



Contents lists available at ScienceDirect

Nutrition

journal homepage: [www.nutritionjrn.com](http://www.nutritionjrn.com)

Applied nutritional investigation

## Maternal age has more pronounced effect on breast milk retinol and $\beta$ -carotene content than maternal dietary pattern



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### ARTICLE INFO

#### Article History:

Received 28 August 2018

Received in revised form 29 January 2019

Accepted 23 February 2019

#### Keywords:

Colostrum

Mature milk

Maternal age

Retinol

$\beta$ -carotene

Maternal dietary and lifestyle habits

### ABSTRACT

**Objective:** The effects on breast milk composition of advanced maternal age and maternal dietary habits during pregnancy and lactation have not, to our knowledge, been investigated in southeastern Europe and the Balkans. The aim of this study was to compare the content of retinol and  $\beta$ -carotene in colostrum and mature milk samples obtained from different maternal age (MA) groups and to assess the potential relationship with maternal and demographic characteristics, dietary patterns, and lifestyle habits during pregnancy and lactation.

**Methods:** Forty-three nursing mothers were divided in two groups according to MA:  $\geq 35$  y of age ( $n = 22$ ) and  $< 35$  y of age ( $n = 21$ ). Total lipid concentrations were determined by gravimetric method, whereas retinol and  $\beta$ -carotene contents were assessed by high-performance liquid chromatography method. Dietary patterns during pregnancy and lactation were assessed using food frequency questionnaires, and principal component analysis (PCA) statistical analysis was performed.

**Results:** Except for retinol levels in mature milk, significantly higher levels of total fats, retinol, and  $\beta$ -carotene were found in the older group. Results of PCA analysis showed that MA was strongly correlated with fat content, retinol, and  $\beta$ -carotene levels in colostrum samples, whereas in mature milk samples MA was highly correlated with fat content and moderately with  $\beta$ -carotene. In terms of dietary patterns, retinol contents in milk samples from both groups were weakly to moderately correlated with consumption frequency of eggs, meat, milk and dairy products, whereas  $\beta$ -carotene contents were weakly to strongly associated with consumption frequency of fruits and vegetables.

**Conclusions:** PCA analysis used in the study clearly confirmed that MA, total lipids, retinol, and  $\beta$ -carotene levels might serve as a good criterion for delimitation of breast milk samples collected in different stages of lactation from mothers of various ages. The present findings could represent key basis for further investigations.

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This research was funded by the Ministry of Education, Science and Technological Development of Serbia, Republic of Serbia (Project No. TR 31060). SS, GK, and MD designed the study. MD, SS, and MJ collected the samples and data. MD, SS, JŽ and TJ performed the laboratory analyses. MD and SS wrote the article. MG performed statistical and principal component analyses. MD had primary responsibility for the final content. This work is part of MD's Ph.D. thesis. All authors read and approved the final version of the manuscript. The authors have no conflicts of interest to declare.

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### Introduction

Adequate nutrition is essential to ensure normal growth and development of newborns. Human milk is the most valuable nourishment for infants because it contains all the nutrients needed during this crucial period. Maternal age (MA) at childbirth has increased in most countries in Europe and worldwide in the past 20 y and there is clear evidence that women are delaying first childbirth to the later reproductive years. Advanced maternal age (AMA) is generally defined as a pregnancy in women  $\geq 35$  y of age [1–3] and in recent years this trend has been quite apparent in

Serbia as well. The reasons for postponement of first childbirth and parenthood are different and depend on geographic area. In general, the main factors include women's higher education level, economic or housing uncertainty, unemployment or temporary work, divorce, lack of child care, and so on. Unfortunately, it is well known that older pregnant women have a higher rate of complications such as pregnancy-induced hypertension, preeclampsia, diabetes mellitus, fetal chromosomal abnormalities, preterm labor, or low birthweight baby. Also, older breastfeeding women have more trouble establishing and maintaining adequate lactation during the entire breastfeeding period [1,4]. For these reasons, there is a need for continuous monitoring of the effects of MA on breast milk composition and quality.

Retinol (vitamin A) and  $\beta$ -carotene (pro-vitamin A) are two essential lipophilic nutrients present in human milk that play important roles in the early stages of the infant's life. Retinol is a fat-soluble vitamin necessary for normal development, growth, immune function, and eyesight.  $\beta$ -carotene is known to have antioxidant and anti-inflammatory activities, so it could be protective against cardiovascular diseases, diabetes, inflammatory diseases, and cancer [5].

The composition of breast milk could be influenced by a number of factors, such as geographic area, stage of lactation, sociodemographic characteristics of mothers, and maternal diet. Among these factors, the effect of MA on breast milk composition and quality is one of the most interesting research targets, but still not studied sufficiently. Recent studies reported that colostrum fat and carbohydrate content in mature milk are higher in older than in younger breastfeeding mothers. The authors speculated that those biochemical changes in milk composition throughout the lactation period could be related to the differences in fat and carbohydrates metabolisms with aging [6,7]. However, in those studies, the authors did not correlate obtained results simultaneously with MA and maternal dietary habits during pregnancy and lactation, which are important factors affecting breast milk composition.

Considering that in recent years there has been an interest in researching the composition of human milk of older women compared with their younger counterparts, especially across the Europe and in the Balkans, to the best of our knowledge, there is a lack of information in terms of retinol and  $\beta$ -carotene content and with its relationships with maternal and demographic characteristics and maternal dietary and lifestyle habits. Therefore, the main objective of the present study was to determine the retinol and  $\beta$ -carotene concentrations in colostrum and mature milk samples obtained from different MA groups and to assess its potential relationship with maternal and demographic characteristics, dietary patterns, and lifestyle habits during pregnancy and lactation.

## Materials and methods

### Data collection

Data and milk samples were collected from 43 nursing mothers, 18 to 40 y of age, who had given birth at the Clinic of Gynecology and Obstetrics of the Clinical Centre of Niš. All participants were healthy, well nourished, and delivered healthy term babies (gestational age  $\geq 37$  wk). The exclusion criteria for this study were maternal complications or acute or chronic diseases (hypertension, eclampsia, diabetes, acute or chronic infective diseases), preterm delivery (gestational age  $< 37$  wk), and fetal malformations. During hospitalization, after delivery and the explanations about the objectives of the study, mothers gave written informed consent to take part in the study, which was approved by the Ethical Committee for Medical Researches of Faculty of Medicine, University of Niš. Through face-to-face interviews, the questionnaire was used to collect socioeconomic characteristics and lifestyle aspects of lactating women including MA, residential area, educational level, occupation, alcohol consumption, and smoking habits, in addition to parity, gestational week at delivery, and infant sex. All participants were divided in two groups according to maternal age:  $\geq 35$  y of age ( $n = 22$ ) and  $< 35$  y of age ( $n = 21$ ).

### Dietary assessment

In addition, dietary patterns during pregnancy and lactation were assessed using a food frequency questionnaire that included seven groups of food characteristic for dietary habits of lactating mothers from the local area (Supplementary Table S): cereal products, milk and dairy products, meat, eggs, vegetables, fruits, and beverages. All women enrolled in the study were asked to indicate the frequency (once a day, 2–3 times/d, once a week, 2–3 time/wk, monthly, rarely or never) of consumption of each foodstuff from the mentioned groups during pregnancy and lactation, to reduce recall bias and to avoid inaccurate estimation of portion size. Each mother completed one of these questionnaires for their pregnancy period and another one for the postpartum period. The first questionnaire was used to assess the effects of the maternal diet on retinol and  $\beta$ -carotene levels in colostrum samples and the second to evaluate the same effects on mature milk samples.

### Milk collection

Milk samples were collected by means of manual expression and each mother contributed two samples (colostrum and mature milk sample). For both types of collected samples, the single breast that had not been previously used for feeding on the collection day was completely emptied of milk and the first ejections were discarded to avoid fluctuations in lipid content. During hospitalization, mothers were instructed from obstetrics and gynecology nurses to avoid breastfeeding from the breast that was used for mature milk collection  $\geq 3$  h before sampling.

The colostrum sample was collected in sterile test tubes in hospital during the first 72 h after labor, in the morning (between 07:00 and 10:00). Samples were aliquoted and kept in the freezer at  $-20^{\circ}\text{C}$  until analysis. The mature milk samples were collected 1 mo after labor in sterile test tubes in home conditions and were individually stored at  $-20^{\circ}\text{C}$  until they were taken by researchers, transported to the laboratory, and analyzed.

### Measurement of total lipids concentration

Total lipid concentration was determined in duplicate for each sample using the slightly modified gravimetric method [8]. The details of the method are given in the supplementary material.

### Retinol and $\beta$ -carotene extraction and determination

Retinol and  $\beta$ -carotene were simultaneously extracted from milk samples and their content was determined by high-performance liquid chromatography (HPLC) method. The details of the extraction procedure and HPLC method are given in the supplementary material.

### Statistical analyses

The results of total lipid, retinol, and  $\beta$ -carotene concentrations were expressed as the mean  $\pm$  SD. The differences between the means of these data found in both groups were analyzed using paired Student's *t* test. Demographic and maternal characteristics of the studied groups were evaluated using descriptive statistics. These statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Probability values  $P < 0.05$  were considered statistically significant.

The relationship between total lipids, retinol, and  $\beta$ -carotene concentrations in milk samples with MA and correlations of these biochemical parameters with dietary patterns (foodstuffs) were assessed using PCA, which was performed using the Excel program plug-in XLSTAT version 2018.3. To establish correlations between total lipid, retinol, and  $\beta$ -carotene concentrations in milk samples with MA, we performed two different PCA analyses: one on a data set combining mentioned variables for colostrum and mature milk samples from both studied age groups and the other on independent data sets for colostrum samples from both groups and for mature milk samples from both groups. In our study of the influence of maternal dietary patterns and lifestyle habits on retinol and  $\beta$ -carotene content, PCA method was applied independently on colostrum and mature milk samples, and two analyses were performed in each case. In the first, we combined data for colostrum or mature milk samples from both age groups. In the second, we used independent data for advanced-aged mothers and younger mothers as variables. We did this to obtain the as-high-as-possible sum of the first and second principal components (F1 and F2 factors) to gain more reliable results. Pearson's correlation coefficient for two compared variables was determined and the magnitudes of coefficients (*r*) were set as follows: very weak (0–0.19), weak (0.2–0.39), moderate (0.4–0.59), strong (0.6–0.79), and very strong ( $> 0.8$ ). Correlations were considered significant for  $P < 0.05$ .

## Results

### Maternal characteristics

Among 43 lactating mothers who were included in the present study, 22 were AMA ( $\geq 35$  y of age) and 21 were  $< 35$  y of age. Demographic and maternal characteristics of both groups are presented in Supplementary Table 2. Approximately 50% of the women in both groups were first-time mothers. The majority of women (95%) in the older group were employed compared with 71% in the younger one. All of the women had either high school or university degrees and only 25% of mothers from both groups came from rural areas. Therefore, it is more likely that studied population represented well-nourished women.

Comparing the demographic and maternal characteristics of analyzed groups, the results did not differ significantly in terms of maternal parity, educational levels, and residence, nor in terms of infant's sex and gestational age. On the other hand, considerable differences were observed in terms of MA and lifestyle (Supplementary Table 2). The mean MA difference between studied groups was 11.4 y ( $P < 0.01$ ). In addition, mothers from the older group were also more likely have smoked cigarettes and consumed alcohol during pregnancy compared with mothers from the younger group. However, a significantly higher percentage of mothers from the younger group lived with a smoker while pregnant.

### Dietary parameters

The list of foodstuffs included in the dietary habits questionnaire is given in supplementary Table 1. All mothers included in the present study completed a questionnaire that represented their dietary habits during pregnancy and another that reflected their dietary habits after delivery. We supposed that dietary habits of mothers during pregnancy mainly affected retinol and  $\beta$ -carotene levels in colostrum samples, whereas nutrition after childbirth affected concentration of these compounds in mature milk samples. Therefore, we used data from the first questionnaire and retinol and  $\beta$ -carotene levels in colostrum samples, and data from the second questionnaire and retinol and  $\beta$ -carotene levels in mature milk samples to assess (by multivariate statistical analysis) the effects of maternal dietary habits on retinol and  $\beta$ -carotene content in human milk samples during different stages of lactation.

### Total fats, retinol, and $\beta$ -carotene content in analyzed colostrum and mature milk samples

Colostrum and mature milk content of total lipids, retinol, and  $\beta$ -carotene are shown in Table 1. Milk total fat concentrations in

colostrum samples differed between groups ( $3.38 \pm 0.31$  and  $2.42 \pm 0.31$  g/L for older and younger women, respectively), and increased during lactation ( $4.51 \pm 0.30$  and  $3.50 \pm 0.31$  g/L for older and younger women, respectively). In colostrum samples, the average retinol concentrations were  $0.96 \pm 0.13$   $\mu\text{g/mL}$  ( $3.35 \pm 0.45$   $\mu\text{mol/L}$ ) and  $0.73 \pm 0.12$   $\mu\text{g/mL}$  ( $2.55 \pm 0.42$   $\mu\text{mol/L}$ ) for older and younger women, respectively. The level of retinol decreased in mature milk to  $0.48 \pm 0.08$   $\mu\text{g/mL}$  ( $1.67 \pm 0.28$   $\mu\text{mol/L}$ ) in the older group and to  $0.46 \pm 0.11$   $\mu\text{g/mL}$  ( $1.60 \pm 0.38$   $\mu\text{mol/L}$ ) in the younger arm. The mean values of  $\beta$ -carotene content in colostrum samples were  $182.62 \pm 18.82$  ng/mL in the older group and  $133.31 \pm 24.11$  ng/mL in the younger mothers. As in case of retinol, the mean concentration of  $\beta$ -carotene decreased with the duration of lactation in both groups ( $76.78 \pm 15.25$  and  $62.48 \pm 17.98$  ng/mL for older and younger women, respectively).

Except for retinol levels in mature milk, significantly higher levels of all analyzed compounds were found in the older group ( $P < 0.05$ ). Moreover, during lactation, in both groups, retinol and  $\beta$ -carotene concentrations considerably decreased ( $P < 0.01$ ), whereas total lipids content noticeably increased ( $P < 0.05$ ).

### Influence of maternal characteristics, dietary patterns, and lifestyle habits on retinol and $\beta$ -carotene content

Multivariate statistical treatment of MA data and colostrum fat content, retinol, and  $\beta$ -carotene levels in analyzed samples revealed strong positive correlations between retinol concentrations and MA ( $r = 0.546$ ,  $P < 0.0001$ ),  $\beta$ -carotene concentrations and MA ( $r = 0.636$ ,  $P < 0.0001$ ), and fat concentrations and MA ( $r = 0.821$ ,  $P < 0.0001$ ). In mature milk samples, MA was highly associated with fat content ( $r = 0.773$ ,  $P < 0.0001$ ) and weakly with  $\beta$ -carotene ( $r = 0.394$ ,  $P = 0.009$ ), but there was no correlation with retinol ( $r = 0.178$ ,  $P = 0.253$ ). In addition, retinol and  $\beta$ -carotene concentrations were mutually positively correlated ( $r = 0.966$ ,  $P < 0.0001$  and  $r = 0.361$ ,  $P = 0.017$  for colostrum and mature milk samples, respectively), as well as with total lipids content (retinol:  $r = 0.668$ ,  $P < 0.0001$  for colostrum and  $\beta$ -carotene:  $r = 0.716$ ,  $P < 0.0001$  and  $r = 0.636$ ,  $P < 0.0001$ , for colostrum and mature milk samples, respectively). Again, there was no statistically significant correlation between total lipids and retinol content in mature milk samples ( $r = 0.301$ ,  $P = 0.05$ ). According to results of statistical analysis, the bioavailability of retinol and  $\beta$ -carotene was higher for infants fed with colostrum milk samples containing higher level of total fats. Moreover, the same could be applied for mature milk samples regarding  $\beta$ -carotene and total fat content. This is an important finding for colostrum samples in particular, where strong positive correlations were observed, because adequate nutrition of newborns is required in first days after birth.

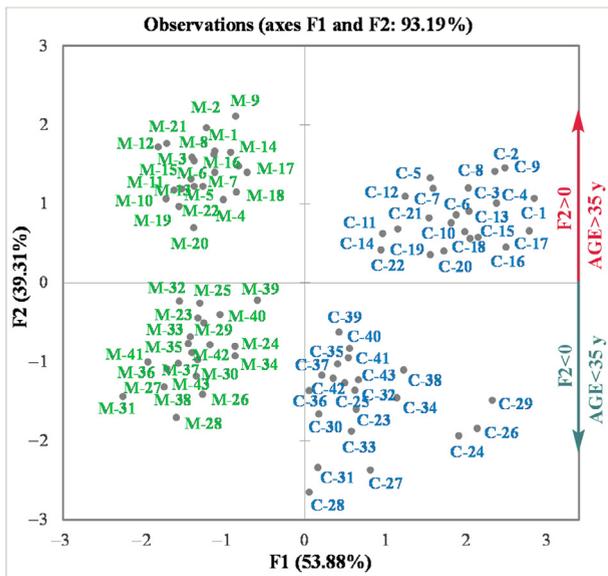
**Table 1**

Total lipid, retinol, and  $\beta$ -carotene content in colostrum and mature milk samples in older and younger women

	$\geq 35$ y (n = 22) Mean $\pm$ SD (range)	$< 35$ y (n = 21) Mean $\pm$ SD (range)	P-value*
Total lipids (g/100 mL)			
Colostrum	$3.38 \pm 0.31$ (2.95–3.82)	$2.42 \pm 0.31$ (2.13–3.04)	$< 0.001$
Mature milk	$4.51 \pm 0.30$ (3.97–4.98)	$3.50 \pm 0.31$ (3.06–3.92)	$< 0.001$
Retinol ( $\mu\text{g/mL}$ )			
Colostrum	$0.96 \pm 0.13$ (0.76–1.23)	$0.73 \pm 0.12$ (0.58–1.01)	$< 0.001$
Mature milk	$0.48 \pm 0.08$ (0.36–0.61)	$0.46 \pm 0.11$ (0.29–0.62)	ns (0.565)
$\beta$ -carotene (ng/mL)			
Colostrum	$182.62 \pm 18.82$ (141.35–211.33)	$133.31 \pm 24.11$ (107.83–188.27)	$< 0.001$
Mature milk	$76.78 \pm 15.25$ (55.32–106.80)	$62.48 \pm 17.98$ (38.14–90.20)	0.003

ns, not significant.

\*Statistical significance.



**Fig. 1.** Principal component analysis (PCA; content of total maternal age and content of total lipids, retinol, and  $\beta$ -carotene used as variables) of 43 colostrum (blue) and 43 mature milk samples (green) collected from breastfeeding mothers from Serbia (observations). F1 and F2 factors (the first and second principal components, respectively) refer to the ordering scores obtained from the samples. F1 accounts for ~54% and F2 for a further 39% of the total variance.

Moreover, the PCA biplot depicted in Figure 1, obtained as the result of PCA analysis performed using maternal age, total fats, retinol and  $\beta$ -carotene content in colostrum and mature milk samples in both studied age groups, delimited four statistically different groups. The sum of F1 and F2 factors, the first and second principal components, in this PCA analysis was quite high (~93%). Colostrum samples from older (upper right quadrant) and younger mothers (lower right quadrant) could be clearly distinguished from corresponding mature milk samples (upper and lower left quadrant, respectively) according to the first principal component (F1; ~54%) which showed very strong positive correlation with retinol and  $\beta$ -carotene content ( $r=0.940$  and  $r=0.953$ , respectively). Further segregation of colostrum and mature milk samples was mainly due to the MA, which showed very strong positive correlation ( $r=0.870$ ) with the second principal component (F2; ~39%). However, the strong positive correlation of this principal component with the lipid content ( $r=0.660$ ) in analyzed milk samples should be noted. Overall, it seems that these four variables could serve as good criterion for delimitation of breast milk samples collected in different stages of lactation from mothers of various ages.

**Table 2**

Correlations of retinol and  $\beta$ -carotene levels in breast milk with dietary patterns and lifestyle habits of mothers in the two groups (Pearson's  $r$  is presented)

	Colostrum				Mature milk			
	$\geq 35$ y (n = 22)		$< 35$ y (n = 21)		$\geq 35$ y (n = 22)		$< 35$ y (n = 21)	
	Retinol	$\beta$ -carotene	Retinol	$\beta$ -carotene	Retinol	$\beta$ -carotene	Retinol	$\beta$ -carotene
Cereal products	-0.187	0.116	-0.132	-0.155	-0.010	0.055	0.002	-0.025
Milk and dairy products	<b>0.424*</b>	0.179	<b>0.321*</b>	0.186	<b>0.379*</b>	0.189	<b>0.308*</b>	0.156
Meat	<b>0.368*</b>	-0.149	<b>0.410*</b>	0.015	<b>0.386*</b>	-0.177	<b>0.321*</b>	0.044
Eggs	<b>0.276*</b>	0.105	<b>0.312*</b>	-0.064	<b>0.255*</b>	0.068	<b>0.204*</b>	0.096
Vegetables	0.143	<b>0.737*</b>	0.179	<b>0.778*</b>	0.044	<b>0.556*</b>	0.015	<b>0.576*</b>
Fruits	0.012	<b>0.386*</b>	0.032	<b>0.401*</b>	-0.136	<b>0.239*</b>	-0.037	<b>0.365*</b>
Drinks	-0.109	-0.134	-0.153	-0.087	0.136	-0.154	-0.059	-0.077
Maternal smoking habits	-0.159	-0.105	-0.023	0.043	-0.120	-0.076	-0.044	-0.028
Maternal alcohol consumption habits	-0.035	-0.167	-0.092	0.075	-0.144	-0.098	-0.011	0.025

Statistically significant values for correlation coefficient ( $r$ ) are highlighted in **bold**.

\*Significant at the level of  $P < 0.05$ .

With respect to retinol levels in colostrum and mature milk samples collected from the older group, statistically significant correlations with milk and dairy products ( $r=0.424$  and  $r=0.379$ ,  $P < 0.05$ ), meat ( $r=0.368$  and  $r=0.386$ ,  $P < 0.05$ ), and eggs ( $r=0.276$  and  $r=0.255$ ,  $P < 0.05$ ; Table 2) were noted in PCA matrix. For the younger group, during both periods of lactation, significant correlations were found with the same types of foods ( $r=0.321$  and  $r=0.308$ ;  $r=0.410$  and  $r=0.321$ ;  $r=0.312$  and  $r=0.204$ ,  $P < 0.05$ ). Comparing the  $\beta$ -carotene levels in colostrum and mature milk samples obtained from both analyzed groups, notable associations with vegetables ( $r=0.737$  and  $r=0.556$ ;  $r=0.778$  and  $r=0.576$ ,  $P < 0.05$ ) and fruits ( $r=0.386$  and  $r=0.239$ ;  $r=0.401$  and  $r=0.365$ ,  $P < 0.05$ ) were observed.

We also performed multivariate statistical analysis on data set consisting of other maternal and infants characteristics (such as parity, gestational week at delivery, infant sex, maternal education level, residence, or occupation) and retinol and  $\beta$ -carotene levels in analyzed colostrum and mature milk samples. Interestingly, neither of these variables showed any statistically important association with analyzed biochemical parameters in studied groups during the both periods of lactation ( $P > 0.05$ ). In addition, there was not any significant correlation between the levels of both examined lipid components and maternal smoking and alcohol consumption habits during pregnancy and lactation ( $-0.2 < r = < 0.2$ , Table 2).

## Discussion

Presently, there is great interest in research on the composition of human milk obtained from nursing mothers  $\geq 35$  y of age compared with younger mothers ( $< 35$  y of age). Considering that breast milk is a compositionally variable fluid that varies within different populations, periods of lactation, maternal age, sociodemographic characteristics, dietary patterns, or lifestyle habits, there is a lack of information about the effects of these factors on the concentrations of important lipid constituents in breast milk such as retinol and  $\beta$ -carotene.

Among the macronutrients in breast milk, lipid content is the most susceptible to variations under the influence of various factors such as different portions (fore-, mid-, or hind-milk) and techniques for milk collection, different analytical methods for fat extraction and its determination, and diverse local dietary habits of nursing mothers during the pregnancy and lactation.

First, as the lactation stage proceeded, total lipid content increased in both groups, which is in agreement with previous reports. For both periods of lactation, we found significantly higher total lipid content in breast milk of lactating women  $\geq 35$  y of age than in younger women (Table 1). Thus, MA was found to be an

important factor showing a strong positive correlation with total lipid content ( $r > 0.74$ ). Total fat content in the colostrum samples were slightly elevated in both groups compared with recently published results [6,7,9]. These authors assumed that these biochemical changes in fat content throughout the lactation period could be related to the differences in fat metabolism with aging. Also, the reduction of milk volume and water content in milk, which occurred in the group of older mothers, potentially could be associated with a relative increase in fat concentration compared with younger mothers. Contrary to previous results, we also found a similar pattern in total lipid content for mature milk samples in both groups of nursing mothers (Table 1). This observation might be due to variable methods for determination of fat content and to different dietary habits of participants, although the exact mechanism of our remark is yet to be determined.

The results of the present study for retinol and  $\beta$ -carotene levels in both groups followed opposite trends from total lipid content as lactation proceeded. The mean concentrations of  $\beta$ -carotene in the milk from younger lactating mothers were significantly lower than those obtained from older lactating mothers during both periods of lactation (Table 1). Equally, retinol levels in colostrum samples were noticeably lesser in the younger group than in the older one, although this tendency was not observed for retinol levels in mature milk samples between the two groups.

In colostrum samples, the average retinol concentration was  $0.96 \pm 0.13 \mu\text{g/mL}$  ( $3.35 \pm 0.45 \mu\text{mol/L}$ ) in the older group and  $0.73 \pm 0.12 \mu\text{g/mL}$  ( $2.55 \pm 0.42 \mu\text{mol/L}$ ) in the younger women, which decreased in mature milk to  $0.48 \pm 0.08 \mu\text{g/mL}$  ( $1.67 \pm 0.28 \mu\text{mol/L}$ ) and  $0.46 \pm 0.11 \mu\text{g/mL}$  ( $1.60 \pm 0.38 \mu\text{mol/L}$ ) for the older and younger groups, respectively. These results are within the range for retinol levels in breast milk collected from unsupplemented nursing mothers published worldwide (Supplementary Table 3). The retinol levels in colostrum found in the present study are considerably lower than those of lactating women from Turkey [10], China [11], Germany [12], and Canada [13]; very close to those of lactating women from Cuba [14] and Japan [15]; but notably higher than those of Brazilian [16], Tunisian [17], and Bangladeshi [18] lactating women. For mature milk samples, the present results are very similar to those obtained for Czech mothers [19], but remarkably lower than those reported in studies carried out in Turkey [20], Germany [12], and Poland [21] (Supplementary Table 3). According to the literature, these considerable variations in retinol levels among different nations during the lactation period might be primarily associated with dissimilar dietary patterns and lifestyle habits and with inconsistency in time of sample collection and analytical approaches for retinol extraction and evaluation.

It has been suggested that retinol concentration in breast milk may be a useful indicator of vitamin A status of women and infants. According to the biochemical criteria [22], a milk retinol concentration  $>0.60 \mu\text{g/mL}$  ( $2.10 \mu\text{mol/L}$ ) in colostrum samples and  $>0.30 \mu\text{g/mL}$  ( $1.05 \mu\text{mol/L}$ ) in mature milk samples were considered sufficient to prevent vitamin A deficiency, and these cutoff values indicated that the mother is well nourished. Regarding colostrum samples collected from mothers in both groups, only one woman from the younger group (2.3% of analyzed population) had retinol content  $<0.6 \mu\text{g/mL}$  in her milk sample. Moreover, just two women, again from the younger group (4.6%), had retinol content  $<0.3 \mu\text{g/mL}$  in their mature milk samples. These results point to an adequate status of lactating mothers who participated in the present research and that there were sufficient amounts of retinol during both periods of lactations in analyzed milk samples, although retinol content in mature milk was significantly lower than in the initial phase of lactation.

Vitamins are transferred from maternal plasma to the mammary gland and secreted into breast milk, whereas milk fats are

synthesized de novo in the gland. Maternal diet is one of the most important determinants for the vitamin content in breast milk. Vitamin A is present in the diet as fatty acid esters of retinol (preformed vitamin A) and it is found in animal products such as organ meats, egg yolks, milk, and dairy products [5]. Statistical comparison of the present results with the data from dietary questionnaires disclosed weak and moderate positive correlations ( $r = 0.204$ – $0.424$ ) among retinol milk concentrations in both groups throughout lactation and the frequency of milk and dairy products, meat, and eggs consumption by mothers (Table 2). These associations were somewhat expected considering that lactating mothers from our region consume, on a daily basis, the foodstuffs from these groups (especially different types of milk and dairy products, meats and eggs, as shown in Supplementary Table 1). As these types of foods are predominant dietary sources of retinol, it can be supposed that considerable amounts of this vitamin in analyzed milk samples are the result of the dietary habits of lactating mothers from the local area.

Concerning  $\beta$ -carotene content in colostrum samples, there was a significant difference between the mean values for the older group ( $182.62 \pm 18.82 \text{ ng/mL}$ ) and the younger mothers ( $133.31 \pm 24.11 \text{ ng/mL}$ ). The concentration of  $\beta$ -carotene decreased with the duration of lactation in both groups ( $76.78 \pm 15.25$  and  $62.48 \pm 17.98 \text{ ng/mL}$  for older and younger mothers, respectively), but the difference among the analyzed groups remained statistically significant. In the present study, the average  $\beta$ -carotene levels in mature milk samples in both groups were slightly elevated compared with levels found in studies carried out in Japan [23] and the United States [24], but considerably higher than those found in studies in Germany [12] and Brazil [25] and also in a multinational study conducted in China, Australia, Canada, Chile, Mexico, United Kingdom, and the Philippines [26] (Supplementary Table 3). Comparing the obtained results for colostrum samples, older lactating mothers from the present study had values very close to those from Japan [23], but notably lower values were obtained in both groups compared with those from Germany [27] (Supplementary Table 3). In general, in the previous studies,  $\beta$ -carotene concentrations varied greatly among the different countries worldwide. These discrepancies in the results from various studies might be primarily due to variability in dietary patterns, lifestyle habits, or genetic differences among the populations involved in the studies. Some methodological factors, such as the postpartum day of collection, collection of fore- or hind-milk, or the collection from a one or both breasts could also contribute to those fluctuations. Moreover, dissimilarities in methods used for extraction of  $\beta$ -carotene from this type of biological matrix could importantly affect its concentration.

Until the end of pregnancy, a balanced diet is very important to guarantee the transfer of the nutrients to the fetus and to prepare it for birth and lactation. Mother's dietary habits during pregnancy and lactation, especially the consumption of fruits and vegetables, have a great effect on the content of  $\beta$ -carotene in breast milk. Humans cannot synthesize this compound, so it must be provided exogenously through dietary intake. In the present study, the significant correlations (PCA analysis) between  $\beta$ -carotene concentration in milk samples from both groups and the frequency of vegetables and fruits consumptions were observed, for both colostrum and mature milk samples (Table 2). The lactating mothers who participated in the present study reported frequent use of tomato, paprika, carrots, potatoes, and dark leafy vegetables such as spinach, lettuce, broccoli, or cauliflower, as well as apple, pear, apricot, peach, orange, or tangerines in their diet during the pregnancy and lactation period (Supplementary Table 1). These foodstuffs are characteristic in the regional dietary pattern of Serbia and can represent significant sources of  $\beta$ -carotene in nutrition. Although observed correlations were in the range from

weak positive to strong positive ( $r = \leq 0.239$  to  $\leq 0.778$ ; Table 2) and most of them were moderately positive, these findings should not be neglected and certainly could have an important effect on the level of  $\beta$ -carotene in analyzed human milk samples from the study region. In addition, statistical analysis indicated that the frequency of consumption of dark leafy vegetables, such as spinach, lettuce, broccoli, or cauliflower, had the most pronounced effect on  $\beta$ -carotene concentration in analyzed milk samples ( $r = 0.613$ – $0.837$ ).

## Conclusions

The present study demonstrated that the levels of total lipids, retinol, and  $\beta$ -carotene in breast milk during both analyzed periods of lactation were significantly higher in the group of mothers  $\geq 35$  y of age, with the exception of retinol levels in mature milk samples. In addition, PCA analysis used in the present study clearly confirmed that four variables (MA, total lipids, retinol, and  $\beta$ -carotene levels) might serve as a good criterion for delimitation of breast milk samples collected in different stages of lactation from mothers with various ages. Contrary to MA, there was not any significant association of retinol and  $\beta$ -carotene content in analyzed human milk samples with other maternal and demographic characteristics investigated in the present study. However, dietary patterns during pregnancy and lactation, especially in terms of consumption of foodstuffs characteristic for the study region, which are predominant dietary sources of retinol and  $\beta$ -carotene, may contribute to their levels in breast milk. The results of the present study only partially confirmed those of previous research, primarily in terms of significantly elevated lipid content in the colostrum of older mothers. Other data revealed in the present study could represent an important basis for further investigations of differences in the composition of human milk between older and younger lactating mothers. Although there are some assumptions, the accurate mechanism through which maternal age affects the differences in concentrations of these lipophilic components in breast milk is still unclear, and the biological significance is yet to be resolved.

## Acknowledgment

The authors acknowledge the women who participated in this study.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.nut.2019.02.019](https://doi.org/10.1016/j.nut.2019.02.019).

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