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SALIVA-INDUCED FLOCCULATION OF MILK FAT GLOBULES STUDIED BY IMAGE ANALYSIS

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Abstract

The present study investigated the effect of changes in the milk fat globule membrane on the emulsion changes and flocculation of milk fat globules under the influence of saliva. Samples of raw and homogenized milk were examined. The flocculation process was characterized through computer image analysis of the emulsion observed with the confocal microscope. As a result, the fractal dimension was calculated, which characterized the extent of flocculation. The applied method allows unbiased quantitative comparison of changes in the milk emulsion system.

Key words: *saliva, flocculation, milk fat globules, image analysis, fractal dimension*

1. INTRODUCTION

Milk fat occurs in milk in the form of emulsion as fat globules. These are highly ordered and complex structures with sizes ranging from 0.1 to 10 μm (Walstra et al. 2006). Beginning from the interior, they are built of: a lipid backbone constituted by triacylglycerols, and a monolayer of polar lipids and proteins from cell cytoplasm. The whole globule is additionally covered by a real, external lipid bilayer composed of proteins, glycoproteins, enzymes, non-polar and polar lipids and phospholipids of the secretory cell (Keenan 2001, Mather 2000). It originates from the apical section of the cellular membrane of secretory cells of the mammary gland. Elements of the cytoplasm of a secretory cell may be entrapped between the mono and bilayer of phospholipids (Mather and Keenan 1998).

On the one hand, the fat globule membrane ensures large interphase surface during milk fat digestion with lipase, and on the other hand it prevents undesirable lipolytic transformations. Owing to its structure, through specific interactions with the reaction medium in the gastrointestinal tract, it ensures optimal digestion of milk fat. By this means, changes in the "quality" of the interphase surface affect bioavailability of milk fat during digestion in the gastrointestinal tract. Homogenization is a process of high pressure treatment of milk that leads to a reduction of the size of fat globules, an increase of their surface and changes in the structure of milk fat globule membrane. These changes were demonstrated to lead to significant differences in the digestion process of milk fat (Berton et al. 2009, Berton et al. 2012).

Fat digestion in the gastrointestinal tract is a complex multi-stage process. It begins as early as in the oral cavity, then fat hydrolysis is initiated in the stomach by gastric lipase. The main stage of digestion proceeds in duodenum and small intestine under the influence of pancreatic lipase. A significant role in this process is ascribed to bile salts which – by modifying the interphase surface and emulsifying lipolysis products – allow lipase to access the interior of globules and hence the substrate, namely triacylglycerols (McClements et al. 2009).

One of the first changes in the emulsion system proceed in the oral cavity under the influence of saliva. Saliva is a complex biological fluid that contains a variety of proteins, electrolytes, organic compounds and water. It serves various functions by interacting with ingested food (Humphrey and Williamson 2001, van Aken et al. 2007). Mucins present in saliva may contribute to changes in the emulsion that lead to flocculation of fat globules and even to their coalescence (van Aken et al. 2005, Silletti et al. 2007). These changes may significantly affect subsequent changes, including the enzymatic ones, under the influence of lipase. One of the potential methods applied to analyze the phenomenon of flocculation and coalescence is image analysis coupled with determination of fractal dimension. The fractal dimension may be used to characterize the structure and phenomena displaying apparently chaotic and unordered character and therefore being difficult to compare and describe. Such structures may, to some extent, exhibit fractal characteristics like self-similarity (Mandelbrot 1982). Fractal dimension may, hence, express quantitatively some traits and enable quantitative comparison of structures or phenomena (Leman et al. 2005, Peleg 1993, Smoczynski and Baranowska 2013).

In view of the above, this study was aimed at quantitative comparison of the effect of changes in the structure of the membrane of milk fat globules on the process of saliva-induced flocculation. Changes in the membrane were induced by milk homogenization, which is a routine process applied in the production of drinking milk. An additional objective was to apply image analysis coupled with determination of fractal dimension as a tool to monitor changes in the emulsion system of milk.

2. MATERIAL AND METHODS

The experimental material included raw and homogenized milk. Raw milk was obtained from a local dairy plant. Next, part of milk was subjected to one-stage homogenization using a two-stage high-pressure homogenizer NS 1001L Panda 1K (GEA Niro Soavi, Parma, Italy) at the pressure of 200 bar. Thus prepared milk samples were analyzed within 12 h.

2.1 Saliva-induced flocculation of milk fat globules

Saliva for analyses was collected from three healthy individuals immediately before measurements. After mixing, saliva was centrifuged for 1 min at 1000 rpm to remove spume. The effect of saliva on the flocculation process of milk fat globules was investigated at three different concentrations. To this end, 0.3 cm^3, 1.5 cm^3 and 3.0 cm^3 portions of saliva were added to 3 cm^3 milk samples (samples denoted as: milk 1, milk 2 and milk 3, respectively). The portions were adjusted so as to reflect possibly a wide range of probable concentrations. Next, immediately after saliva addition to milk and 5 minutes after saliva addition, particle size distribution was determined and particles were subjected to microscopic analysis coupled with image analysis. Determinations were repeated three times in 3 different samples of raw milk and homogenized milk.

2.2. Particle size measurement

The mean particle size and particle size distribution were determined by light scattering measurement in a Mastersizer apparatus (Malvern, Malvern Instruments Ltd., Worcestershire, UK). Milk samples were instilled into a measuring cell to achieve obscurance of 10-15%. Refraction indices for water and milk reached 1.33 and 1.46, respectively. The following parameters were determined in the above measurements: surface-weighted diameter (D_{32}) defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, available surface of globules in milk samples, and total particle size distribution. Each time, measurements were conducted in 5 replications for each milk sample.

2.3. Microscopic analysis

Microstructural analysis of fat globules was conducted with the use of Nikon Eclipse reversed confocal microscope (Nikon, Tokyo, Japan) using an oil immersion objective at magnification of 60x. During observations, series of 10-15 photographs of randomly selected sites of a preparation were taken for each milk sample. They were used in the further stage of the study in image analysis.

2.4. Image analysis coupled with determination of fractal dimension.

The photos of microstructure were subjected to image analysis using NIS-Elements Basic Research Software (Nikon, Tokyo, Japan). Contrast was increased on the photos applying the same parameters of grey hue cut-off points (80 for low and 81 for high), which allowed obtaining binary black-and-white images. It enabled easier marking of contours of the examined objects. Analyses were conducted for 10 to 15 microphotographs. All objects in the visual field of the lens were marked, and their perimeters and surfaces were measured. The achieved series of correlations between perimeter and surface area allowed determining the fractal dimension of the perimeter of the analyzed objects. The fractal dimension was computed from the slope of a simple logarithmic correlation between the surface area and perimeter of the analyzed objects.

3. RESULTS AND DISCUSSION

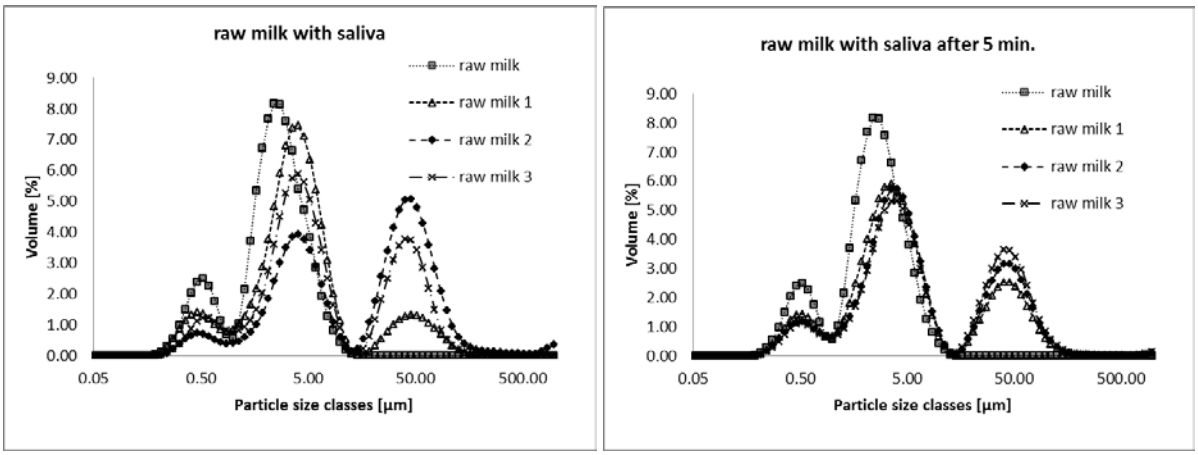
In the homogenization process, fat globules are disintegrated into smaller granules, which results in an increased surface area of the interphase. In consequence, there is not enough membrane material to surround the newly-formed surface, and thus adsorption of other surface-active milk components, mainly casein micelles and whey proteins, proceeds (Michalski and Januel 2006). The newly-developed and modified interphase surface should display significantly different properties compared to the native membrane. Therefore, in the presented study we tried to established whether these changes affect differences in interactions with saliva and differences in the flocculation process of milk fat globules.

In this study, the surface area of milk fat globules increased over three times after homogenization, which was reflected in similar changes of the surface mean diameter (Sauter's diameter) (Tab. 1).

Milk sample	Time 0 min			Time 5 min		
	D3,2 (µm)	Surface area (m^2/g fat)	Fractal dimension	D3,2 (µm)	Surface area (m^2/g fat)	Fractal dimension
Raw milk	2.6	4.41	1.07			
Raw milk 1	11.6	2.72	1.24	5.2	3.81	1.22
Raw milk 2	36.8	1.38	1.49	23.9	2.05	1.46
Raw milk 3	18.8	2.04	1.55	18.2	1.98	1.53
Homogenized milk	0.8	14.20	1.12			
Homogenized milk 1	3.6	13.85	1.43	5.48	1.40	1.40
Homogenized milk 2	27.7	8.19	1.38	15.6	11.24	1.39
Homogenized milk 3	21.6	9.20	1.63	16.6	11.09	1.50

Tab. 1 Physicochemical properties of the analyzed milk samples.

Changes in the distribution of fat globules of raw and homogenized milk immediately after and 5 min after saliva addition are depicted in Fig. 1.



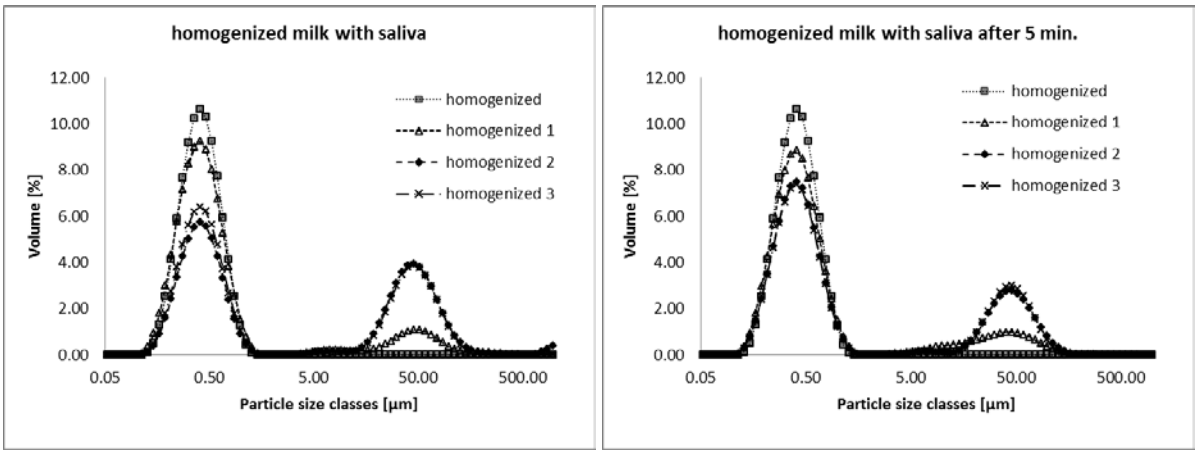


Fig. 1 Particle size distribution of raw milk and homogenized milk after saliva addition

The mechanism of interactions between emulsion particles and saliva is affected by such factors as: depletion-inducing forces, van der Waals forces or bridging interactions via electrostatic forces (Silletti et al. 2007). In the case of both raw and homogenized milk, flocculation or/and coalescence of milk fat globules occurred upon saliva addition. It was indicated by reduced sizes of fat globules occurring in milk and, simultaneously, by the appearance of larger agglomerates with sizes of ca. 50 µm. In samples of raw and homogenized milk, the most intensive flocculation was observed at a milk to saliva ratio of 1:0.5. Also in this case, values of Sauter's diameter were the highest, which points to large sizes of the aggregates being formed. The increased content of saliva in the third sample had no effect on the intensification of flocculation and/or coalescence process. The least intensive flocculation was observed at the lowest saliva concentration in a milk sample.

In samples of raw milk, no differences could be noticed after 5 min since saliva addition that could be indicative of saliva effect on the flocculation process. In turn, in homogenized milk again the most intensive process occurred in the second sample with milk to saliva ratio of 1:0.5. In general, these results may indicate that interactions between saliva and fat globules were more intensive in raw milk, i.e. in the case of native fat globules. It may be due to evolutionary adjustment of milk and fat globules to the optimal interaction with elements of the gastrointestinal tract enabling effective digestion and absorption of milk fat in the alimentary tract of man. In homogenized milk, the structure of milk globule membrane modified by homogenization, may exhibit slightly different properties that affect different behavior in the gastrointestinal tract. Interestingly, despite differences in the initial size of fat globules in raw and homogenized milk samples, the size of aggregates formed in these milk samples was similar and reached ca. 50 µm. This shows that the extent of flocculation and size of the formed aggregates (flocs) depend not on the initial size of globules undergoing flocculation, but rather on saliva content leading to complex interactions as a result of balance between binding forces and forces causing destabilization of too large flocs.

Slight process reversion might additionally be observed 5 minutes after saliva addition. A similar trend could be noticed in values of Sauter's diameter. In general, they were reduced after 5 min, which points to reduction of flocs sizes and increased concentration of free milk fat globules. After saliva addition, the samples were mixed and then left to stand for 5 min. Afterwards, a sample was collected for analyses. This may indicate a significant role of mixing in interactions between fat globules and saliva.

It is common knowledge that the charge occurring on particles surface influences the mechanism of flocculation (Blijdenstein et al. 2004), and that in both raw and homogenized milk a low negative charge (from -12 to -20mV) occurs on the surface of fat globules (Berton et al. 2012). In turn, Silletti et al. (2007) demonstrated that for particles bearing a low negative charge, a reversible flocculation might proceed according to the mechanism of depletion flocculation. Here, the main role is ascribed to negatively-charged proteins of mucin. In the case of positively-charged particles, the irreversible flocculation is likely to occur according to the mechanism of electrostatic bridging between positively-charged emulsion droplets and negatively-charged molecules of mucin proteins (Blijdenstein et al. 2004). Results of our study point also to the reversible flocculation, which – considering a low negative charge of fat globules – is consistent with findings of other authors (Silletti et al., 2007).

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The flocculation process was additionally characterized by image analysis of photos of milk fat globules taken using a confocal microscope. Examples of microstructure images were presented in fig. 2.

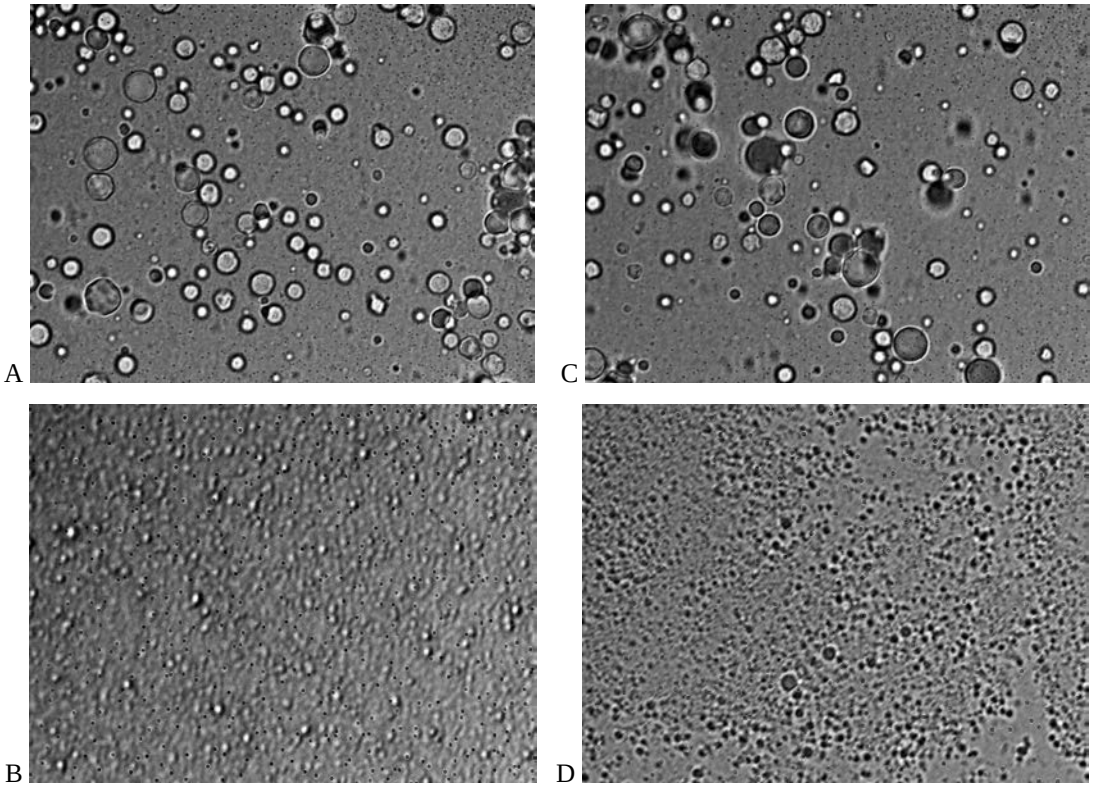


Fig.2 Examples of microstructure of fat globules and raw and homogenized milk (A and B) and in the same milk samples after saliva addition (C and D).

The image analysis consisted in the measurements of perimeter and surface area of the examined milk fat globules. As the perimeter (P) is proportional to surface area (A), the plot of a correlation log (A) = f (log P) is a straight line. The slope of this straight line is used to calculate the fractal dimension of a perimeter (contour) of globules as $D_L = 2/a$, where D_L - is a fractal dimension, and a - direction coefficient (Dziuba et al. 1999). By this means, this dimension characterizes the extent of flocculation, as for individual globules it should reach around 1.0. In turn, when globules undergo flocculation, the value of the fractal dimension should increase because the perimeter of such structures starts to diverge from spherical shape. Figure 3 presents exemplary logarithmic correlation between the perimeter and surface area of milk fat globules with a straight line equation and determination coefficient. In all cases, the coefficients of determination were higher than 0.9, which indicates good fit of the mathematical model to achieved results of measurements.

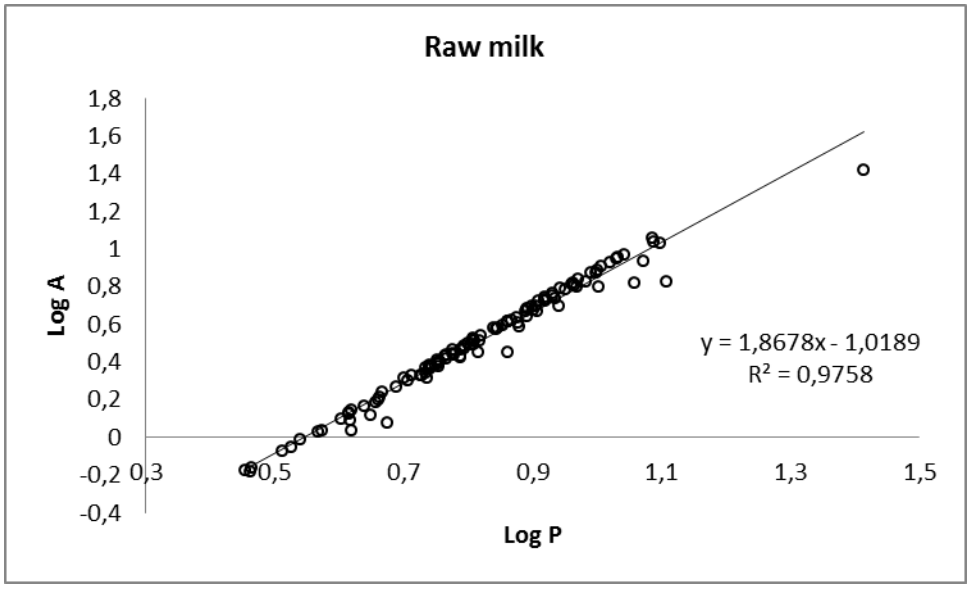


Fig. 3 Example of logarithmic correlation between perimeter and surface area of fat globules for raw milk

As shown from data in Table 1, the values of fractal dimension for both raw and homogenized milk without saliva were close to unity and reached 1.07 and 1.12, respectively. It is consistent with theoretical assumptions, however a slightly higher value obtained for homogenized milk may result from lesser accuracy at smaller sizes of fat globules. The observed trend in changes of the fractal dimension was, generally, similar to results obtained from light scattering measurements. However, in this case, the highest value of fractal dimension was reported for milk sample 3, at milk to saliva ratio of 1:1. This may point to partial process of coalescence in milk sample 2. Comparing values of fractal dimension determined in milk immediately after mixing and 5 min after saliva addition, an insignificant reduction of fractal dimension may be observed in practically all samples, which shows the possible reversion of the flocculation process, meaning the reversible mechanism of flocculation. It seems to confirm results obtained from laser light scattering measurements in the Mastersizer apparatus. Nevertheless, consideration should be given to difficulties in the measurement of fat globules in homogenized milk. At the applied magnification, the sizes of globules were small and this could cause some inaccuracies affecting study results. Greater magnification could be possible upon the use of electron microscopy, but this type of microscopy requires more complex procedures of specimens preparation, which in turn may influence subtle interactions between globules that lead to flocculation and, thereby, affect results of image analysis.

4. CONCLUSION

The presented study result provide quantitative characteristics of the flocculation process. Differences were observed in the flocculation process between native milk fat globules and globules with the modified interphase surface in homogenized milk. The results indicate that saliva concentration may influence the effectiveness of flocculation process and that this process may be the most effective at its specified concentration. They additionally point to a significant role of the mechanical impact on interactions between fat globules and saliva. The applied method of image analysis, leading to determination of fractal dimension, constitutes a novel and unique approach to the analysis of phenomena of this type as it provides new, wider and more complete information on the observed processes.

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