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Effect of convection and microwave heating on the retention of bioactive components in human milk

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ABSTRACT

Bioactive substances are very important components of human milk (HM), especially for premature newborns. The effects of convection (CH) and microwave heating (MWH) at 62.5 and 66 °C, on the level of selected bioactive components of HM: lysozyme (LZ), lactoferrin (LF), secretory immunoglobulin A (sIgA), basal lipase (BL), cytokine TGF-2, vitamin C and total antioxidant capacity (TAC) was compared. Regardless of the used heating methods the TAC of HM, determined by TEAC and ORAC-FL assay, proved to be insensitive to temperature pasteurization, in contrary to BL. MWH in the conditions of 62.5 for 5 min and 66 °C for 3 min are ensuring microbiological safety with a higher retention of most of the tested active HM proteins compared to CH. Only in the case of LZ the MWH had a more degradative effect on its concentration. Controlled conditions of MWH preserve the bioactive components of the HM better than CH.

1. Introduction

International health organizations and scientific societies such as the World Health Organization (WHO) recognize human milk (HM) as the normative standard of nutrition for newborns, especially premature infants (WHO, 2011). Breastfeeding reduces of occurrence of necrotizing enterocolitis, sepsis and other infections, and severe retinopathy and improves long-term neurocognitive development of infants (Belfort et al., 2016; Tudehope, 2013). The HM components that positively influence the development of infants include: adaptive immunity compounds (immunoglobulins sIgA, IgG, IgM, IgE, IgD), anti-inflammatory factors (IL-10, cytokine TGF-β2); antimicrobial factors (lactoferrin LF and lysozyme LZ), antioxidative factors (vitamin C, tocopherols) and enzymes, both antioxidant and digestive (Ballard & Morrow, 2013; Fichter et al., 2011).

A great challenge for a neonatal intensive care unit is nutrition of infants who are born extremely preterm. The first choice for these children feeding is an own mother's milk. When there is not enough milk from the own mother, donor human milk obtained from a human milk bank (HMB) is the best alternative (WHO, 2011). Considering the health status and development level of the premature infants, providing microbiologically safe milk is a priority. In most HMBs, donor milk is pasteurized by heating at 62.5 °C for 30 min (Holder pasteurization, HoP). This method guarantees the inactivation of microbiota and viruses, but also results in significant degradation of many nutritional and bioactive components (Peila et al., 2016).

Due to the negative influence of HoP on HM components, there is the need to develop alternative preservation techniques, which allow to keep bioactivity of HM components and thus its high nutritional and immunological quality. The most promising ones are using high pressure without heating (Wesolowska et al., 2019) and microwaves heating in controlled temperatures and time (Malinowska-Pańczyk et al., 2019).

The objective of this study was to compare the effects of convective heating (CH) and microwave heating (MWH) on the concentration or activity of selected bioactive components of HM. We hypothesized that the use of MWH, which allows to obtain the desired pasteurization temperature and the assumed pasteurization effect in a significantly shorter time than conventional CH, will have a less degradative effect on bioactive proteins of HM such as LZ, LF, sIgA, basal lipase BL and cytokine

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TGF-β2. The effect of both preservation techniques on the total antioxidant capacity (TAC) of HM and vitamin C levels was also compared.

All the experimental procedures were approved by the Local Ethics Committee of the Medical University of Gdansk. The patients gave written consent to participate in the study.

2. Materials and methods

2.1. HM collection

Samples of mature HM secreted between the 21st and 30st day after delivery were obtained from 23 healthy mothers who gave birth on the scheduled date and without complications at the Department of Obstetrics of the Clinical Hospital in Gdańsk. All newborns were in good health (Apgar score ≥ 9 in the first minute of life), and their body weights were within the norm (3100 – 4200 g). Characteristics of study participants are presented in Table 1. The samples were collected by the mothers within 24 h at home with an electric breast pump (Symphony, Medela Polska Sp. z o.o., Warsaw, Poland) and stored in a refrigerator at around 5 °C. HM was sampled into sterile containers designed for food contact, according to standard hygiene requirements. Milk samples from different mothers were delivered to the laboratory at the same time; they were immediately pooled and divided into smaller samples. Milk was frozen and stored at −80 °C until analysis, but not longer than three weeks.

2.2. Convective heating (CH)

Convection heating was carried out on a non-commercial equipment, but in a way that faithfully reflected the HoP pasteurization in industrial pasteurizers STERiFEED T30 routinely used in HMB. Milk samples (50 mL) were transferred to screw-cap laboratory bottles DURAN® and heated to the appropriate temperature in a water bath. The temperature of milk during processing was controlled with a digital laboratory thermometer placed into a control bottle containing the same volume of HM. A control bottle was placed in the central point of water bath. The temperature 62.5 °C was achieved within 18.1 ± 0.8 min and 66 °C within 18.3 ± 1.0 min. The milk samples were held at each temperature for 30 min. Samples for analysis were taken after reaching the appropriate temperature (time 0) and then every 10 min. Separate samples were prepared for each time interval. The processed milk was immediately cooled to below 20 °C in 5–10 min by immersion in an ice/water bath. All treatments were replicated three times.

2.3. Microwave heating (MWH)

MWH was achieved by using a prototype microwave device where the temperature was kept constant for a limited time (2450 MHz, 800 W, Enbio Technology, Poland). Milk samples (50 mL) were transferred to a laboratory bottles DURAN® and placed in a microwave pasteurizer. During the process, the HM was mixed by pumping in a closed circuit (Supplemental Figure). The temperature 62.5 and 66 °C were reached within 2.6 ± 0.5 and 2.9 ± 0.5 min, respectively. Milk samples were held at each temperature for 1, 3 and 5 min. After pasteurization, the milk was immediately cooled, in the same way as after CH, by immersion in an ice/water bath. The temperature and process time were selected based on our previous research (Malinowska-Pańczyk et al., 2019) and Patent No. PL235534-B1 (Martysiak-Żurowska et al., 2020). All heating treatments were performed in three replications.

Table 1
Demographic and obstetrical characteristics of study participants.

Parameter	Value*
No. of subjects	23
Maternal age (years)	28.3 ± 5.44 (19 – 39)
Parity (n)	1.6 ± 0.73 (1 – 4)
Gestational age (weeks)	40.0 ± 1.31 (38 – 42)
Infant gender	female 9, male 14
Maternal weight at delivery (kg)	76.8 ± 8.76 (59 – 93)
Birth weight (g)	3 516 ± 282.1 (3 090 – 4 170)
Apgar score at 1st min (points)	9.8 ± 0.40 (9 – 10)
Lactation time (day)	30.1 ± 4.95 (25 – 49)

*Values presented as arithmetic means ± (SD) and ranges (in parenthesis).

2.4. Determination of lactoferrin LF

The content of LF in samples of HM was determined by reversed phase high-performance liquid chromatography (RP-HPLC) with detection at 210 nm. The fat was removed from milk by centrifugation (4 000 *g*, 20 min, 4 °C). The aqueous fraction of HM was diluted 1:5 with deionized water. HPLC was performed with a 1220 Infinity LC chromatograph with diode-array detector DAD (Agilent Technologies, USA). The LF separation was performed at 35 °C on the Zorbax SB-C18 chromatographic column (4.6x150 mm, 5 μm, 100 Å) with guard column SB-C18 (4.6 × 12 mm) (Agilent Technologies, USA). The mobile phase was 0.1% solution of trifluoroacetic acid in water (A.) and 0.1% solution of trifluoroacetic acid in acetonitrile (B.) with a flow rate of 0.8 mL/min. The gradient elution applied 65% A: 35% B in 0–2 min, initial 35% B, final 50% B in 2–15 min and initial 50% B, final 35% B in 15–20 min was used. Quantification was done by external standardization using the standard solutions of LF from HM (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) with concentrations varying from 0.15 to 1.00 mg/mL. Parameters of the calibration curve and average coefficient of variance (CV) are presented in Supplemental Table. The average recovery of the analytical method of LF determination equal 98.15% by the internal standard method, using of three independent solutions of LF in five replicate and the same sample of HM as a matrix was taken into consideration in the final calculations. The results were expressed in g LF/L of the sample. All samples were analyzed in triplicate.

2.5. Determination of BL activity

The activity of BL in the tested samples of HM was determined using the commercial QuantiChrom™ Lipase Assay Kit (BioAssay Systems, Hayward, CA USA, Cat. No. DLPS-100), following the manufacturer's instruction attached to the test (Christen et al., 2013; Wesolowska, et al., 2019). Milk samples were diluted 1:499 with deionized distilled water. The lipase activity of the samples was measured and calculated according to the QuantiChromTM procedure. The activity of lipase was expressed in unit (U) per mL ($Ux10^3$/L), where one activity U represents the amount of enzyme that reduces the formation of superoxide radicals by 50%.

2.6. Determination of TGF-β2 concentration

The concentration of TGF-β2 in the tested samples of HM was determined by using the commercial test ELISA Kit Cloud-Clone Corp. (Park Row, TX, USA, Cat. No. SEA218Hu) following the instructions provided with the test by the manufacturer. The test was performed using the supernatant of the milk after centrifugation for 20 min at 3 000 *g* at 4 °C. The concentration of TGF-β2 in the milk samples was determined by comparing the absorption of the samples to the standard curve in the range from 15.6 to 1000 ng/L made using the stock solution provided with the kit.

2.7. Determination of sIgA content

sIgA was measured in raw or processed HM using sIgA ELISA kit (Immundiagnostic AG, Bensheim, Germany, Cat. No. K8870) following

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the manufacturer's protocol. Data were expressed as g per liter (g/L) of milk. All analytical determinations were carried out three times in duplicate.

2.8. Determination of LZ concentration

The concentration of LZ in the HM samples was determined using Lysozyme ELISA kit (Immundiagnostic AG, Bensheim, Germany, Cat. No. K6902) and calculated on the basis of calibration curve made with the use of standard solutions attached to the test. Data were expressed as a mg per L (mg/L) of milk. All analytical determinations were carried out three times in duplicate.

2.9. Determination of LZ activity

Turbidimetric procedure using *Micrococcus lysodeikticus* recommended by Sigma Chemical Co. (Sigma, St. Louis, MO) was used to determination of LZ activity. Lyophilized cells of *M. lysodeikticus* were suspended in 66 mM potassium phosphate buffer (pH 6.3) to the final absorbance 0.6–0.7 at 450 nm. Samples of HM were diluted in 66 mM potassium phosphate buffer (pH 6.3) (1:20) and 30 μL of dilution was transferred to 800 μL suspension of *M. lysodeikticus.* Immediately after mixing the decrease in absorbance at 450 nm was measured for 10 min at 1 min intervals using a Jenway 6305 UV–Vis spectrophotometer against the blank containing the 800 μL suspension and 30 μL of buffer. Each sample was measured in triplicate. One unit of LZ activity was defined as the amount of the enzyme that produces a ΔA450nm/min of 0.001 per min, at pH 6.3 using a suspension of *M. lysodeikticus* as substrate.

2.10. Determination of ascorbic acid content

Vitamin C content in HM was determined by the method described by Martysiak-Żurowska et al. (2017) which relies on reverse-phase high performance liquid chromatography with UV detection (RP-HPLC/UV). To determine the total content of vitamin C dehydroascorbic acid (DHsA), was reduced to ascorbic acid (AsA) with the use of DL-dithiothreitiol (DTT). PerkinElmer Series 2000 chromatograph (PerkinElmer, Waltham, MA, US) a C-18 column (125 × 4.0 mm I.D., d_p 5 μm) with a guard cartridge (C18, 5 μm) (Merck, Darmstadt, Germany), column temperature of 25 °C, detection at 245 nm, injection volume 25 μL and flow rate 0.7 mL/min was used. The mobile phase was a mixture of 0.1% acetic acid and methanol (95:5, v:v) with isocratic elution. Using a standard of AsA (Sigma-Aldrich, Merck KGaA, Darmstadt, German) a series of dilutions in the range of 0.01 to 100 mg AsA /L was made. Parameters of the calibration curve and average coefficient of variance (CV) are presented in Supplemental Table. The average recovery of the analytical method of vitamin C determination equal 97.65% was taken into consideration in the final calculations. The results were expressed in mg AsA/L of the sample.

2.11. Determination of TAC

2.11.1. TAC measure by ABTS assay

The TAC was determined by the ABTS assay designed for HM (Martysiak-Żurowska et al., 2017). The stock solution of the ABTS radical cation was prepared by dissolving 38.4 mg of ABTS (2,2-azino-bis (3-ethylbenzthiazoline-6-acid) in 10 mL of a sodium persulphate ($K_2S_2O_8$) solution (2.45 mM). The working solution was obtained by diluting the stock solution of the ABTS radical cation with methanol to obtain an absorbance of 0.7 ± 0.005 at 734 nm. HM samples (0.02 mL) were reacted with 2 mL of the $ABTS^{\bullet+}$ working solution for 10 min at room temperature. After centrifugation (3 000 *g*, 5 min) absorbance of transparent solution was measured at 734 nm against the methanol as the reference sample on a BioTek Synergy HT Plate Reader (Biokom, Poland). As a standard the solution of Trolox a water-soluble vitamin E analog (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used. The average coefficient of variance (CV) and standard curve parameters were calculated (Supplemental Table). The TAC of the HM samples was expressed in mM of Trolox and presented as Trolox equivalent antioxidant capacity (TEAC) per L of HM.

2.11.2. The TAC measure by ORAC-FL

The TAC of milk was determined in the oxygen radical absorbance capacity assay with fluorescein (ORAC-FL) (Tijerina-Sáenz et al., 2009). In the ORAC-FL method, peroxyl radicals are generated during the thermal decomposition of 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) dissolved in a phosphate buffer (pH = 7.4). The free radical damage to fluorescein (FL) leads to a decrease in fluorescent intensity. The presence of antioxidants inhibits free radical damage to the FL compound. Reduction the degradation rate of FL over time is proportional to the antioxidant content of the solution. The ORAC-FL assay of milk was conducted in 96-well black microplate. Milk samples were diluted 1:199 with a phosphate buffer. 150 μL of 0.4 nM FL solution and 25 μL of diluted milk sample or Trolox standard solution (0.0–20 μM) were added to the wells. After incubation at 37 °C by 30 min, 25 μL of 153 mM fresh solution of AAPH was added to each well. All measurements were performed with a microplate reader (Synergy HT, BIOKOM, Janki k /Warszawy, Poland). The microplate was shaken for 10 s and fluorescence was read every minute for 60 min at excitation of 485 nm and emission of 528 nm. The average coefficient of variance (CV) and standard curve parameters were presented Supplemental Table. The value of ORAC-FL was expressed in mM of Trolox (TE) per L of HM.

2.12. Statistical analysis

The results were presented as mean values and standard deviations (mean ± SD). The statistical analysis was conducted using SigmaPlot 11.0 software (Systat Software, Inc., USA). The normality of all variables was evaluated with the Shapiro-Wilk test. The significance of differences in the content or activity of the compounds measured in raw and processed HM was determined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-hoc test. Differences between means were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

The pasteurization effect depends not only on the temperature and processing duration used but also on the time required to reach the set pasteurization temperature. The set temperature of HM can be achieved in significantly shorter time than CH by using MWH in the Enbio Technology prototype pasteurizer used in this work. Our previous work has shown that the factor determining the efficiency of the pasteurization is the appropriate microbiological quality of HM. One criterion for accepting milk into HMB is the initial level of microbiological contamination, which must not exceed 10^5 colony forming units (cfu)/mL. For such good quality raw material, it is possible to eliminate the vegetative microbiota (detection below 1 cfu/mL) after the application of HoP or CH at 66 °C for 20 min as well as MWH at 62.5 °C for 5 min or 66 °C for 3 min (Malinowska-Pańczyk et al., 2019; (Martysiak-Żurowska et al., 2020). The paper presents the effect of both heating techniques (CH and MWH) carried out at the above temperatures on the bioactive components of HM.

3.1. Concentration of LF

LF is a glycoprotein found in the body fluids and secretions such as milk, tears and saliva. LF exhibits bacteriostatic, bactericidal, anti-inflammatory, antitumor, antioxidant and antiviral properties and is a major HM whey protein supporting the underdeveloped immune sys-

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tem of an infant. Epithelial cells synthesize this protein in the mammary gland, and prolactin regulates its expression. LF content in HM varies depending on the lactation phase and ranges from about 7 g/L in colostrum to about 2 g/L in mature milk (Montagne et al., 2000). In this study in the raw pooled HM sample, the mean concentration of LF was 1.8 g/L, which is characteristic of mature milk (Table 2). After 30 min of keeping at these temperatures LF level dropped by a further 40 and 20%, respectively.

It was shown that the LF is highly sensitive to high temperatures, and its level of denaturation depends on the temperature and duration of the process. CH of milk to the temperature 62.5 °C and 66 °C caused a decrease in LF content by approximately 20 and 60%, respectively, compared to raw milk. After 30 min of keeping at these temperatures LF level dropped by a further 40 and 20%, respectively. Similar losses of LF in HM after HoP were found by Goldsmith et al. (1983), while Klotz et al. (2017) showed 80% reduction of this protein. The level of LF degradation in HM after MWH was significantly lower. At 62.5 °C for 5 min, the LF concentration in HM did not change significantly (Fig. 1). However, using a higher MWH temperature increased the level of reduction of this protein. In HM heated at 66 °C for 5 min a 50% decrease of LF concentration was observed.

3.2. Concentration and activity of LZ

LZ is an enzyme present in all tissues and organs and in most tissue fluids (e.g., saliva, plasma, tears, milk). In the body, it is found in granulocytes, monocytes and macrophages, where it takes part in a non-specific immune response, which is the first line of defense against pathogens. Unlike other immune components such as LF and immunoglobulins, the content of LZ in milk increases with the time of lactation. In a mature HM LZ accounts for 8% of the total milk protein (Montagne et al., 2000) and in tested HM of this study was about 100 mg/L (Table 2). Similar concentration of lysozyme in mature HM showed Trend et al. (2016), but Klotz et al. (2017) demonstrated nearly half the content of this enzyme. These differences in the lysozyme concentration may result from the individual variability of HM.

LZ is a heat stable enzyme in an acidic environment, but in neutral pH its susceptibility to inactivation at elevated temperature increases. Unfortunately, the data regarding changes in the content/activity of LZ after CH are ambiguous. Many authors have shown that after heating at 62.5 °C for 30 min the amount of lysozyme decreases drastically. Koenig et al. (2005) detected that LZ content decreased by about 75% under these conditions but Czank et al. (2009) showed 53% reduction. In our studies after HoP the level of lysozyme decreased by about 44% comparing to raw HM. At temperature 66 °C, losses were even greater and amounted to about 55% after 20 min of treatment (Table 2). Retention of LZ was on a similar level when MWH was used. Under conditions where the complete pasteurization effect was achieved (62.5 °C for 5 min and 66 °C for 3 min), the LZ retention was 44 and 30%, respectively (Fig. 1).

We also determined the activity of LZ and in tested HM it was 31,555 Ux10^3/L. Lima et al. (2017) demonstrated a significant loss of LZ activity after HoP, as much as 50%, while Viazis et al. (2007) showed only 21% decline. In our studies, the LZ activity after HoP decreased by 26% and at 66 °C for 20 min by about 60%. MWH resulted in retention of LZ activity at a level comparable to that of HoP. At 62.5 °C for 5 min and 66 °C for 3 min reduction in LZ activity was about 30% (Fig. 1). Quan et al. (1992) showed, that microwave treatment at low temperatures (20 °C to 53 °C) reduced LZ activity in HM by about 19%. MWH at higher temperatures (72 °C to 98 °C) caused a drastic decrease in LZ activity, to a level of about 2% of the activity in raw HM.

3.3. Concentration of sIgA

sIgA is classified as a soluble pattern recognition receptor (sPRR) and plays a key role in humoral immunity. This immunoglobulin inhibits the colonization of pathogens in the infant's intestinal epithelium, which prevents bacteria from entering the bloodstream (Maxson et al., 1995). HM used in experiments came from donors who were breastfeeding in the first month and contain about 830 mg of sIgA per L (Table 3). According to Trend et al. (2016) content of sIgA in mature HM (26th-30th day of lactation) amounted to about 700 mg/L. The sIgA content in HM changes with the duration of lactation. Colostrum and mature milk after the second year of lactation contain higher concentration of sIgA than transitional and mature milk from the first year of lactation (Trend et al., 2016).

The concentration of sIgA in HM decreased by about 21% after CH at 62.5 °C by 30 min. Reduction of sIgA about 58% was noted already after reaching 66 °C. Extending the holding time for 20 min at 66 °C led to retention only 37% of sIgA. Changes described by other research groups regarding the quantity of this immune factor in HM after pasteurization at 62.5 °C for 30 min are variable. According to Lima et al.,

Table 2
The concentration of lactoferrin (g/L), lysozyme (g/L) and activity of lysozyme (U × 10^3/L) in raw human milk (control) and human milk processed by convection and microwave heating under different conditions (temperature and time).

Heating time (min)	Content/activity of			Heating time (min)	Content/activity of		
	Lactoferrin (g/L)	Lysozyme			Lactoferrin (g/L)	Lysozyme	
		(g/L)	(U × 10^3/L)			(g/L)	(U × 10^3/L)
	after convection heating at				after microwaves heating at		
				62.5 °C			
Raw milk	1.83 ± 0.176^{a}	100.4 ± 1.48^{a}	31555 ± 1806^{a}	Raw milk	1.83 ± 0.176^{a}	110.2 ± 6.75^{a}	31555 ± 1806^{a}
RST*	1.50 ± 0.057^{b}	91.4 ± 12.14^{a,b}	28333 ± 2357^{a,b}	RST*	1.84 ± 0.132^{a}	63.8 ± 1.24^{b}	29620 ± 1958^{a}
10	1.12 ± 0.186^{c}	87.9 ± 9.60^{b,c}	24925 ± 3509^{b,c}	1	1.70 ± 0.192^{a}	59.6 ± 5.56^{b}	29611 ± 1178^{a}
20	0.99 ± 0.271^{d}	69.9 ± 2.93^{c}	24958 ± 907^{c}	3	1.69 ± 0.167^{b}	34.5 ± 1.03^{c}	24293 ± 1657^{b}
30	0.74 ± 0.096^{d}	56.1 ± 9.51^{d}	23404 ± 1156^{c}	5	1.65 ± 0.191^{b}	44.4 ± 3.46^{c}	21974 ± 3022^{b}
	66 °C						
Raw milk	1.83 ± 0.176^{a}	100.4 ± 1.48^{a}	31555 ± 1806^{a}	Raw milk	1.83 ± 0.176^{a}	110.2 ± 6.75^{a}	31555 ± 1806^{a}
RST*	0.75 ± 0.104^{b}	112.8 ± 2.59^{a}	24414 ± 2989^{b}	RST*	1.57 ± 0.098^{b}	48.8 ± 3.5^{b}	29030 ± 196^{b}
10	0.62 ± 0.136^{b}	82.2 ± 1.81^{b}	19037 ± 1204^{c}	1	1.43 ± 0.255b	32.8 ± 6.4^{c}	22528 ± 196^{c}
20	0.49 ± 0.114^{c}	45.8 ± 4.76^{c}	12929 ± 2052^{d}	3	1.05 ± 0.120^{c}	29.3 ± 4.94^{c}	22389 ± 2950^{c,d}
30	0.41 ± 0.117^{c}	27.6 ± 9.69^{d}	11254 ± 1628^{e}	5	0.92 ± 0.076^{c}	19.2 ± 1.44^{d}	17260 ± 3206^{d}

*RST – Reaching the set temperature. Each value is expressed as mean ± SD of three samples per triplicate.
^{a,b,c} – Means value within a column with different superscripts are significantly different at a given temperatures ($p < 0.05$).

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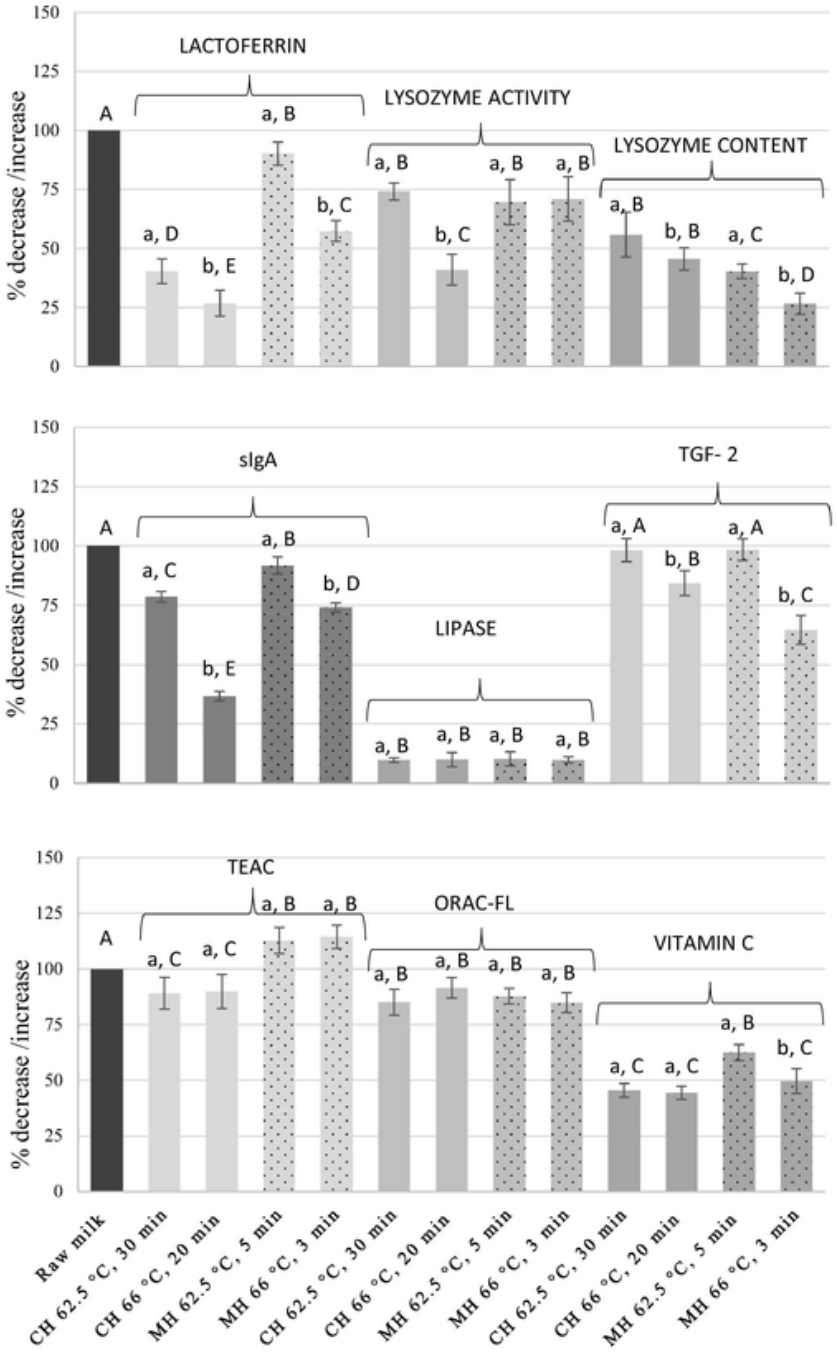

Fig. 1. Changes in mean concentration/activity of bioactive components in human milk after convention heating (CH) at 62.5 ˚C, 30 min; 66 ˚C, 20 min; and after microwaves heating (MWH, pattern with dots) at 62.5 ˚C, 5 min; 66 ˚C, 3 min; compared with raw human milk (HM, 100% value). The data are presented as mean values with the standard deviation (±SD). Statistically significant differences were observed and marked: a, b, .. between data in the same pasteurization methods (CH, MWH); A, B, … between data in the different pasteurization methods and raw milk ($p < 0.05$).

(2017) the amount of sIgA in HM after pasteurization lowered only slightly but Czank et al. (2009) showed that the concentration of this protein decreased by about 30%. Even greater losses in IgA content, by about 64% after HoP, was reported by Braga & Palhares (2007).

MWH at 62.5 ˚C did not significantly affect the sIgA level in HM. After 5 min of heating at 62.5 ˚C, changes in sIgA content were statistically insignificant and ranged from 3 to 9%. Unfortunately, increasing the process temperature to 66 ˚C led to a gradual decrease in the content of this immunoglobulin. After heating at 66 ˚C for 3 min (conditions allowing for microbial safety) 74% retention of this component was obtained. Extending the heating time to 5 min led to 37% reduction of sIgA (Table 2). Some authors have also shown that IgA activity decreases with increasing temperature during microwave heating. This immunoglobulin was stable up to about 60 ˚C and almost no activity was detected at 77 ˚C (Ovesen et al., 1996; Quan et al., 1992).

3.4. Concentration of cytokine TGF-β2

Cytokine TGF-β family constitutes the most abundant cytokines of HM, and comprises of three isoforms TGF-β1, TGF-β2, TGF-β3, of which TGF-β2 predominates. TGF-β2 is the major form in colostrum and mature milk, representing up to 95% of all TGF- β (Hawkes et al., 1999).

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Table 3
The concentration of sIgA (mg/L), TGF-β2 (ng/L) and BL activity (Ux10^3/L) of raw human milk (control) and human milk processed by convection and microwave heating under different conditions (temperature and time).

Heating time (min)	Content/activity of			Heating time (min)	Content/activity of		
	sIgA (mg/L)	TGF-β2 (ng/L)	BL (U × 10^3/L)		sIgA (mg/L)	TGF-β2(ng/L)	BL(U × 10^3/L)
	after convection heating at				after microwaves heating at		
				62.5 °C			
Raw milk	830.0 ± 27.0^a	217.7 ± 17.68^a	131.1 ± 2.02^a	Raw milk	827.9 ± 30.0^a	217.7 ± 17.68^a	131.1 ± 2.02^a
RST*	730 ± 15.6^b	242.3 ± 8.11^b	14.4 ± 0.88^b	RST*	805.0 ± 16.8^{a,b}	280.8 ± 6.24^b	66.7 ± 6.34^b
10	747 ± 22.0^b	214.2 ± 11.36^a	13.7 ± 1.02b	1	806.1 ± 29.7^{a,b}	262.1 ± 12.95^c	29.4 ± 5.63^c
20	594.0 ± 26.6^c	210.4 ± 15.63^a	13.7 ± 1.13^b	3	766.7 ± 22.7^b	268.4 ± 8.97^c	15.7 ± 3.65^d
30	652.5 ± 18.6^c	213.9 ± 10.45^a	12.8 ± 1.27^b	5	759.6 ± 29.4^b	214.2 ± 9.90^a	13.5 ± 3.85^d
	66 °C						
Raw milk	830.0 ± 27,0^a	217.7 ± 17.68^a	131.1 ± 2.02^a	Raw milk	827.9 ± 30.0^a	217.7 ± 17.68^a	131.1 ± 2.02^a
RST*	483.3 ± 19.9^b	214.9 ± 6.48^a	11.3 ± 0.90^b	RST*	682.1 ± 20.2^b	164.6 ± 4.46^b	32.7 ± 7.64^b
10	357.5 ± 23.1^c	215.4 ± 8.26^a	13.7 ± 0.84^b	1	650.1 ± 31.6^{b,c}	140.2 ± 8.55^c	16.4 ± 1.00^c
20	305.0 ± 16.7^d	183.5 ± 9.63^b	13.1 ± 3.84^b	3	613.3 ± 16.4^c	140.7 ± 8.54c	12.9 ± 1.73^c
30	236.0 ± 19.4^e	146.6 ± 11.23^c	6.40 ± 3.37^b	5	524.2 ± 19.1^d	126.2 ± 9.52^a	7.6 ± 1.33^d

*RST – Reaching the set temperature. Each value is expressed as mean ± SD of three samples per triplicate.
^{a,b,c} – Means value within a column with different superscripts are significantly different at a given temperature ($p < 0.05$).

TGF-βs are an important family of growth factors involved in maintaining homeostasis in the intestine, regulating inflammation and allergy development, and promoting oral tolerance development in infants. TGF-β2 is considered as a one for a key immunomodulatory factor in HM. Breast milk cytokines have the potential to regulate the immune response to food antigens in infants. Early oral antigen exposure may be beneficial for promoting tolerance development during introduction of food antigens (Hawkes et al., 1999; Kalliomäki et al., 1999)

The concentration of TGF-β2 is significantly higher in colostrum (1376 to 5394 ng/L, average 3325 ng/L) compared with mature milk (592 to 2697 ng/L, average 1644 ng/L) (Kalliomäki et al., 1999). According to the authors, a wide range of TGF concentration in milk from both stages of lactation, detected at each time in the study, indicates a significant influence of individual features of women (Hawkes et al., 1999; Kalliomäki et al., 1999). In our studies the pooled sample of raw HM the presence of transforming growth factor TGF-β2 was found at the level of about 220 ng/L (Table 3).

The cytokine TGF-β2 present in HM is resistant to the temperature of 62.5 °C transmitted convective. The CH at 62.5 °C for 30 min did not cause statistically significant changes in the concentration of the tested protein in relation to raw milk (Table 3). Similar results were obtained by Escuder-Vieco et al. (2018). In the presented study an unexpected result was a slight, about 10%, increase in TGF-β2 content in HM convectively heated to 62.5 °C. MWH of HM to 62.5 °C increased TGF-β2 level by up to nearly 30%. At the time and temperature values, at which the microbiological safety of HM by MWH is achieved (62.5 °C; 5 min), the concentration of the tested cytokine was comparable to that in unheated milk (Fig. 1). An increase in the concentration of TGF-β2 in HM because of heating was also found in samples subjected to heating using the HTST (High Temperature Short Time) technique. In samples heated at 70 °C for 5–25 s, the level of the factor increased by about 20% compared to the content in the samples before the process (Escuder-Vieco et al., 2018). According to these authors, the increase of the relative TGF-β2 concentration after heat treatment were probably due to either TGF-β2 release from milk cells during frozen storage, to the partial heat activation of TGF or to the release of TGF-β2 from chondroitin sulfate-containing proteoglycans by different known activators (Escuder-Vieco et al., 2018; Witkowska-Zimny & Kaminska-El-Hassan, 2017). In our opinion, the speed of generating high milk temperature was also of great importance, and hence the rapid degradation of milk cells.

A different course of changes in the content of TGF-β2 in HM was observed as a result of heating the milk at 66 °C. CH milk samples at this temperature for 30 min resulted in over 30% decrease in cytokine concentration. The use of MWH led to the degradation of TGF-β2 by about 25% already at the stage of obtaining the desired milk temperature.

3.5. Influence on the activity of BL

Lipases breaks down triacylglycerol into monoacylglycerides and free fatty acids. However, the levels of lipases are low in newborn infants and in preterm infants in particular. In breast-fed infants, digestion of milk triglycerides is catalyzed by a coordinated action of gastric lipase, colipase-dependent pancreatic lipase, and BSSL (Hernell & Bläckberg, 1994). Human milk contains the major part of BSSL and the lesser part originates in the infant's pancreas. Formula-fed neonates and preterm infants fed pasteurized HM have been shown to have a lower fat absorption and growth rate compared to neonates fed raw HM (Andersson et al., 2007; Hernell & Bläckberg, 1994).

We found the activity of BL in pooled raw HM in the average of 130 U/mL. The obtained results are analogous to those presented in the available literature data. Christen et al. (2013) using the same test as in the presented study, determined the activity of BL in raw HM at the level of about 120 U/mL. Lipases are enzymes very sensitive to high temperature. BSSL inactivation starts at 45 °C (Wardell et al., 1984). Some authors (Czank et al., 2009; Christen et al., 2013, Wesolowska et al., 2019) showed that HoP almost completely eliminated BSSL activity. In the cases of CH, when the temperature of the milk samples reached 62.5 °C and 66 °C, an approximately 90% decrease in the activity of the tested enzyme was found (Table 3). The effect of high temperature generated by MWH also led to a drastic decrease in lipases activity in the tested samples of HM, although the process was less violent. When the temperature of 62.5 °C and 66 °C was reached, BL activity was found lower by 50 and 75% compared to the activity in raw HM, respectively. Continued heating resulted in a further reduction of enzyme activity. The applied pasteurization methods allow only about 9% of the activity of HM lipase to be preserved.

3.6. Influence of CH and MWH on the antioxidant potential of HM

HM contains many ingredients with antioxidant effects. Antioxidative properties of HM refer to the sum of activities derived from: active nonenzymatic antioxidants (such as vitamin C and A, tocopherols, glutathione, cysteine, arginine, citrulline, taurine, creatine, selenium, zinc,

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β-carotene, carotenoids coenzyme Q10, flavonoids), antioxidative enzymes (superoxide dismutase, glutathione peroxidase, catalase, reductase) and other bioactive factors (e.g. urate, LF) (Zulueta et al., 2009). A competent antioxidant system of the infants is strongly dependent on the intake of free radical deactivating molecules from feeding. Determining the content and activity of each of these substances individually is very complicated and time-consuming. Therefore, several methods have been developed that allow the overall determination of the antioxidant activity of milk and other biological matrices (plasma, blood, saliva) as well as food (meat, juices, etc.). The techniques such as ABTS or ORAC assays allow the determination of the nonenzymatic activity of matrices, defined as TAC (Marinković et al., 2016; Zulueta et al., 2009). Level of TAC in mature HM from healthy mothers depends on many factors such as diet, live style, health and individual characteristic (Nikniaz et al., 2013; Tijerina-Sáenz et al., 2009).

3.7. TEAC of HM

The TEAC value in the tested samples of raw HM was 1.66 mM TE/L. Nikniaz et al. (2013) shown that TEAC in mature HM is on average 0.3 mM while Turoli et al. (2004) using the same technique found TEAC at the level above 3 mM per L of HM.

Our previous research (Martysiak-Żurowska et al., 2017) as well as presented by Lorençoni Silva Júnior & Fronza (2020) showed that HoP has no significant effect on the TAC of HM. We showed also that the activity of antioxidant enzymes in HM samples pasteurized by MWH was comparable or higher than in samples subjected to CH (Martysiak-Żurowska et al., 2019). In the present study, CH carried out for 10 min at 62.5 and 66 °C caused a slight decrease of the TEAC value, of about 10%. The longer heating did not result in a further significant decrease in the level of antioxidant capacity of milk (Table 4). Using MWH increased TEAC level in HM already at the time the milk reached the 62.5 °C and 66 °C by about 13 and 7%, respectively. As with the use of CH, extension of heating time did not generate any further changes in the TEAC value. The increase in the antioxidant activity of milk after MWH has probably a similar source as for the temporal increase in the level of TGF-β2. It can be assumed that it is related to the release of antioxidant factors from the cells contained in the milk as a result of their breaking under the influence of rapid temperature increase.

3.8. ORAC-FL of HM

Raw HM showed ORAC-FL value at about 6.5 mM TE /L. This is quite a high level of ORAC-FL for mature milk. Research indicates that ORAC-FL colostrum ranges from approximately 2.5 to 5.5 mM TE/L and from about 1.8 to 3.0 mM TE/L in mature milk (Marinković et al., 2016).

The greatest changes in the ORAC-FL level in CH milk occurred at the stage of heating the sample to the pasteurization temperature (Table 4). After reaching 62.5 °C and 66 °C the level of ORAC-FL was about 12 and 10% lower, respectively, than in raw milk. Keeping samples at the heating temperature for 30 min did not change the ORAC-FL level. Using WMH, the most significant changes in the ORAC-FL level was found between 0 and 1 min of HM heating at both temperatures. Similar to CH pasteurization, extension MWH time did not significantly affect the total nonenzymatic antioxidative capacity of HM. The greatest change in ORAC-FL was found after MWH of HM for 5 min at 66 °C (Fig. 1).

Marinković et al. (2016) also found no significant effect of thermal pasteurization on total nonenzymatic antioxidative capacity measured by the ORAC-FL. Importantly, however, it was found that specific components and features of HM were altered. Processing shifted the load of antioxidative activity from ascorbate to urate and led to a decreased the capacity of milk to diminish hydroxyl radical.

3.9. Concertation of vitamin C

Vitamin C acts as a cofactor for several human enzymes among others that synthesize and metabolize collagen, hormones (such as adrenaline and serotonin) and carnitine, facilitate the synthesis of corticosteroids, the conversion of cholesterol to bile acids and the biosynthesis of connective tissue components, including elastin. Moreover, due to its strong reducing properties, vitamin C effectively neutralizes reactive oxygen species (ROS), reactive nitrogen species (RNS), singlet oxygen and hypochlorite, thus protecting tissues that are subjected to high oxidative stress. Vitamin C also offers indirect antioxidant protection by regenerating other biological antioxidants, such as glutathione and α-tocopherol, to their active state (Arrigoni & De Tullio, 2002).

Vitamin C is the most abundant vitamin in HM. Due to its high sensitivity to external factors, vitamin C is one of the key indicators of changes in the properties of foods during processing and storage. About 48 mg of AsA/L was found in the pooled raw HM, but already reaching

Table 4
The concentration of vitamin C (mg AsA/L) and total antioxidant activity by TEAC and ORAC-FL assay (mM TE/L) in raw human milk (control) and human milk pasteurized by convection and microwave heating under different conditions (temperature and time).

Heating time (min)	Content/activity of			Heating time (min)	Content/activity of		
	ORAC-FL (mM TE/L)	TEAC (mM TE/L)	Vitamin C (mg AsA/L)		ORAC-FL(mM TE/L)	TEAC(mM TE/L)	Vitamin C (mg AsA/L)
	after convection heating at				after microwaves heating at		
				62.5 °C			
Raw milk	6.46 ± 0.309 ^{a}	1.66 ± 0.190^{a}	48.38 ± 1.13^{b}	Raw milk	6.46 ± 0.309^{a}	1.66 ± 0.071 ^{a}	48.38 ± 1.13 ^{a}
RST*	5.74 ± 0.089^{b}	1.60 ± 0.187^{a}	29.57 ± 1.40^{b}	RST*	6.48 ± 0.272^{a}	1.87 ± 0.091^{b}	38.19 ± 1.16^{b}
10	5.82 ± 0.364^{b}	1.45 ± 0.196^{b}	27.13 ± 1.46^{bc}	1	6.14 ± 0.b429^{a}	1.79 ± 0.139^{a}	35.57 ± 2.79^{bc}
20	5.88 ± 0.330^{ab}	1.55 ± 0.177^{b}	24.78 ± 1.58 ^{cd}	3	6.03 ± 0.334^{b}	1.76 ± 0.078^{a}	33.90 ± 1.94 ^{cd}
30	5.49 ± 0.391^{b}	1.48 ± 0.194^{b}	22.03 ± 0.68^{d}	5	5.66 ± 0.704^{b}	1.87 ± 0.108^{b}	30.15 ± 1.07^{d}
	66 °C						
Raw milk	6.46 ± 0.309^{a}	1.66 ± 0.268^{a}	48.38 ± 1.13^{a}	Raw milk	6.46 ± 0.309^{a}	1.66 ± 0.060^{a}	48.38 ± 1.13^{a}
RST*	5.83 ± 0.177^{b}	1.63 ± 0.229^{a}	25.23 ± 1.16^{b}	RST*	6.08 ± 0.289^{a}	1.78 ± 0.082^{a}	31.43 ± 0.49^{b}
10	6.15 ± 0.515^{ac}	1.45 ± 0.258^{b}	24.41 ± 0.80^{b}	1	5.56 ± 0.190^{b}	1.84 ± 0.169^{b}	28.76 ± 2.26^{b}
20	5.91 ± 0.441^{bc}	1.49 ± 0.304^{b}	21.37 ± 0.64^{b}	3	5.48 ± 0.414^{b}	1.90 ± 0.099^{b}	23.92 ± 1.33^{c}
30	5.65 ± 0.486^{b}	1.57 ± 0.354^{b}	20.20 ± 0.62^{b}	5	5.23 ± 0.401^{b}	1.77 ± 0.122^{a}	20.40 ± 0.63^{c}

*RST – Reaching the set temperature. Each value is expressed as mean ± SD of three samples per triplicate.
^{a,b,c} – Means value within a column with different superscripts are significantly different at a given temperature ($p < 0.05$).

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a temperature of 62.5 °C using CH and MWH caused a decrease of this vitamin content by about 40 and 20%, respectively. After it reached 66 °C by using CH and MWH 50 and 35% decrease of vitamin C was obtained, respectively (Table 4). Heating the samples at the applied, constant temperatures resulted in a further, mild decrease in the vitamin C content in HM. After the longest exposure to CH (30 min) at both analyzed temperatures, vitamin C content was to about 55–58% lower than in unheated milk. Almost the same losses in concentration of vitamin C was reported by Van Zoeren-Grobben et al. (1987), by about 40%. In comparison Moltó-Puigmartí Permanyer Castellote & López-Sabater (2011) detected a lower degradation of vitamin C concentration, by about 20% after HoP.

The use of MWH at 66 °C influenced on vitamin C content in the almost same way as CH. But the use of MWH at 62.5 °C had much less effect on this vitamin degradation. After 5 min MWH of milk the retention of concentration of vitamin C was about 60% to that in raw milk (Fig. 1).

4. Conclusion

The bioactive components of HM are very important for infants, especially for preterm newborns. They play a crucial role in promoting healthy growth and development of infants. The retention of bioactive components such as BL, TGF-β2, and ORAC-FL in HM MWH at 62.5 °C for 5 min was at a comparable level to that in HM after HoP. However, the content of LF, sIgA, vitamin C and antioxidant activity expressed as TEAC in milk after MWH was higher than that after HoP. Only in the case of LZ the use of MWH had a more degradative effect on its concentration and activity. Although we have shown in our previous research that the level of degradation of microorganisms analogous to that obtained by HoP can be achieved by using a temperature of 66 °C in CH for 20 min and using of MWH at 66° C for 3 min the current study shows that these conditions have various effects on the level of HM bioactive components. At a higher temperature (66 °C) and despite the shorter pasteurization time (20 min in CH, 3 min in MWH) retention of active proteins were usually lower than in 62.5 °C (30 min in CH, 5 min in MWH). MWH and CH at higher temperatures caused more degradation of LF, LZ, sIgA and TGF-β2. Only BL characterized by highly sensitive to heating was at least 90% degraded, regardless of the conditions of pasteurization of HM. Results derived from this study suggest that MWH of HM at 62.5 °C for 5 min could be a practical and efficient alternative to HoP in HMB resulting in HM with higher capacity to confer immunological protection to infants. Strict adherence to the temperature and time of the MWH process is an extremely important element affecting HM quality. Even a slight increase in temperature can result in a greater depletion of bioactive components in the HM. Additionally, HM can be a transmission source of the cytomegalovirus, human immunodeficiency virus, human T cell leukaemia virus and other viruses. Therefore, further research is needed to confirm that MWH can effectively eliminate virus particles.

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CRediT authorship contribution statement

Dorota Martysiak-Żurowska: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Edyta Malinowska-Pańczyk:** Conceptualization, Methodology, Validation, Investigation, Visualization, Writing – original draft. **Małgorzata Orzołek:** Methodology, Investigation, Data curation. **Bogumiła Kiełbratowska:** Resources, Data curation. **Elena Sinkiewicz–Darol:** Methodology, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131772.

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