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Designer milk: In the view of molecular genetics

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Abstract
By using nutritional and genetic techniques, dairy biotechnology is quickly making progress in the area of modifying milk composition for processing and/or animal and human health. In terms of processing, casein primary structure modification, lipid profile modification, enhanced protein recovery, milk containing nutraceuticals, and infant formula substitution all offer benefits. Some possibilities for "designing" milk for human health advantages include reducing the amount of fat in it, changing the fatty acid profiles to include more beneficial fatty acids like CLA and ω-fats, improving the amino acid profiles, adding more protein, lowering the amount of lactose, and removing β-lactoglobulin (β-LG). Farm animals developed through transgenic technology also secrete human lactoferrin, lysozyme, and lipase in their milk, simulating human milk in terms of both the quality and quantity of these components that are protective to infants. By removing the β-LG gene from cows, the likelihood that children may become allergic to cow milk could be decreased. Genetically modified animals are used to provide milk that contains therapeutic substances for human health, including insulin, plasma proteins, medications, and vaccines. The eventual acceptability of "designer" commodities will be influenced by moral considerations like animal welfare and safety, in addition to improved health benefits and enhanced profitability of goods produced using revolutionary procedures.

Keywords: Designer milk, β-lactoglobulin, milk protein, transgenic, gene

Introduction
In the modern era consumers are very much aware about their health. As a result, the demand for functional foods is rising globally every day. Numerous institutions and health-related groups, including the American Dietetic Association, concur with the upbeat predictions of rising demand for functional foods. Milk, which contains all the nutrients, is the main dietary source for young mammals among all other foods. Lactation is the process through which the mammary secretary cells of female mammals produce milk, a white fluid secretion. It is considered a comprehensive and well-balanced diet for both people and animals. The nutritive value of milk is very high due to the complete blend of nutrients like water, carbohydrates, proteins, fats and minerals. It carries out an essential role by providing all the nutrients needed to sustain life and growth of infants. To avoid bone diseases like osteoporosis, milk is an important source of calcium (Guetouache *et al*., 2014) [74]. Keeping in mind the significance and nutritional value of milk for human health, changes in social and eating behavior, milk should hold a special place in the competition with other food products.

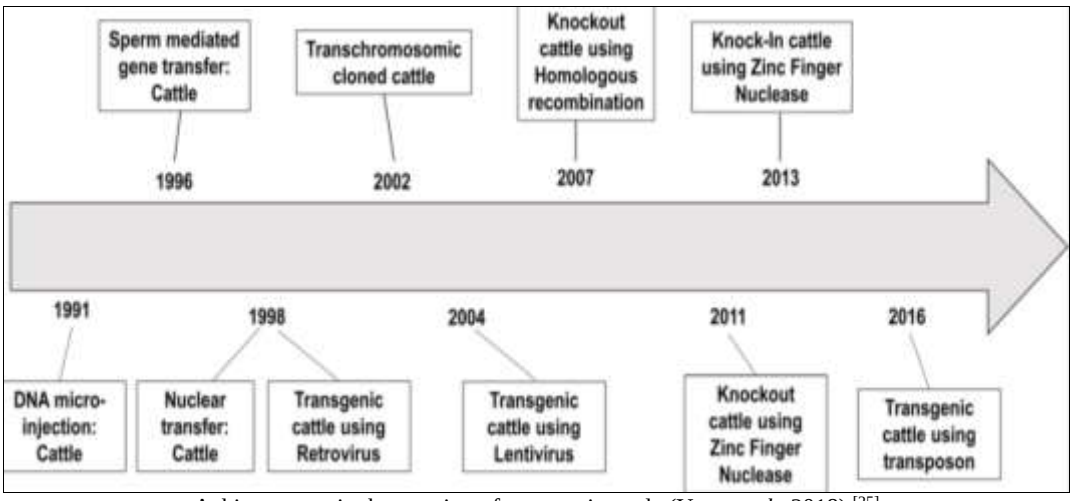
Not only bovine milk, non-bovine milk like goat, sheep, camel, donkey, and yak also have some specific therapeutic and medicinal properties. Bovine milk is allergic to some infants but goat milk is less proven to be allergenic and easier to digest because of smaller fat globules. It also contributed to platelet count progression and also for the treatment of cardiovascular problems. Sheep milk contains a large amount of amino acids as well as total solids compared to bovine and goat milk. Camel milk was also found to be having high medicinal properties and is good for type I diabetic, cancer, tuberculosis, hepatitis C and B patients.

Knowing the importance of milk in human health, milk has to be designed according to the need of individuals. Designed milk means changing the property of milk and also improves the value of milk. Designer milk or enriched milk is known for altering the normal composition of milk and adding some nutritive and pharmaceutical value to the milk. Simply called as value added milk. Milk composition can be changed either by nutrition or by altering the gene makeup of the animal.

In this review, it is dealt about altering the milk composition by genetic approach. Major milk proteins were encoded by monogenic, so it is easy to manipulate the gene sequence. Milk composition can be altered by adding foreign DNA to an animal's germline or by directly altering an animal's germline, as well as by giving milk additional therapeutic, nutraceutical, and dietary benefits.

Production of transgenic animals through genetic engineering by insertion and deletion of genes of interest is important. Initially, DNA micro injection technique was used but it causes mosaics. After that somatic cell nuclear transfer technique was introduced for the production of cloned animals. But this technique was very slow and had low gene targeting efficiency. In light of this, recent advances in genome editing technologies, such as Zinc Finger Nucleases (ZFN), Transcription Activator like Effector Nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and CRISPR- associated nuclease -9 (CRISPR -Cas9), have been applied to a variety of fields, including bioreactors and disease models (Reinoso *et al*., 2021, Yum *et al*., 2018 and Lee *et al*., 2020) [4, 25, 9]. By using a specialized genome editing approach on dairy cows, it is possible to create *in vivo* protein-coding genes and produce novel milk with improved nutritional qualities. Pharmaceutical firms are presently utilizing recombinant technology to produce therapeutic proteins for the treatment of human ailments.

Designer milk or bio-pharmocological proteins are produced by applying specific genome-editing technologies into genome-edited cattle. Bio pharmacological proteins produced from transgenic animals have existed for prolonged and also three recombinant proteins (Aytrin® from goats, Ruconest® from rabbits and Knuma® from chickens) have been accepted by the Food and Drug Adulteration (Hay *et al*., 2022) [10]. Targeted protein is inserted using genome editing technologies into a target region with high expression (i.e., the whey acidic protein). Advanced genome editing technologies like Zinc Finger Nuclease, TALEN and Clustered Regularly Interspaced Short Palindromic Repeats Cas-9 were introduced into livestock for the generation of recombinant proteins in milk (P. Singh and S. A. Ali, 2021, Shepelev 2018, and A.N. Hay *et al*., 2022) [11, 20, 10].



Achievements in the creation of transgenic cattle (Yum *et al*., 2018) [25]

Technologies adapted for development of Designer milk from transgenic animals

The nutritional and technological qualities of milk are greatly influenced by proteins and lipids, which are essential milk components (Bauman *et al*. 2006) [2]. The total protein in bovine milk majorly comprised of six proteins (95%). According to biochemical properties, the major proteins were casein and whey proteins. Caesin is classified as CASAS1, CASA S2, CASB and CASK. Around 20% of the total protein in milk is made up of whey proteins. Some significant whey proteins include β-lactoglobulin (BLG), α-lactalbumin (LALBA), immunoglobulins, serum albumin, lactoferrin and lactoperoxidase (D.W. Threadgill and J. E. Womack. 1990) [16] [16]. The genetic map of the major bovine milk proteins has considerable for the development of breeding programs and also to budding individuals with encoded genotypes. The physical linkage of the casein genes was tightly linked together and inherited as a cluster, so they have an important role in marker - assisted selection (Lien *et al*. 1993) [30]. However, encoded genotypes or creating transgenic animals with the haplotypes would be considerably more effective at introducing the desired alleles into breeding populations than utilizing this to produce individuals with desirable casein haplotypes.

Table 1: Principle proteins in human and bovine milks (g/l)

Protein	Bovine	Human
α- casein	12.6	0.4
β-casein	9.3	2.0
Κ-casein	3.3	0.8
α-lactalbumin	1.2	2.8
B-lactoglobulin	3.2	ND[b]
Lactoferrin	0.1	2.0
Lysozyme	ND	0.4
Serum albumin	0.4	0.6
Immunoglobulin	0.7	0.4

ND- not detectable

Source: S. Kumar and D. Kumar, 2015

Genetic modification of milk proteins

The expression of additional copies of one or more milk proteins led to changes in the composition of milk. Foreign genes that encode casein or whey proteins were expressed heavily in the majority of the experiments. A Study by Lie *et al*. 2014 [79] showed that the beta lactoglobulin gene is expressed in 50 percent of milk protein. Transgenic animal production technology, is not able to enhance the protein content of milk but it is possible to alter the property of milk.

Introducing extra copies of the beta and kappa casein gene into female fibroblasts to raise the casein content of milk. This method resulted in transgenic calves that produced milk

with levels of beta-casein that were increased by 8 to 20%, a two-fold increase in kappa casein levels (Brophy *et al.*, 2003 and Laible *et al.*, 2016) [1, 18].

The monogenic encoded the primary milk protein. During pregnancy and lactation, they were highly and specifically expressed in the secretory epithelial cells of the mammary gland. In bovines, all the casein and whey protein genes were characterized at the sequence level. Most of the milk protein genes were small (<20kb). But alpha and beta caseins were huge and more complicated intronic and exonic regions than whey proteins (A.J. Clark., 1996) [45].

The most prevalent milk protein, beta-casein, which aids in binding calcium phosphate, regulates the level of calcium in milk. Through genetic engineering technology, transgenic cows secreted milk with two-fold levels of beta and kappa casein.

Bovine kappa-casein (K-CN) gene variants have been associated with milk protein content and have a big impact on milk firmness, cheese yield, and rennet clotting time. Higher kappa casein in milk is associated with better heat stability, smaller micelles and better cheese-making properties (Othman *et al.*, 2011) [29]

In a beta-casein locus, lysostaphin was integrated, leading to high expression and significant lysostaphin synthesis in ZFN-treated cows. Gene-targeted secretion of lysostaphin in milk kills *Staphylococcus aureus* infection and also increases the resistance towards this bacterial infection which leads to mastitis and causes decreased milk production. So lysostaphin secretion in milk had some significant effect on dairy farming (Bauman *et al* 2006) [2].

Table 2: Bovine milk protein genes

Protein	Gene	Exon	Reference
αS1-casein	17507	19	Koczan. D, *et al*., 1991 [46]
αS2-casein	18483	18	Groenen M.A.M, *et al*., 1993 [47]
β-casein	8498	9	Bonsing J. *Et al*., 1998 [48]
K-casein	13000	5	Alexander L.J, *et al*., 1988 [49]
α-lactoalbumin	2007	4	Vilotte J.L, 1987 [50]
β-lactoglobulin	4722	7	Mackinlay A.G. 1989 [51]

Knocking out β-lactoglobulin (BLG) in milk

Numerous allergen proteins, including casein, β-lactoglobulin (BLG) and α-lactalbumin are present in bovine milk and BLG is considered as a major milk allergen. It is allergic to infants. The prevalence of allergies to cow's milk is increasing and affects about 2 to 3 percent of babies and young children (Wal J.M *et al*., 1988) [44]. Now a day's breast milk feeding is decreased, and bovine and non-bovine milk is used as substitutes for human milk.

MiRNA is designed to knockdown the activity against the BLG variant, so transgenic calves produced by targeted micro-RNA mediated expression produced double the amount of alpha and beta-casein protein and the absence of allergenic beta lactoglobulin in their milk, so knocking out beta lactoglobulin reduces the allergy caused by this protein (Jabed *et al*., 2012) [15]. Previously transgenesis, gene knockout, SCNT, microinjection and TALEN work were carried out to remove this gene. DNA-free beta-lactoglobulin gene knockout transgenic cow was produced hypoallergenic milk for the first time by zinc finger nuclease mRNA genome editing technology (Sun *et al*. 2018) [21]. Zhu, H. *et al*. 2016 [23] reported that BLG deletion in goats using TALENs-mediated gene editing and SCNT led to targeted mono- and bi-allelic gene exchange of BLG against either human alpha-lactalbumin (hLA) or human lactoferrin (hLF). Human lactoferrin (hLF) was expressed while goat BLG was rendered inactive using TALENs to integrate the hLF gene into the β-lactoglobulin (BLG) endogenous gene location in the genome of primary goat fibroblasts with 13% efficiency. The milk allergen BLG gene was absent in goats developed from the tailored cells, which lowered the allergenicity of the milk produced and demonstrated the efficiency of the BLG gene as a gene integration site for mammary gland-specific expression (Cui, *et al*. 2015) [26].

Wei *et al*., 2018 [28] produced transgenic cattle by zygote-mediated knockdown of beta-lactoglobulin gene through using TALEN-mediated gene editing technology, it produces hypoallergenic milk. Now advanced technology like CRISPR cas9 gene editing technology is effectively used for the introduction of mutant BLG gene in cattle. These transgenic cows created by CRISPRcas9 technology produce hyper allergenic milk (Silaeva *et al*., 2020) [5]. Zhou *et al*., (2017) [13] generated transgenic goat producing hypoallergenic milk by knockdown of beta-lactoglobulin using CRISPR cas9 technology.

Table 3: Potential targets of genome editing in cattle

Si. No	Gene target	Function	Reference
1	Beta lactoglobulin knockout	Elimination of milk allergen	Yu. *et al*. (2011) [54] Wei *et al*. (2018) [28]
2	Insertion of Lysostaphin / lysozyme transgene	Resistance to mastitis	Lie *et al*. (2014) [79]
3	ASIP gene editing	Alter lipid composition	Xie *et al* (2022) [3]

Modification in milk fat

The optimum milk fat composition for human health should contain >82% monounsaturated fatty acids (MUFA), <8% saturated fatty acids (SFA), and <10% polyunsaturated fatty acids (PUFA). The amount of palmitic acid in the sn-2 position of the triglyceride is the main difference between human and bovine milk (O'Donnel J.A., 1989) [41].

But it is very difficult to attain ideal milk fat composition. Therefore, it is feasible to alter the milk composition by changing feeding procedures and/or using genetic engineering techniques.

Decreasing the saturated fatty acid level in the milk

Ma *et al*., 2021 reviewed the genetic markers associated with milk production traits in dairy cattle. Many genes control the milk production traits. The precision of animal selection is increased by the identification of candidate genes for milk production traits. On bovine chromosome 29, there is a gene called fatty acid desaturase 2 which is a candidate for milk fatty acid traits. Manipulation of the stearoyl desaturase gene to produce milk with decreased levels of desaturated fatty acids was studied by Reh *et al*., 2004.

Conjugated linoleic acid (CLA)

Milk fat CLA is a cis-9 trans -11 isomer, it is associated with the inhibition of carcinogens, leukemia proliferation, and colon, prostate, ovary and breast cancers. Denaturation of vaccenic acid (trans-11 octadecenoic acid) by the enzyme stearoyl coA desaturase is an important source of linoleic acid in milk fat. Further research is needed to understand the

genetic factors involved in desaturation and the control of SCD expression.

Reducing the fat content in the milk

Reduced acetyl CoA carboxylase activity controls the rate of fat synthesis in the mammary gland, which lowers milk fat content and lowers energy requirements for milk production. Milk from transgenic animals with less than 2% fat would result in a 22% feed cost reduction per kilogram of milk (Wall *et al*., 1997) [73]. Finding the enzymes that control the synthesis of fat is crucial for modifying the fat content of milk.

Alteration in lactose content in the milk

Lactose is milk sugar; lactose is enzymatically hydrolyzed into glucose and galactose by beta-galactosidase enzyme, which is transported into bloodstream. As age progresses, the beta galactosidase enzyme starts to decrease and is absent in adulthood. When milk and milk products are consumed by these people, it causes indigestion, malabsorption, and water retention in the gut as a result of its osmotic effect. This water retention along with bacterial production in large volumes leads to intestinal distress and dehydration (Saavedra, J. M., 1989: J. Leaver and A.J.R. Law, 2003) [31, 76]. Milk is an important constituent for human health since it contains calcium, which is necessary to maintain strong bones. Using methods that manipulate genes, such as using β gal replacement (Pre-harvest) or hydrolyzing low lactose output (Post-harvest), it is possible to lessen the effects of lactose intolerance.

Pre-harvest techniques for lowering lactose content in milk include removing alpha-lactalbumin, using gene knockout techniques, or adding lactase enzyme through the production of genes unique to the mammary gland. However, the overall sugar content of milk is substantially reduced by these methods, increasing milk viscosity. Studies in mice (Stacey, A., *et al*., 1995 and 1994) [33-34] showed that milk's lactose concentration is decreased by deleting alpha-lactalbumin, but it causes hampered milk volume regulation. These mice's milk was high in viscosity, protein, and fat but low in alpha-lactalbumin and lactose (Karatzas and Turner, 1997) [77]. In mice, the UDP-gal gene was turned off by Vilottee 2002 generated milk with a high viscosity but no lactose.

The post-harvest methodologies for decreasing the lactose content in milk by over-expression of beta-galactosidase in milk. They created transgenic mice that produced a specific amount of beta-galactosidase in their milk. The lactose level of milk has been cut in half in transgenic mice. The author states that at least double the amount of lactose content was reduced. In some other studies by Karatzas and Turner, transgenic mice produced less amount of lactose and preserve most of the monosaccharide content of milk. Further, it did not affect the protein content of milk. Producing livestock with this transgene would also be possible theoretically. The transgenic animal did not affect the milk protein level but helps to maintain a balanced nutrient supply **(**Hettinga, 1989) [32].

Table 4: Potential changes in milk through genetic engineering

Changes in Gene	Changes in milk
Casein	Increase protein
Engineered casein	Manufacturing properties
Anti-sense β-lactoglobulin	Reduce/remove
Anti-sense acetyl CoA carboxylase	Reduce/remove fat
B-Galactosidase, lactase	Increase solids content
Antibodies of pathogens	Safer food, mastitis prevention

Source: Bremer *et al*., 1989 [78]

Boost lactoferrin production in milk

Lactoferrin is the primary whey protein found in human milk. It is an iron-binding protein with both immunological and antimicrobial properties. Its level in human milk is about 1 to 2 g/l but the level of lactoferrin in bovine is very low 1/10th of human milk (Lonnerdal, B 1995) [36]. Pharming NV (Leiden, The Netherlands) created the first transgenic bull in the late 1980s and a range of transgenic cows that manufacture a variety of proteins, including human lactoferrin (Subramanian 2004 and Umaraw *et al*., 2015) [42, 75].

Synthesis of Lysozyme in milk

Lysozyme enzyme has antibacterial properties. Human milk contains 0.4 g/l of lysozyme (LZ). So, milk of transgenic mice has been produced human lysozyme at concentrations of 0.78 g/l (Maga, E.A and Anderson, G.B., 1995) [37] The prevalence of mastitis in cows may also be decreased by a transgenic cow that expresses both human lactoferrin and lysozyme in milk (Sui *et al*, 2014 and Bawden, W. S., 1994) [19, 38]. The line of transgenic goats designed to produce human lysozyme in the mammary gland, in addition to lower somatic cell count implied good udder health (Maga *et al*., 2006) [14]. Transgenic cattle that can secrete human lysozyme into their milk to inhibit microorganisms like *Staphylococcus aureus* are created by integrating the human lysozyme gene into the cattle casein locus in mammary epithelial cells (Bauman *et al*., 2006) [2].

Humanization of milk

Nature's ideal food for infants is breast milk. It provides all the essential nutrients and also prevents infections. Breast milk was replaced by bovine milk by changing the property of bovine milk with genetic engineering produces similar to human milk composition (Lonnerdal, B. 1996) [35]. By gene replacing technique, the alpha-lactoalbumin gene in mice is replaced by its human counterpart. It can be used as a substitute for infant formula. So, it is possible to express the human protein in the milk of transgenic animals.

Knocking out of ASIP gene in milk

Though the ASIP gene is coat color related, studies showed that CRISPERcas9 editing of the ASIP gene leads to a change in the lipid composition of milk. It increases the saturated and polyunsaturated fatty acids and decreases the monounsaturated fatty acids (Xie *et al.*, 2022) [3].

α anti –trypsin production from sheep milk

The role of alpha anti-trypsin protein was inhibition of neutrophil derived elastase. Congenital absence of alpha anti-trypsin protein leads to the formation of emphysema, and also

it was associated with cystic fibrosis and other lung-related problems. Production of alpha antitrypsin from bacteria, yeast, mammalian cell culture and human plasma donations does not meet out the demands of the medical field. So significant large amounts of human alpha anti-trypsin are produced from transgenic sheep milk. The generation of five founder sheep produced the human alpha anti-trypsin gene (A. colman, 1996) [8].

Table 5: Examples of transgenic animals create recombinant proteins in milk

Species	Protein	Genetic engineering technology	Reference
Cow	Human lactoferrin	Pronuclear injection and SCNT	Van berkel *et al*. 2002 [58] Wang *et al*. 2017 [63]
	Human serum albumin	TALENs /SCNT	Luo *et al*. 2016 [61]
Goat	Human granulocyte colony stimulating factor	Pronuclear injection	Ko *et al*. 2000 [64]
	Human glycoprotein alpha fetoprotein	SCNT	Parker *et al*. 2004 [66]
	CuZn and EC-SOD	SCNT	Lu *et al*. 2018 [67]
	Human lactoferrin	TALENs/SCNT	Cui *et al*. 2015 [53]
	Antithrombin (ATyrn)	Pronuclear injection	Adiguzel *et al*. 2009 [68]
Rabbit	Human alpha glucosidase	Pronuclear injection	Van den Hout *et al*.2001 [59]
	Human plasminogen activator	Pronuclear injection	Song *et al*. 2016 [69]
	C1 esterase inhibitor	Pronuclear injection	Van veen *et al*. 2012 [60]
Pig	Human blood clotting factor VIII	Pronuclear injection	Paleyanda *et al*. 1997 [70]
	Human erythropoietin	Pronuclear injection	Park *et al*. 2006 [66]
	Human furin enzyme and Factor IX	SCNT	Zhoa *et al*. 2015 [71]

Milk with human therapeutic proteins

GTC Biotherapeutics uses both cows and goats to create therapeutic proteins, such as plasma proteins, monoclonal antibodies, and vaccines. Recombinant antithrombin III, an anti-coagulant protein found in human blood was produced from goat milk. GTC researchers working on the development of a malaria vaccine from goat milk. A transgene in the transgenic goat secretes human protein into the goat's milk under the control of a promoter region that regulates expression in the mammary gland. Production of transgenic protein from mammalian cell culture was costly compared to production from goat milk. Human haemophilia patients used the milk secreted by transgenic animals, which also contained proteins such as blood clotting factors (A. Colman 1996 and O. G. Maksimenko *et al*., 2012) [8, 27]

GTC Biotherapeutics is currently constructing a method for the production of various routinely used mAbs (Rituximab®, Herceptin®, Humira®, and Erbitux®) in transgenic goats.

The production of specific proteins and growth factors that are lacking in milk may be improved through transgenic alteration of the milk composition. The enhanced expression of a few of these proteins in milk may enhance the developing offspring's growth, development, health, and ability to survive. These include lactoferrin, epidermal growth factor (EGF), transforming growth factor (TGF), and insulin-like growth factor 1 (IGF-1).

Expression of recombinant lysozyme, lactoferrin and lactoalbumin in the milk had a significant effect on the health of infants and also the prevention from infection. A current trend in the development of biotechnologies is the use of transgenic animals as bioreactors for the manufacture of recombinant proteins secreted into milk. The development of targeted genome editing technologies, in particular, has opened up new possibilities and significantly enhanced efficiency in the breeding of animals that generate recombinant proteins in milk, along with other economically significant animals. Transgenic animals produce specific antibodies in mammary glands will be helpful for the prevention of mastitis in cows, sheep and goats and pigs.

Monzani *et al*., 2016 [24] discussed about the use of recombinant proteins has increased in various production sectors. There are many systems available for producing proteins, but due to its high level of expression and ability to achieve post-translational modifications, the mammary gland is thought to be a highly promising system for the synthesis of recombinant proteins. Cows are an ideal target for recombinant protein production in milk because they secrete large quantities of milk over a prolonged lactation period. The insufficiency of transgenic techniques, the lengthy times required for transgene detection, the expression of recombinant proteins, and the fact that each pregnancy produced a single calf make the generation of transgenic cows more difficult. Promising approaches are being developed that could assist overcome this obstacle and allow the use of transgenic cattle as bioreactors for the production of protein in milk for the industry.

Conclusion

Although population genetics, breeding strategy, and nutritional management have increased milk production, the composition of the milk has not significantly changed as a result. There will be many opportunities to develop new value-added products for designing milk for the benefit of human health as genetics advances and geneticists identify candidate genes in cows for diseases, and desirable traits such as milk fat synthesis and milk protein synthesis, especially in livestock. Numerous transgenic cows have been described for a range of purposes, including the expression of medicinal proteins, antibodies, hypoallergenic milk, enhanced milk casein protein, decreased milk fat content, greater disease resistance, and improved animal welfare.

Future

In the upcoming stages, we can look forward to producing cows with low milk fat in nature, which can complete through the incorporation of marker-assisted selection and targeted genome-edited transgenic animals. In any case, for the public to be convinced of the advantages of recombinant products sufficiently to buy the resulting dairy products, the advantages of these products must be established, and protection concerns must be addressed in a clear and objective manner. However, despite the contribution and future importance of genome editing technology, it has inherent risks, morals and social acceptance are important factors linked with the implement of these technologies. It would be better to focus efforts on keeping records of gene-edited livestock and taking notice of how this livestock is reproduced and consumed, as opposed to trying to stop the development of transgenic animals. It would

be beneficial to raise knowledge of the advantages and disadvantages of these transgenic animals. Before this technology can be used to its full potential, numerous obstacles must be overcome.

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