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REVIEW

The assesment of quality and traceability biomarkers in the buffalo milk primary production

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Abstract

Tracking of foods (traceability) is a complex process made possible by the assessment of suitable biomarkers, stable and traceable along the entire food chain. In this particularly dairy step, the primary production, we rely on the assessment of the most common biochemical markers such as: fat, protein, lactose, somatic cell count, fatty acids composition, retinol, cholesterol and tocopherol levels. The findings in this area concerned the fatty acids profile which vary according to the seasonal changes, the highest amounts being found in the C10:0, C14:0, C16:0, C18:0 and C18:1 levels. The tocopherol and retinol levels were dependent also to the season and were found to be in higher values than of those stated in cow milk. Also the innovative part of our study consisted in the evaluation of the individual's genetic polymorphism in regard to the leptin hormone. The results indicated that the leptin TT genotype is associated with increased milk and protein yield compared to the leptin CC genotype.

Keywords: marker, buffalo milk, leptin, protein, fatty acids.

Introduction

Buffaloes are predominant dairy animals in some countries contributing at a major share of the world's milk production. Compositional aspects of buffalo milk have been studied and reviewed by many workers [1,2,3,4] given the fact that milk and dairy products are major components of the human diet, providing about 30% of dietary proteins and lipids and about 80% of dietary calcium. Nowadays, the marker-assisted selection may provide the opportunity to improve important economic traits in livestock. In this study we took into analysis some of the most important qualified markers in the milk production such as: the fat percent, the protein percent, the lactose percent, the somatic cell count, the retinol level and the tocopherol amount.

Given the fact that the genome analysis opens new possibilities for a more accurate evaluation of the economically important traits, we investigated also the genetic polymorphism of each individual in concern to leptin hormone. The fact known until now is that this hormone is a 16 kDa protein, produced by the adipose tissue which inhibits the feed intake [5] and down regulates the adipose tissue deposition [6]. Furthermore, there is evidence that leptin positively influences fertility. For example, leptin was reported to restore fertility in leptin deficient *ob/ob* mice [7] and to accelerate the onset of puberty in normal female rodents [8]. Studies made in this field indicate that this hormone can also influence milk's quality at the following species: bovine, ovine and caprine [9,10,11]. No studies have been made concerning this aspect at buffalo milk. The results revealed in the former studies show that the

leptin TT genotype is associated with increased milk and protein yield, without changing the yield of milk fat [12].

Materials and methods

The research was conducted on 80 buffalo milk samples, collected from private households in the north and central region of Transylvania and also from large scale farms situated in the same area. The collecting of the sample was done each season, consisting in 20 milk samples for every season. The samples were kept under proper analysis conditions, maintaining their quality and features.

The genotype analyses were performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. This technique consists in repeated cycles of heating and cooling so as to melt and enzymatically replicate the DNA. The short fragments of DNA that have complementary sequences, along with a DNA polymerase region are the key components in allowing the selective and repeated amplification. Using the PCR method we obtain a progress, in generated DNA this being used as a replication model, establishing in movement a chain reaction in which the DNA pattern is exponentially amplified. The PCR can be modified on a large scale so as to make a large amount of genetic manipulations.

The DNA extraction was done using the following protocol: 40µl raw milk was immersed in Eppendorf tubes and centrifuged at 2000 rotations/min for a period of 30 minutes. After centrifugation the fat layer and the supernatant was averted, and afterwards the pallet was suspended in one ml PBS solution (phosphate-buffer-saline) at a pH of 7,4, and centrifuged again at 400 rotations/minute for 10 minutes. After this step the pallet was immersed again in a 49 ml lyses solution (0.32 M sucrose, 10mMTris-HCl, 7.5mM MgCl1%, TritonX-100). The pallet's nuclei were centrifuged at 2465gxg for 10 minute and washed twice with 10ml NaCl and 0,025 M EDTA. The obtained sample was mixed in 3 ml 10mM Tris-HCl solution with a pH of 8, and 2mM EDTA, and then added 100ml 10% SDS and 40ml proteinase K 910mg/ml). The nuclei lyses obtained were incubated for an hour at 65°C. After this incubation period 500ml of NaCl solution was added, and the precipitated proteins were centrifuged at 1800 gxg 10 minutes. The supernatant was then averted and 6 ml isopropanol added, after which we centrifuged for 10 minutes at 1800 gxg. The pallet was dried at room temperature and re dissolved it in 500ml Tris-EDTA (10 Mm Tris-HCl, 1mM EDTA solution, pH 7.5. After following these steps the total quantity values of the DNA read at the Nanodrop spectrophotometer ranged between the values of 50ng/µl-400ng/µl.

The PCR technique followed these main steps:
In the Eppendorf tubes 16.2 µl water, 2.5µl buffer, 1µl DNTP, primers (amcrse): sense primer 1µl, antisense primer 1 µl; TAQ-polymerase 0.3µl, $MgCl_2$-1µl, extracted genomic DNA were mixed. Forward (5'-TGGAGTGGCTTGTTATTTCTTCT-3') and reverse (5'GTCCCCGCTTCTGGCTACCTAACT- 3') primers were used in the amplification. The amplifying program used at the Termocycler was: 1x95^{o}C→5 minutes (the alteration) the attaching of the primers in several stages at 40x95^{o}C→1 minute, at 40x64^{o}C→1 minute were the attachment takes place usually, and at 40x72^{o}C→1 minute. After this attachment protocol the final extraction took place at 1x72C →10minutes.

The enzyme restriction was done with the Kpn21 (BspEi) enzyme and followed the next steps: we added H_2O 6,5ml, after which a tampon solution 2,5ml and afterwards Kpn21 (BspEi) enzyme 1µl. The reaction mix was added then in 20µl PCR product applied by the PCR. The enzyme has an activity at 55° C; the samples were put in the thermostat for 4 hours

in a water bath. The restriction's role was to digest the amplified fragment from the leptin gene in order to differentiate the implicated allele in the buffalo's milk quality.

The DNA sample's migration was made by an electrophoresis reaction in agars gel using 2μl ethidium bromide.

The fat, protein and lactose contents were evaluated with the help of the Milkoscan 134 (Foss-Electric A⁄C, Hillord, Denmark) (IDF standard 141 B:1996) apparatus and the somatic cell count with the Fossomatic™ FC.

For the fatty acids profile the following method was used:

Milk fat extraction: About 2ml of milk samples were mixed with 0,6 ml ammonia 25%, 2ml EtOH, 4ml Ethyl ether and 4 ml hexane and then agitated for 2-3min. After this process the lower layer (the ammonia layer) was discarded. Following this step the mixture was passed through a cellulose filter with Na_2SO_4 and then brought to dryness.

The transesterification:

Fatty acids were converted to methyl esters by reaction with boron trifluoride/methanol at 80ºC for two hours in a closed Pyrex glass tube. The content was transferred into a separator funnel.

The methyl ester extraction:

The extraction was made using 10 ml hexane. The hexane fractions collected were dried using anhydrous sodium sulfate, filtered, concentrated under a nitrogen stream and finally re-eluted in 1 mL hexane. Fatty acids were analyzed by gas chromatography (GC) with flame ionization detection (FID). A 1µL sample was injected into the Shimadzu GC-17A series gas-chromatograph, equipped with a 30m polyethylene glycol coated column (Alltech AT-WAX, 0.25mm I.D., 0.25µm film thickness). Helium was used as the carrier gas at a pressure of 147 kPa. The injector and detector temperatures were set at 260ºC. For the oven temperature the following program was used: 70ºC for 2 min. then raised to 150ºC at 10ºC/ min. rate and held at 150ºC for 3min., then further raised up to 235ºC at a 4ºC/min.

The retinol and β carotenoids determination was made using the following protocol: A 20 ml milk volume treated with 5 ml ammoniac solution 25% and 20 ml ethanol 96%. In order to extract the retinol and tocopherol a mixture of 40 ml ethylic ether (that contained 0.0025 % butilat hidroxitoluen) and 40 ml of petrol ether was agitated for 5 minutes. The superior ether phase was retaken in a balloon and evaporated at a rotating evaporator, at a temperature of 35ºC. The sample's saponification was made by adding at the obtained residue a volume of 30 ml potassium hydroxide 5 % in 96 % ethanol. The samples were agitated using a magnetic spinner for 3 hours, in the dark. The samples were transferred in a separation cone with 30 ml water and 30 ml hexane. The hexane was removed and the watery phase again extracted with hexane. The hexane phases were rejoined, the separation cone washed with water and then evaporated at dry. The samples were kept at the deep freezer until the following use. The quantitative analysis of the retinol was made using a gauge curve made with retinol-trans-total (Sigma) solution of concentrations in between 10 and 80 µg/ml. the standard solutions and the samples were injected in a HPLC system with a Shimadzu LC-20 AT, Waters 990 detector with a data analysis soft, Rheodyne injector with a 25 cm Spherisorb RP-18 curl, inferior diameter of 4,6 mm and the particle dimensions of 5 µm. The mobile phase consisted in acetonitril : methanol 85:15, in a isocratic system.

The milk's tocopherol content was determined using the same extract made for the caroteneoid and retinol analysis. The mobile phase consisted in 100% methanol with a flow of 1ml/min. The chromatographic monitoring was made at 298 nm and the quantitative determination was based on gauge curve made with solutions of 10µg/ml – 100 µg/ml concentration of α-tocopherol (Sigma).

Results and discussion

In what concerns the leptin genotype, the presence of the C and T alleles of leptin gene were revealed.

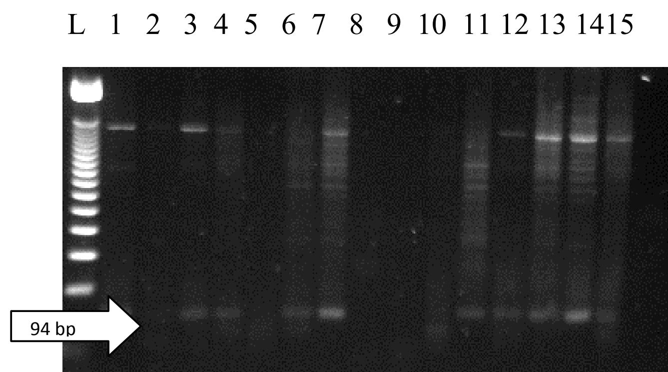


Figure 1. Genotyping for buffalo leptin gene, lane L is a marker for molecular weight (Gene Ruler, 100 bp). The lines 1 – 15 reveal a 94 bp fragment indicated as TT genotype.

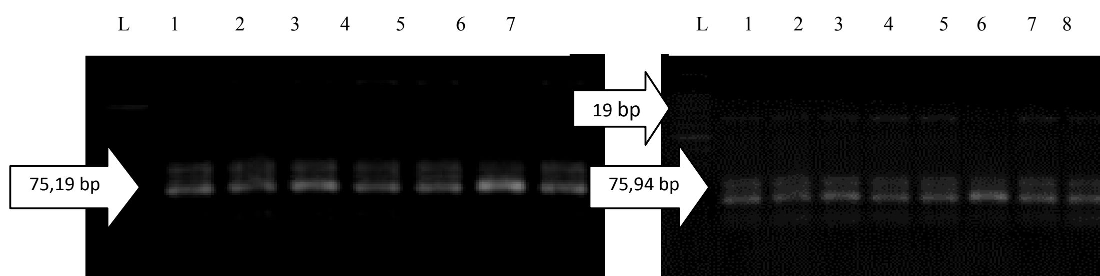
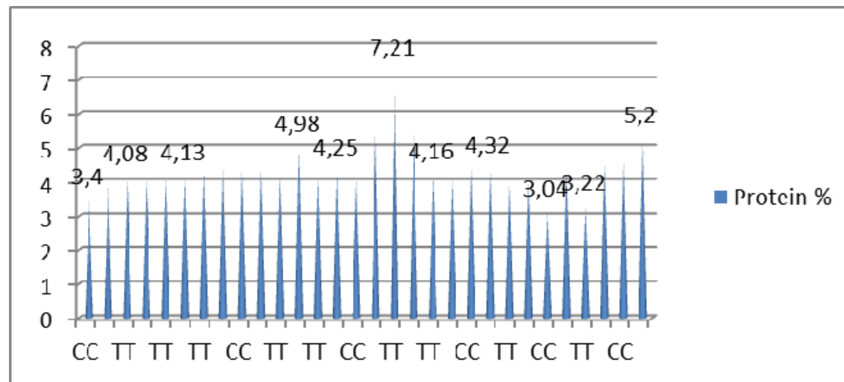


Figure 2, 3. Genotyping for buffalo leptin gene, lane L is a marker for molecular weight (Gene Ruler, 100 bp). The lines 1 – 7 from reveal the 75 and 19 bp fragments corresponding to CC genotype, and the lines 1 – 8 represent the CT genotype with a 19, 75 and 94 pb.

The three leptin genotypes corresponding to the alleles identified: the TT genotype, CC genotype and CT genotype were revealed. The frequency of TT genotype was higher than of the CC and CT ones, accounting for 85% in the population studied. The CC genotype was found in only 2.5% percent of the population studied, the remaining percentages being represented by the CT genotype. In recent studies on Iranian Holstein cows, the TT genotype was associated with higher milk, fat and protein yield compared to other genotypes [13]. The same theory is sustained by Abdul Raoof Alashawkany et al. in a study made on Holstein cows suggesting also the possible influences that the genotype TT might have in the higher production and quality of milk. Buchanan et al. reported that the T allele was associated with fatter carcasses and the C allele with leaner ones. Our data sustain some of the researches made on other species. The protein percent was found to vary according to the individual reaching the lowest value at the CC genotype and the highest in the case of the TT genotype, as it is shown in graphic 1. In what concerns the fat and lactose percentage no significant differences were noticed among these genotypes, the values ranging from 5.2% to 15.92% respectively 4.7% - 5%. Significant changes in these biochemical markers were found depending on the season, among the average value recorded in spring (8.88%) and the one recorded in winter (9.5%) ($P < 0.05$) , but no significant changes among the rest of them (autumn: 8.34%, summer: 8.20%) ($P > 0.05$). This difference in the fat content is probably due to the feeding frequency

of low fiber, high grain diets which increase milk fat levels during the winter and autumn period and the lack of herbage which was not available, only in the spring and summer seasons. The same results were obtained by Anderson and Pollak (1980) who have reported that the percentage of fat in bovine milk rose in countries with the same climate conditions has been influenced by the seasonal variations. The lactose average percentage during the period studied did not show significant changes (P > 0.05), varying very little from one season to another (autumn: 5.4%, winter: 5.13%, spring: 5.11%, summer: 4.85%).



Graphic 1. Protein percent representation from the milk samples analyses according to the individual's leptin genotype

Comparing these SCC values to the genotypes of each individual, buffalos homozygous for the T allele revealed an increased number in SCC linear score in contrast with the buffaloes homozygous for the C allele, and heterozygous CT. Higher SCC can be symptomatic for mastitis infection but also this may reflect a possible increase in mastitis incidence associated with higher milk yield influenced by leptin or the role of leptin in modulating an immune response. The samples gathered from private households revealed also a higher SCC value compared to the ones collected from large scale farms.

In the fatty acids composition analysis the five most important fatty acids in quantitative terms were the C10:0, C14:0, C16:0, C18:0 and C18:1 which accounted for more than 80% of the total fatty acids in all the samples studied, no matter the season in which they were collected. Overall the concentration of short chain fatty acids (<14:0) in milk were highest in the winter season and the lowest in the spring season.

These seasonal variation tendencies were noticed by Book and Banni in 1996. Later Lock and Gransworthy in 2003 reported also concentrations with 10% less C12:0, C14:0 and higher C16:0 content with up to 23% percent in milk during summer period compared to winter. These changes can, in this respect, present important benefits for human health, thus it is known that only C12:0, C14:0 and C16:0 affect the plasma cholesterol levels LDL [19,20]. The C18:0 content varies along the entire year as it is revealed by the field studies, being significantly lower in the summer period, a fact possible due to the changes in the food supplement of polyunsaturated fatty acids (PUFAs).

More authors reported that extensive hydrogenation of PUFAs can cause a growth in C18:0 content from milk [21; 22]. The main concentration of C18:1 *trans* from milk in the study made on buffaloes (2.08g/100g), are similar to the values reported previously by Aneja and Murthi, 1990, at buffaloes (2.10), and by Peterson et al., 2000, at cow (2.18). The C18:1 *trans* fatty acids are formed similarly with the final hydrogenation intermediary elements PUFAs [21]. In normal diet conditions C18:1 *trans* is the predominant element [23]. The possible motives for C18:1*trans* concentration include: the growing of deposit consumption

(linoleic (LA) and linolenic (LN) and/or the decreasing in the final hydrogenation step of the *trans* – 11 C18:1 from the rumen.

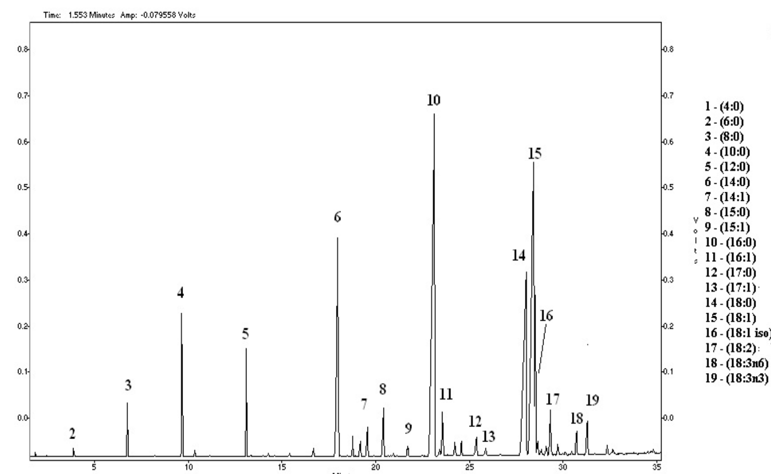


Figure 4. The chromatogram of a milk representative sample collected in autumn (September)

The buffalo milk has a high retinol level with an average of 44.1 μg/100 g), being superior from this point of view to cow milk, which was reported by Hulshof P.J.M et al. to have a media concentration of 40 μg/100 g . This 13 *cis* isomer of retinol is generally present in very small amount in the freshly collected raw milk, its concentration increasing with time, along with the heat treatments and environmental factors, being among the most important variables to be considered in the study of the dairy food chain traceability. The amount of retinol was higher in winter than in summer and no differences among the retinol level and the individual's leptin genotype was noticed.

The level of α – Tocopherol in the buffalo milk was of 134.4 μg/100g, higher than the average quantity found in cow milk and reported by Mihaiu et al. to be of 88.2 μg/100g. a fact known by now is that the α – Tocopherol absorption capability is dependant also on the quantity of lipids from the nutrients and the chemical nature of the fat (German and Dillon, 2006).

Our attempt to find and evaluate the β carotene level in buffalo milk was unsuccessful given the fact that it lacks, the white color of this particularly milk is a strong suggestion of that fact.

Conclusions

The association between leptin polymorphism with milk production traits suggests that this marker may be useful for selection based on molecular information. Furthermore we were able to establish a new protocol for DNA extraction and fatty acids extraction from milk, which helps future investigations in this area. Among the genetic marker we identified and analyzed the other biochemical components, which compared to the values obtained by other researches on bovine milk state that they can be assessed as traceability indices and quality parameters. The fatty acids profiles, along with the tocopherol and retinol level are influenced by the seasonal variations. The β – carotene cannot be found in the buffalo milk, being a characteristic of this product.

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