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CHARACTERIZATION OF BUFFALO MILK FAT GLOBULES USING THE CONFOCAL LASER SCANNING MICROSCOPY

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ABSTRACT. Milk fat globules are biologically essential components due to their functional and health properties. Milk fat composition has been thoroughly studied in cow milk but still it remains unclear in buffalo milk. We have used confocal laser scanning microscopy (CLSM) to investigate the structure of the fat globules in cow and buffalo milk, using two types of fluorescently-labelled dyes for phospholipid and triglyceride constituents. Using this technique, we have observed heterogeneities in the distribution of these lipids both in the membrane as on the surface relating to the specie and size of the globules. The statistical analysis has shown that there are significant differences ($p<0.05$) among the average fluorescence intensity (13.68±9.98 AU/μm^2) found at buffalo milk fat globules in comparison to cow ones (16.88 ± 4.3 AU/μm^2). The statistical comparison of the phospholipids quantification values in both species revealed the fact that there are no significant differences ($p>0.05$), the average found at cow milk being 31.07 AU/μm^2 and at buffalo 34.85 AU/μm^2. The use of specific dyes may be essential in the evaluation of the unsaturated lipid milk fraction, the buffalo milk fat globules being easily differentiated from cow milk by OilRed quantification.

Keywords: *milk, buffalo, confocal laser scanning microscopy, fluorescence, dye*

INTRODUCTION

Milk fat globules play an essential role in the processing and technology characteristics of dairy products, leading to different particularities according to the specie. The size of these fat globules is essential in milk

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separation, cheese technology and their further processing [1-3]. The protein quantity absorbed per surface unit, the emulsion stability as well as their optical, rheological (colour and viscosity) [4] and conductibility features [5] are influenced by fat globules. On the other hand, the variation in size, distribution, microstructure and rheological properties according to the animal species [6,7] leads to the characteristic of each dairy product.

The distribution of the fat globules' sizes was previously evaluated using various methods such as classical microscopy, turbidity measurements, and electronic impulses [8]. The classical microscopy was mainly used to establish the quality damage degree of milk fat globules. Until now, the heat treated milk or cream was investigated by using the classical [9], electronic [10] and confocal microscopy [11,12,13]. Recently, Evers et al. (2008) [14] have introduced the laser confocal microscopy method (CLSM) as a non-invasive technique for studying the cow fat globule' membrane using lipophilic and lectin colorants. Lopez et al. (2011) [15] has used a fluorescent staining for phospholipids analogue with lectines in order to visualize their distribution in the milk fat globule membrane. In Romania this is the first study performed on buffalo milk that characterizes the biochemical aspects of fat globules through confocal laser microscopy in comparison to cow milk.

RESULTS AND DISCUSSION

The unsaturated lipid fractions evaluation found in the fat globules of buffalo and cow milk was performed by using the lipid fluorescent dye OilRed.

The images presented in figure1 reveal the fluorescence emission of the dispersed dye in the spherical areas of cow milk. The use of this dye with affinity for unsaturated lipid fractions has confirmed the fact that in both species these molecules are found exclusively in the fat globules. This is not very surprising given the fact that the class they belong to, triglycerides, are hydrophobic molecules. This method has allowed the making of 2D and 3D images of these fat globules.

The distribution of fat globules in cow milk was measured also, varying from a 0.10 μm in diameter to 10 μm, with a final average of 4,33 μm. The distribution was revealed in three peaks, corresponding to the diameters: small sized (0,13 – 2,84 μm); average sized (3,5 – 7,87 μm) and large sized globules (much lower in number) with values in between 8 and 10,2 μm.

The fat globules' sizes evaluated in buffalo milk have varied considerably in diameter, registering a minimal value of 0.45 μm and a maximal value of 18.2 μm. The average registered was of 9.41 μm. Our study has revealed a higher value than the one found by El-Zeini (2006) [16] (8,7 μm).

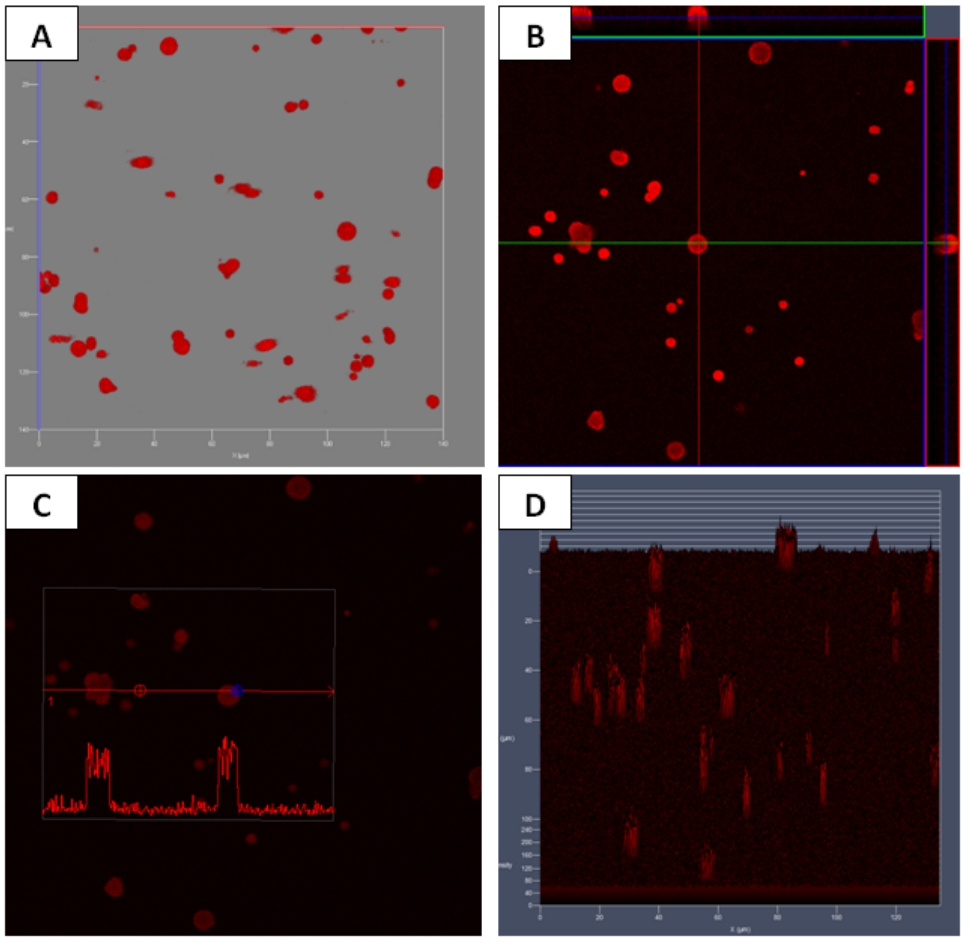

Figure 1. CLSM representing buffalo milk fat globules, colored with selective staining for fats Oil Red (Obx63, Apocromat immersion); A - tridimensional reconstruction; B – section analysis; C- fluorescence peak compared to the minimal background fluorescence; D – 2,5 D triglyceride fluorescence

The use of OilRed dye for these lipid fractions has allowed the statistical evaluation of fluorescence values measured in these stained fat globules and the interpretation of their fluorescence degrees. Their distribution is structurally and chemically heterogeneous in cow and also buffalo milk. Some of the results at the triglycerides' quantification from buffalo milk are shown in table 1.

Table 1. Triglycerides' fluorescence quantification in fat globules of buffalo milk

No.	Mean intensity (AU/Arbitrary units)	SD*	Pixels	Area
1.	4,67	3,95	1188	82,53
2.	3,4	3,57	331	23
3.	4,28	2,1	251	17,44
4.	2,14	3,62	502	34,88
5.	1,78	2,72	483	33,56
6.	2,18	2,31	461	32,03
7.	3,56	7,12	335	23,27
8.	2,85	3,97	326	22,65
9.	6,56	5,82	491	34,11
10.	50,72	9,53	861	59,82
11.	77,03	13,83	343	23,83
12.	8,17	3,67	494	34,32
13.	10,25	1,04	208	14,45

*SD – standard deviation

As shown also in table 1, buffalo milk fat globules have revealed a much lower intensity in the triglycerides' fluorescence. The statistical analysis has shown the fact that there are significant differences ($p<0.05$) among the average fluorescence intensity found at buffalo milk fat globules in comparison to cow ones. The average value of the fluorescence obtained in cow milk samples was 16.88 ± 4.3 AU/μm^2, with a minimum registered of 9.3 AU/μm^2 and a maximum of 32.57 AU/μm^2. In case of buffalo milk, the intensity was lower revealing an average of 13.68±9.98 AU/μm^2 with a minimum of 1.78 AU/μm^2 and a maximum of 77.03 AU/ μm^2. At the comparison of the fat globules' surface area, a significant difference ($p<0.05$) was noticed among the two species. This time, the surface area was higher in the case of buffalo milk (435,89 μm) and lower in the case of cow milk (182.98 μm).

These results are in accordance with other studies made on cow milk fat globules evaluated through the same technique [17,14]. Although there are innovative studies that characterize these fat globules lipid constituents, there aren't any that show a fully detailed comparison among different species. The correlation found in this experiment show that the fat globules' higher degree of fluorescence (OilRed) in the unsaturated lipid fraction at cow milk is due to the higher proportion of unsaturated fatty acids proved previously in our studies [18]. Although at the statistical analysis of the percentages of unsaturated fatty acids in cow milk compared to buffalo milk there were no significant changes revealed, we proved that based on their fluorescence degree quantification there are ($p<0.05$).

Milk fat globules were stained also with Rhodamine, an exogenous dye. This substance is a headgroup labelled phospholipid probe which can be incorporated with minimal perturbation into the phospholipids layer of the milk fat globule membrane.

We observed that the staining of the milk fat globule membrane was heterogeneous. In figure 2 it is also shown that two phases coexist within the buffalo milk fat globule membrane, a phase stained with rhodamine (reddish) and a phase where rhodamine is absent (green) (Figure 2). This fact was also observed in the case of cow milk fat globule, where the structure of the membrane formed by phospholipids were revealed in a disorganized liquid phase coexistent with the organized one. When comparing the intensities of fluorescence at the two species it was revealed the fact that there were no significant differences ($p>0.05$), the average found at cow milk being 31.07 AU/μm^2 and at buffalo 34.85 AU/μm^2. No matter the size of the fat globules, the rhodamine fluorescence dependant on the phospholipids substrate remains in the range at both species (28.05 – 36.9 AU/μm^2).

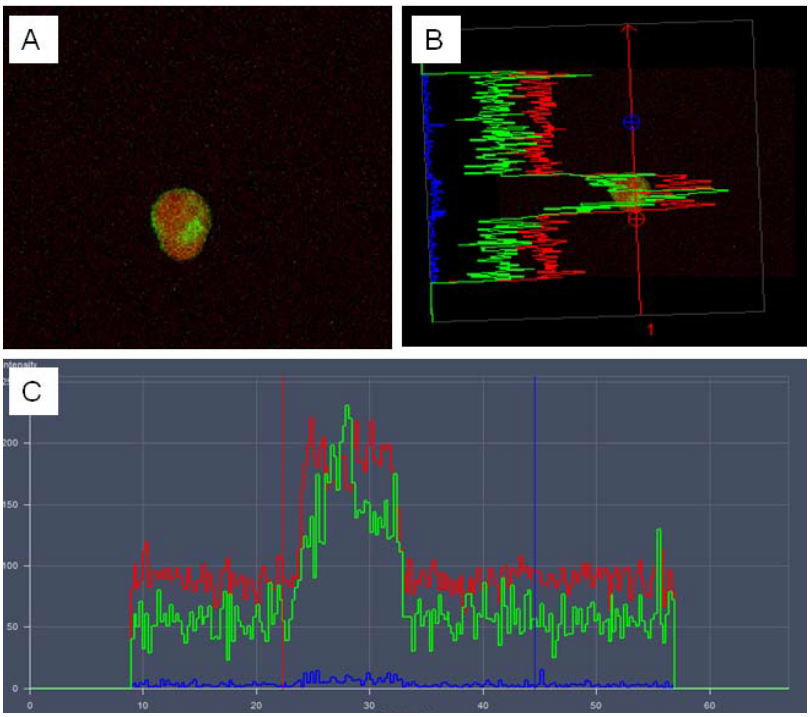

Figure 2. CLSM representing buffalo milk fat globules, stained with selective staining for triglycerides and phospholipids (Obx63, Apocromat immersion); A – plane image; B – fluorescence intensity profile on axis 1; C- Graphical representation of the fluorescence; Ch2 Rhodamine

CONCLUSIONS

The use of specific dyes in the evaluation of the unsaturated lipid fractions has revealed particularities at buffalo milk fat globules. The intensity of tryglicerides' fluorescence found in milk fat globule can be used as a particular marker in assessing the specie. Given the fact that the intensity of fluorescence is dependent on the amount of tryglicerides found in the fat globules we can conclude that in buffalo milk the tryglicerides quantity is lower than in cow milk. Regarding the phospholipids evaluation, the intensity of the fluorescence was not statistically different, being able to affirm that buffalo milk fat globules have similar phospholipids values as cow milk fat globules, not being able to use this method for possible differentiations.

EXPERIMENTAL SECTION

The study was conducted on 84 samples of buffalo milk and 87 cow milk samples, respectively. The samples were collected in sterile recipients and kept at refrigerating temperatures until their further analysis. No samples were kept longer than 6 hours until their analysis.

The confocal laser scanning microscopy analysis

The samples were analysed with the Confocal Laser Zeiss LSM 710 microscope, adjusted to an inversed microscope Acio Observer Z1. The specific visualization of the emitted fluorescence by the complex lipid-Oil red O was made by the laser exciting of the samples at a wave length of 596 nm and the use of an absorption filter between 578 and 637 nm (wave length for exciting/emission of the Texas Red fluorochrome). In order to visualize the Rhodamine 123 fluorescence a fluorochrome excitation of 511 wave length was used and the emission filters were in between 535-623. In order to visualize the entire image spectrum a Zeiss Plan-Apochromat objective (63x/1.40) was used. The images were processed and analysed by using the ZEN software, standard version.

The calibration at the beginning of the experiment was made according to the standard curve provided by the producers of the confocal system.

The quantification of the Oil Red and Rhodamine fluorescent signal

The quantitative assessment of triglycerides and phospholipid was performed with the mentioned dyes: Oil Red and Rhodamine, using the previously published protocol by Bhirde (2009) [19].

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