 | 0.205 | 0.326 | 0.215 | 0.302 | 0.357 | 0.079 |
| **Fat, g/day** | 0.172 | 0.411 | 0.120 | 0.567 | 0.118 | 0.575 | 0.195 | 0.350 | 0.258 | 0.212 |
| **Carbohydrate, g/day** | 0.326 | 0.112 | **0.611** | **0.001** | **0.600** | **0.002** | 0.260 | 0.210 | 0.034 | 0.873 |

## Attachment G - Spearman's correlation between Human Milk Donors' energy and macronutrient intake and Donor Human Milk's nutritional composition after pasteurization (n=25)

Legend: p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; Kcal/ day: kilocalories per day; g: grams; g/day: grams per day; ml: millilitres
The values in bold represent statistically significant correlations, with $p<0.05$
*Using the Miris HMA$^{TM}$ (Human Milk Analyzer, Miris AB, Upsala, Sweden)

| | Donor Human Milk composition after pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **Energy and macronutrient intake** | | | | | | | | | | |
| **Energy, kcal/day** | 0.252 | 0.224 | 0.367 | 0.072 | 0.388 | 0.056 | 0.254 | 0.220 | 0.190 | 0.364 |
| **Protein, g/day** | 0.182 | 0.385 | 0.127 | 0.544 | 0.084 | 0.689 | 0.208 | 0.319 | 0.262 | 0.206 |
| **Fat, g/day** | 0.128 | 0.542 | 0.109 | 0.603 | 0.105 | 0.619 | 0.188 | 0.367 | 0.221 | 0.288 |
| **Carbohydrate, g/day** | 0.309 | 0.133 | **0.499** | **0.011** | **0.590** | **0.002** | 0.231 | 0.267 | 0.093 | 0.658 |

## Attachment H - Spearman's correlation between Human Milk Donors' eating habits and Donor Human Milk's nutritional composition before pasteurization (n=25)

Legend: p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; Kcal/ day: kilocalories per day; g: grams; g/day: grams per day; ml: millilitres
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)
The values in bold represent statistically significant correlations, with $p<0.05$
[1] Whole milk, semi-skimmed milk, skimmed milk, yogurt and cheese
[2] Chicken, turkey, rabbit meat, beef, pork and lamb
[4] Fatty, lean and canned fish, cod fish, squid, octopus and shellfish
[5] Apple, pear, orange, tangerine, banana, kiwi, strawberries, cherries, peaches, plum, melon, watermelon, persimmon, figs, loquats and grapes
[6] White or savoy cabbage, bunch, kale, broccoli, cauliflower, brussels sprouts, turnip greens, spinach, green beans, lettuce, watercress, onion, carrot, turnip, tomato, pepper, cucumber
[7] Almonds, hazelnuts, walnuts, pistachios and peanuts
[8] White or wholemeal bread, toast, cornbread
[9] Rice and pasta
[10] Fried, boiled, roasted, stewed and mashed potatoes
[11] Butter
[12] Olive oil, oil, margarine

| | Donor Human Milk composition before pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **Food groups intake, g/day** | | | | | | | | | | |
| **Dairy[1]** | -0.127 | 0.546 | -0.068 | 0.748 | -0.068 | 0.747 | -0.177 | 0.397 | 0.306 | 0.137 |
| **Meat, fish and eggs** | -0.167 | 0.426 | -0.356 | 0.081 | -0.357 | 0.080 | -0.183 | 0.382 | 0.312 | 0.130 |
| ***Eggs*** | -0.213 | 0.307 | **-0.497** | **0.012** | **-0.479** | **0.015** | -0.204 | 0.328 | **0.507** | **0.010** |
| ***Meat[2]*** | 0.072 | 0.733 | -0.166 | 0.428 | -0.171 | 0.414 | 0.788 | 0.057 | 0.060 | 0.776 |
| ***Fish and seafood[4]*** | **-0.473** | **0.017** | -0.266 | 0.199 | -0.258 | 0.213 | **-0.480** | **0.015** | 0.315 | 0.125 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fruits**[5] | 0.048 | 0.818 | 0.356 | 0.081 | 0.349 | 0.087 | 0.013 | 0.952 | 0.009 | 0.965 |
| **Vegetables**[6] | -0.045 | 0.832 | 0.063 | 0.763 | 0.010 | 0.962 | -0.048 | 0.818 | -0.093 | 0.659 |
| **Nuts**[7] | -0.132 | 0.529 | 0.244 | 0.240 | 0.236 | 0.256 | -0.115 | 0.583 | -0.213 | 0.307 |
| **Bread, rice, pasta and potatoes** | 0.369 | 0.069 | 0.368 | 0.070 | 0.350 | 0.086 | 0.304 | 0.140 | **0.401** | **0.047** |
| ***Bread***[8] | 0.133 | 0.526 | 0.245 | 0.238 | 0.244 | 0.239 | 0.073 | 0.730 | 0.137 | 0.514 |
| ***Rice and pasta***[9] | 0.316 | 0.123 | 0.100 | 0.633 | 0.095 | 0.652 | 0.271 | 0.191 | **0.659** | **<0,001** |
| ***Potatoes***[10] | 0.207 | 0.321 | **0.484** | **0.014** | **0.466** | **0.019** | 0.168 | 0.422 | 0.056 | 0.789 |
| **Fats** | -0.018 | 0.931 | -0.277 | 0.181 | -0.275 | 0.183 | 0.016 | 0.940 | 0.373 | 0.066 |
| ***Saturated fats***[11] | 0.092 | 0.664 | -0.065 | 0.758 | -0.077 | 0.713 | 0.044 | 0.835 | 0.306 | 0.137 |
| ***Unsaturared fats***[12] | -0.122 | 0.562 | -0.193 | 0.355 | -0.181 | 0.387 | -0.090 | 0.670 | 0.285 | 0.167 |

## Attachment I - Spearman's correlation between Human Milk Donors' eating habits and Donor Human Milk's nutritional composition after pasteurization (n=25)

Legend: p25: 25th percentile; p75: 75th percentile; kcal: kilocalories; Kcal/ day: kilocalories per day; g: grams; g/day: grams per day; ml: millilitres
*Using the Miris HMA™ (Human Milk Analyzer, Miris AB, Uppsala, Sweden)
The values in bold represent statistically significant correlations, with $p<0.05$
[1] Whole milk, semi-skimmed milk, skimmed milk, yogurt and cheese
[2] Chicken, turkey, rabbit meat, beef, pork and lamb
[4] Fatty, lean and canned fish, cod fish, squid, octopus and shellfish
[5]Apple, pear, orange, tangerine, banana, kiwi, strawberries, cherries, peaches, plum, melon, watermelon, persimmon, figs, loquats and grapes
[6] White or savoy cabbage, bunch, kale, broccoli, cauliflower, brussels sprouts, turnip greens, spinach, green beans, lettuce, watercress, onion, carrot, turnip, tomato, pepper, cucumber
[7] Almonds, hazelnuts, walnuts, pistachios and peanuts
[8] White or wholemeal bread, toast, cornbread
[9] Rice and pasta
[10]Fried, boiled, roasted, stewed and mashed potatoes
[11]Butter
[12] Olive oil, oil, margarine

| | Donor Human Milk composition after pasteurization* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy (kcal/100 ml) | | True Protein (g/100 ml) | | Crude protein (g/100 ml) | | Fat (g/100 ml) | | Carbohydrate (g/100 ml) | |
| | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| **Food groups intake, g/day** | | | | | | | | | | |
| **Dairy[1]** | -0.188 | 0.368 | -0.144 | 0.589 | -0.144 | 0.492 | -0.201 | 0.335 | 0.249 | 0.230 |
| **Meat, fish and eggs** | -0.209 | 0.317 | -0.349 | 0.087 | **-0.427** | **0.033** | -0.171 | 0.414 | 0.156 | 0.456 |
| ***Eggs*** | -0.215 | 0.303 | -0.383 | 0.059 | -0.362 | 0.075 | -0.193 | 0.355 | 0.266 | 0.199 |
| ***Meat[2]*** | -0.011 | 0.957 | -0.134 | 0.522 | -0.242 | 0.243 | 0.009 | 0.967 | -0.039 | 0.855 |
| ***Fish and seafood[4]*** | **-0.527** | **0.007** | -0.268 | 0.196 | -0.300 | 0.145 | **-0.452** | **0.023** | 0.355 | 0.082 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fruits**[5] | 0.066 | 0.755 | 0.269 | 0.194 | 0.371 | 0.068 | 0.026 | 0.903 | -0.197 | 0.346 |
| **Vegetables**[6] | -0.033 | 0.875 | 0.049 | 0.817 | 0.079 | 0.708 | -0.081 | 0.699 | -0.008 | 0.971 |
| **Nuts**[7] | -0.086 | 0.682 | 0.192 | 0.359 | 0.260 | 0.209 | -0.090 | 0.668 | -0.203 | 0.330 |
| **Bread, rice, pasta and potatoes** | 0.366 | 0.072 | 0.307 | 0.136 | 0.308 | 0.135 | 0.322 | 0.116 | 0.370 | 0.069 |
| ***Bread***[8] | 0.122 | 0.562 | 0.310 | 0.132 | 0.262 | 0.206 | 0.044 | 0.834 | 0.255 | 0.219 |
| ***Rice and pasta***[9] | 0.348 | 0.088 | 0.124 | 0.554 | 0.107 | 0.611 | 0.340 | 0.096 | **0.495** | **0.012** |
| ***Potatoes***[10] | 0.191 | 0.360 | 0.350 | 0.086 | **0.408** | **0.043** | 0.154 | 0.461 | 0.146 | 0.487 |
| **Fats** | 0.103 | 0.625 | -0.126 | 0.549 | -0.120 | 0.566 | 0.093 | 0.657 | 0.197 | 0.345 |
| ***Saturated fats***[11] | 0.016 | 0.941 | 0.011 | 0.957 | -0.020 | 0.924 | 0.018 | 0.930 | **0.487** | **0.014** |
| ***Unsaturared fats***[12] | 0.003 | 0.988 | -0.076 | 0.719 | -0.082 | 0.697 | -0.008 | 0.969 | 0.052 | 0.805 |