Goats producing biosimilar human lactoferrin

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Abstract. Herd of goats producing biosimilar human lactoferrin has been created. The nature of transmission of the transgene through a number of generations, productivity according to target protein, as well as technological parameters of milk containing recombinant human lactoferrin (rhLF) have been studied. The frequency of transgene transmission from parents to offspring was recorded to be in the range of 42.4–52.6%. The median rhLF content in milk of F1 animals was 2.60 g/L, F6 − 2.00±0.85 g/L. In terms of physical and chemical and technological parameters, milk with rhLF meets quality standards. The somatic cells count, being the marker of mammary inflammation (mastitis), has been decreased in milk of producing goats.

1. Introduction
Recombinant human lactoferrin, synthesized by mammary cells of transgenic goats belonging to the RUE Research and Practical Center of the National Academy of Sciences of Belarus for Animal Breeding, is a structural and functional biological analogue of lactoferrin (LF) contained in human milk and is an important component of innate immunity [1,2]. Lactoferrin obtained from milk of producing goats has properties similar to natural lactoferrin: antibacterial, antiviral, immunomodulatory, regenerative, and also has a positive impact on intestinal microflora. Most of the unique and beneficial properties of LF are due to the ability of the molecule of this glycoprotein to bind iron ions [3, 4, 5, 6].

The antiviral and immunomodulatory properties of LF have gained particular importance in connection with COVID-19 pandemic, since there is an active search for medicine around the world that could help in fight against the SARS-CoV-2 virus and pathological changes in the body caused by coronavirus infection. According to research, LF can be considered as a potentially antiviral agent in fight against COVID-19 similar to mechanism of activating nonspecific immunity due to its two properties: 1 — modulation of immune response (activation of NK-natural killer immune cells, increased aggregation and adhesion of neutrophils, as well as binding iron ions and some inflammatory factors); 2 — ability to bind to heparan sulfate proteoglycans (HSPG) on the surface of the host cell and to change the spatial configuration of these complexes, which, in turn, leads to suppression of virus binding to the cell and delayed binding of the viral spike protein to the ACE-2 receptor [7, 8].

Technology for producing biologically active medicinal human proteins using transgenic animals (goat, sheep, cow) producing such proteins in milk as bioreactors can be classified as one of the most promising biotechnologies of the 21st century. The global market for recombinant proteins was
estimated at 125.8 billion in 2020 and the average annual growth rate is expected to make 11.2% in the forecast period of 2021-2026 [9].

The method of pharmaceutical production using transgenic animals as bioreactors producing medicinal human proteins with milk has a number of advantages over microbial synthesis. In some cases, microorganisms cannot carry out post-translational modifications of human proteins similar to mechanism inherent in animal cells, which significantly reduces the biological activity of proteins. Great part of human proteins is synthesized in a bacterial cell in an insoluble form or form aggregates, which makes it difficult to isolate them. It is also known that medicines based on microbial synthesis, even in case small residual amounts of the bacterial component is left in the final product, carry the risk of allergic reactions in patients. Mammals have biochemical metabolic mechanisms similar to humans. Therefore, transgenic mammals are able to synthesize, process, and produce nearly any human protein in active form. Such proteins can be used with whole milk, as a food additive, or isolated for preparation of various dosage forms [10, 11].

Despite the growing efficiency of mammalian transgenesis technologies and a sufficient number of reports for production of transgenic animals producing rhLF, there are practically no published data on long-term monitoring of transgene transmission and expression nature in a number of generations [12, 13, 14]. The aim of this research was to characterize the stock of transgenic goats producing rhLF in terms of the nature of the transgene transmission in a number of generations, productivity of the target protein, as well as technological parameters of produced milk containing biosimilar human lactoferrin.

2. Creating stock of producing goats

The first transgenic goat carrying the human lactoferrin gene in its genome under the control of the goat beta-casein promoter [15] was obtained in 2007 at RUE Research and Practical Center of the National Academy of Sciences of Belarus for Animal Breeding (Zhodino, Republic of Belarus) within the framework of implementation of the Union State Research and Technical Program “BelRosTransgene”. As a result of microinjection of an expression vector developed by Russian colleagues (Institute of Gene Biology, Moscow, Russian Federation) into fertilized goat oocytes, primary transgenic animals were obtained – the founders of the transgenic line LAK1 and LAK2 (F0). For over 10 years, work has been carried out to obtain and reproduce the descendants of primary transgenic animals (F1-F6), which currently make up the stock of 207 rhLF producing animals.

3. The human lactoferrin gene is stably inherited and expressed over a number of generations

PCR analysis of goatlings (over thousand samples), born as a result of fertilization of Saanen goats with the sperm of transgenic goats, showed that the gene encoding human LF was stably transmitted from generation to generation. The frequency of transgene transmission from parents to offspring was recorded to be in the range of 42.4-52.6% throughout the entire research period (13 years).

The monitoring of rhLF content in milk of producing goats confirms that the transgene is transmitted to the offspring of primary transgenic goats with no effect on its functionality, and is stably expressed in the mammary gland of lactating animals.

As a result of the study of analogue pairs (n=120), no negative effect of transgene was shown on health and reproductive traits of producing goats, all experimental animals grew and developed similarly to non-transgenic goats, and their withdrawal from stock was carried out exclusively for natural reasons.

4. RhLF products in producing goats’ milk for generations

Continuous monitoring of rhLF concentration both in individual samples and in a collective sample from all lactating experimental animals was an important element of a comprehensive assessment of producing goats and was carried out by the method of enzyme-linked immunosorbent assay (ELISA) using Tecan Sunrise photometer (Tecan, Austria) and reagents kit for determination of concentration of lactoferrin manufactured by the Institute of Experimental Medicine (St. Petersburg) according to the method proposed by the kit manufacturer. Milk intended for research was obtained from rhLF producing goats with body weight of 30-40 kg, with udders processed in accordance with hygienic requirements.
before and after milking. Milk sampling was carried out during daily milking from the first weeks of lactation, excluding the colostrum period, twice in each month from each animal. Milking method – using an individual mobile milking machine. Statistical and graphic data processing was carried out using the program GraphPad Prism ver.9.1.0. (GraphPad Software, San Diego, California USA).

Figures 1-4 show rhLF indices in goat milk F1, F3, F4, F5, F6, measured over 9 months (July-March) 2020, as relevant representative results of study of the level of biosimilar human lactoferrin production.

As shown in figure 1, the differences in the medians of rhLF concentration in milk of goats of different generations were not statistically significant (One-way ANOVA followed by Dunnett’s multiple comparisons, p=0.89), which confirms our hypothesis of stable inheritance and expression of the human lactoferrin gene in a number of generations of producing goats. The median rhLF content in milk of F1 animals was 2.60 g/L, F3 — 2.33±0.64 g/L, F4 — 2.58±0.86 g/L, F5 — 2.25±0.21 g/L, F6 — 2.00±0.85 g/L.

Correlation analysis of the dependence of productivity of goats by rhLF on the season of the year (month of lactation) showed the absence of causal correlation between the concentration level of the biosimilar lactoferrin in milk of producers of generations F1-F6 and seasonality (figure 2). The exception was the late winter-early spring period (February-March), when the level of rhLF production in milk was significantly increased in all groups of animals (p<0.05). This phenomenon may be due to physiological characteristics of producing animals (changes in the level of hormones, ratio of catabolic and anabolic metabolic processes, response to changes in the length of daylight hours) associated with spring onset.

The monthly average values of rhLF content in milk were 1.9 g/L in July and August, 2.70 g/L in September, 2.60 g/L in October, 2.10 g/L in November, 3.10 g/L in December, 2.10 g/L in January, 3.6
4 g/L in February and 4.2 g/L in March. There was no correlation between the level of rhLF production and goat milk productivity (milk yield) (figure 3). It should be noted that in February-March there was a sharp increase in lactoferrin productivity with no increase in overall milk productivity (milk yield).

Thus, each of the analyzed goats produced recombinant protein into milk at a significantly higher level compared to other platforms (transgenic rice, cell culture), meeting the criteria for profitability of commercial use as a bioreactor producing biologically active compounds.

5. Isolation of rhLF from milk
Due to the positive charge of the lactoferrin molecule (isoelectric point 8.7), isolation of the target product from milk can be carried out by liquid chromatography using conventional cation-exchange sorbents. There is no need to use expensive affinity chromatography (His-Tag, GTS-Tag), which significantly reduces the cost of the isolation and purification process. Figure 4 shows the result of SDS-PAGE electrophoresis of rhLF isolated from milk of producing goats using the Bio-Rad Process Chromatography Skid liquid chromatography system (Bio-Rad, USA).

6. Physical-and-chemical and technological parameters of milk
Comprehensive comparative analysis of biochemical and physical-and-chemical parameters of milk from goats producing rhLF and milk from ordinary goats showed no statistically significant differences in the content of total protein, as well as density, electrical conductivity, pH and freezing point of milk. All of the above parameters were within the physiological standard. In addition, a statistically significant increase in lactose content (p<0.05, df = 145) and SMR (dry skimmed milk residue) (p<0.05, df = 137), as well as statistically significant decrease in somatic cells count (p<0.05, df = 204) in milk of transgenic animals compared to ordinary goats, which probably indicates a local anti-inflammatory effect of the biosimilar human lactoferrin synthesized in the mammary gland of goats, since the increased somatic cells count is a marker of mastitis.

7. Conclusion
Created herd of goats producing biosimilar human lactoferrin is characterized by stable transmission of the gene encoding human LF from generation to generation. The frequency of transgene transmission from parents to offspring was recorded to be in the range of 42.4-52.6%.

There were no signs associated with the impact of inserted gene construct and presence of rhLF in milk of producers on the lactating status and state of health of animals.

The median rhLF content in milk of F1 animals was 2.60 g/L, F3 - 2.33±0.64 g/L, F4 - 2.58±0.86 g/L, F5 - 2.25±0.21 g/L, F6 - 2.00±0.85 g/L, which gives reason to use producing goats as a bioreactor producing biologically active compounds.

In terms of physical and chemical and technological parameters, milk containing recombinant human lactoferrin meets quality standards.
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