

Production and Processing of Milk from Transgenic Goats Expressing Human Lysozyme in the Mammary Gland

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ABSTRACT

The potential for applying biotechnology to benefit animal agriculture and food production has long been speculated. The addition of human milk components with intrinsic antimicrobial activity and positive charge to livestock milk by genetic engineering has the potential to benefit animal health, as well as food safety and production. We generated one line of transgenic goats as a model for the dairy cow designed to express human lysozyme in the mammary gland. Here we report the characterization of the milk from 5 transgenic females of this line expressing human lysozyme in their milk at 270 $\mu\text{g/mL}$ or 68% of the level found in human milk. Milk from transgenic animals had a lower somatic cell count, but the overall component composition of the milk and milk production were not different from controls. Milk from transgenic animals had a shorter rennet clotting time and increased curd strength. Milk of such nature may be of benefit to the producer by influencing udder health and milk processing.

Key words: lysozyme, transgenic, udder health, milk

INTRODUCTION

The genetic engineering of livestock to yield improved animals for the dairy and ranching industries has been frequently discussed since it was first suggested in 1982 (Palmiter et al., 1982; Jimenez-Flores and Richardson, 1988; Yom and Bremel, 1993; Maga and Murray, 1995). Most applications of transgenic technology in dairy animals have been with the intent of recovering the desired protein from the milk for other uses. For example, the mammary gland can be used as a bioreactor to produce pharmaceuticals (reviewed in Clark, 1998) and nutraceuticals (van Berkel et al., 2002) in milk. The concept of using genetic engineering as a means to alter the properties of the milk itself was proven using transgenic mice. In several transgenic mouse models, the function-

ality of milk in terms of its processing properties, antimicrobial activity, and lactose levels was significantly altered (Maga et al., 1995, 1998; Gutierrez et al., 1996; Jost et al., 1999; Kerr et al., 2001). Although progress has been made in the production and commercialization of transgenic crops to benefit the producer, the promise of genetic engineering has yet to be realized in commercially important livestock species. Recently, the production of transgenic dairy cattle resistant to mastitis by the expression of the bacterial enzyme lysostaphin was reported (Wall et al., 2005). Here we report the characterization of transgenic dairy goats producing human lysozyme (**HLZ**) in their milk, with the potential to have an impact on not only the producer but also the consumer and the animal.

Lysozyme is a positively charged, antimicrobial molecule that exists naturally in avian egg whites and mammalian secretions such as tears, saliva, and milk (Jolles and Jolles, 1984). Lysozyme specifically catalyzes the cleavage of the glycosidic linkage between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine in the peptidoglycan component of bacterial cell walls (Phillips, 1966), causing leakage of the cell's interior components (lysis). Lysozyme is naturally present in human milk at concentrations 1,600 to 3,000 times greater than in livestock milk, with human milk containing 400 $\mu\text{g/mL}$ of lysozyme, whereas bovine and caprine milk contains only 0.130 and 0.250 $\mu\text{g/mL}$ of lysozyme, respectively (Chandan et al., 1968). Lysozyme, along with lactoferrin and secretory IgA, is one of the protective compounds responsible for the antimicrobial properties of human milk (Levy, 1998). Due to its intrinsic antimicrobial nature, lysozyme is commonly used as a preservative in a wide range of food products including meat and cheese as well as in cosmetics (Proctor and Cunningham, 1988; Hughey et al., 1989; Gill and Holley, 2000).

The human gene for lysozyme was chosen for introduction into livestock because no allergic response was anticipated due to its ubiquitous presence in the tears, saliva, and milk of humans. Dairy goats were chosen as the experimental animal because they are small ruminants that have a shorter gestation period and gener-

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ation interval than cattle; thus, results can be obtained faster and more economically. Due to the strong similarities between goat and cow lactation, milk composition, and methods of processing, results obtained on the functional properties in our genetically engineered goat model are likely to be applicable to the dairy cow.

The application of genetic engineering to produce dairy animals expressing HLZ in their milk would be an efficient manner in which to deliver multiple benefits with a single gene modification. Due to the properties of HLZ, changes in milk processing, food safety, and udder and animal health are expected. This work confirms earlier proof-of-principle studies conducted in mice (Maga et al., 1995) and was designed to demonstrate the potential of expressing and delivering the benefits of human milk components via livestock milk with the ultimate goal of benefiting both the producer and consumer.

MATERIALS AND METHODS

Transgenic goats were generated by standard pronuclear microinjection with a DNA construct consisting of 23 kb of the promoter and 3' regulatory elements of the bovine α_{S1} -casein gene coupled to the 540-bp cDNA for HLZ (Maga et al., 2003). This transgene was previously expressed in the milk of transgenic mice (Maga et al., 1994, 1995). All animals were housed and cared for under the Association for Assessment and Accreditation Laboratory Animal Care-approved conditions. To maintain the line and generate offspring, transgenic females of age were bred to nontransgenic males, and transgenic males were bred to nontransgenic females. Transgenic offspring were identified by PCR of umbilical and ear samples and confirmed by Southern blot (Maga et al., 2003). The numbers of male and female transgenic and nontransgenic offspring generated were recorded to track transgene transmission and segregation.

Five transgenic does from this transgenic line were studied over multiple lactations (10 combined lactations among the 5 animals). Expression of HLZ mRNA in the mammary gland was confirmed by Northern analysis of RNA isolated from sloughed somatic cells in milk. Milk was collected from lactating transgenic and nontransgenic control animals and immediately centrifuged at $1,500 \times g$ for 10 min at 4°C to pellet the sloughed mammary epithelial cells. Cream and supernatant were removed, and the pellet washed in PBS at $1,500 \times g$ for 5 min at 4°C . Total RNA was extracted by adding 1 mL of Trizol solution (Invitrogen, Carlsbad, CA). Total RNA (5 μg) was run on a 3-(N-Morpholino) propane-sulfonic acid (MOPS)-formaldehyde gel, transferred to a nylon membrane (Hybond N+, Amersham

plc, Little Chalfont, UK), and probed with the complete 540-bp HLZ cDNA labeled with ^{32}P by random priming. After standard washings, the blot was exposed to autoradiographic film overnight.

Expression at the protein level was quantified by Western blot. Standardized amounts of milk from lactating transgenic and nontransgenic control females were subjected to 15% SDS-PAGE and transferred to a polyvinylidene difluoride Hybond membrane (Amersham) for Western blotting with a 1:2,500 dilution of a primary rabbit anti-HLZ polyclonal antibody (DAKO, Glostrup, Denmark) followed by a 1:20,000 dilution of a secondary goat antirabbit-horseradish peroxidase antibody (BioRad, Hercules, CA). Signal was detected by enhanced chemiluminescence (ECL Plus, Amersham). Quantification of HLZ protein expression in goat milk was carried out by image analysis (Image Pro Plus Software, Silver Spring, MD) of Western blots with known amounts of HLZ standard purified from human milk (Sigma, St. Louis, MO) as control. This method was chosen so as to be able to distinguish between HLZ and endogenous goat lysozyme. In addition, activity of HLZ in milk was verified as previously described (Maga et al., 1995). Standardized amounts of milk were run on a 15% SDS-PAGE gel with incorporation of 0.4% *Micrococcus lysodeikticus*. After electrophoresis, gels were placed in renaturation buffer (50 mM sodium phosphate, 1% Triton-X100) at 37°C overnight. Zones of clearing in the incorporated bacteria indicated HLZ activity.

Milk Collection

Milk samples were collected from individual transgenic (5 animals) lactating females over successive lactations and nontransgenic (13 animals) lactating females of the same age, parity, and stage of lactation. Milk samples were taken from each half of the udder of an individual animal and combined before all analyses. Milk was collected at parturition and then once a month for a period of 3 mo. The values for daily milk production, weight percent fat, weight percent protein, and SCC were determined through the California DHIA testing program analysis using an automated infrared test method with a Fossomatic milk analyzer calibrated to cow milk. The SCC values reported are the combined means of the monthly readings taken in 2001, 2002, 2003, and 2004 for all lactating transgenic or control animals at midlactation.

Processing Properties

The effects of in vivo-produced HLZ on several processing properties of milk were characterized as pro-

Table 1. Transgene transmission and segregation in human lysozyme transgenic goats

Year	Animals bred	Kids born	Females born (%)	Males born (%)	Transgenic kids (%)	Transgenic females (%) ¹	Transgenic males (%) ¹
2001	1	3	2 (67)	1 (33)	2 (67)	1 (50)	1 (50)
2002	9	15	8 (53)	7 (47)	5 (33)	2 (40)	3 (60)
2003	9	19	10 (53)	9 (47)	9 (47)	6 (67)	3 (33)
2004	5	11	4 (36)	7 (64)	5 (45)	2 (40)	3 (60)
Total	24	48	24 (50)	24 (50)	21 (44)	11 (52)	10 (48)

¹Percentage of transgenic animals.

viously described (Maga et al., 1995). The rennet clotting time and gel strength of milk from transgenic and nontransgenic control animals were determined using published laboratory-scale methods that were developed based on industry standards (Renner-Nantz and Shoemaker, 1999). The rennet clotting time of milk samples was determined by incubating 20 mL of fresh milk (20°C) with vegetable rennet (New England Cheese Making Supply Company, Ashfield, MA) at a final dilution of 1:4,000 rennet:milk at 32°C. Samples were examined for formation of curd at 5-min intervals. The rennet clotting time was taken as the time that passed before a solid gel was formed at the bottom of the flask. Milk from 5 transgenic and 5 nontransgenic control animals was used to conduct 12 individual trials each carried out in duplicate.

A controlled stress rheometer (AR1000 Rheometer; T. A. Instruments, New Castle, DE; version 5.3 software) was used to evaluate the overall firmness of rennet-induced casein gels. Twenty milliliters of fresh, unrefrigerated 22°C whole milk samples were mixed with vegetable rennet (1:4,000 vol/vol) at 20°C and placed on the rheometer under a 4-cm diameter plate with a gap of 625 μ m. The temperature was raised and held at 32°C for 30 min for the formation of a gel, and then lowered and held at 20°C for 10 min before measurements were carried out. An oscillatory torque-sweep was carried out over the range of 1 to 10 Pa at a frequency of 1 Hz to determine the values of G^* , a measure of the overall firmness of the gel, and $\tan\delta$, a measure of the degree of viscoelasticity or the type of bonds present. Two complete sets of measurements were taken for each gel formed. Milk from 4 transgenic and 5 nontransgenic control does was used. For each animal, all assays were conducted in duplicate and repeated at least 3 times.

Statistical Analyses

Statistical significance was evaluated by using a standard 2-tailed, nonpaired Student's *t*-test on the means of the data collected from DHIA analysis of production data (daily production, percentage fat, percentage protein, and SCC), rennet clotting time, and gel-

strength assays (mean G^* and $\tan\delta$ values) at the 95% confidence level. Mean G^* and $\tan\delta$ values comprised readings taken over the entire torque sweep (20 points). Transgene transmission data were analyzed by χ^2 . The standard deviation was reported for each value as a measure of variation about the mean.

RESULTS AND DISCUSSION

We report the characterization of transgenic goats expressing HLZ in their milk. One line of transgenic goats was generated that produced HLZ in their milk under control of the bovine α_{S1} -casein promoter (Maga et al., 2003). Goats from the founding (1 female), F_1 (1 female), and F_2 (3 females) generations were used to evaluate HLZ expression as well as the phenotypic traits of the resulting milk.

Transgene Transmission

To date, we have generated 21 (11 female and 10 male) hemizygous transgenic dairy goats that carry and express the α_{S1} -HLZ transgene (Table 1). Animals were generated by the natural breeding of transgenic females to nontransgenic males and vice versa, and are all descendants of the founding female. As expected, equal percentages of male and female offspring were born, and almost half (44%) of the animals born were transgenic (Table 1). Because the founding animal was hemizygous for the transgene, we would expect the transgene to be passed to 50% of all offspring. Our results were not significantly different than the expected 1:1 ratio ($\chi^2 = 0.375$; $P > 0.5$). Therefore, it is likely that this line transmits the transgene in a Mendelian fashion. In addition, the transgene segregates equally among male and female offspring (Table 1) as expected.

HLZ Expression

Expression of HLZ mRNA in the mammary gland was verified by Northern blot analysis of total RNA purified from the sloughed somatic cells in the milk of lactating animals (Figure 1a). Western blot results

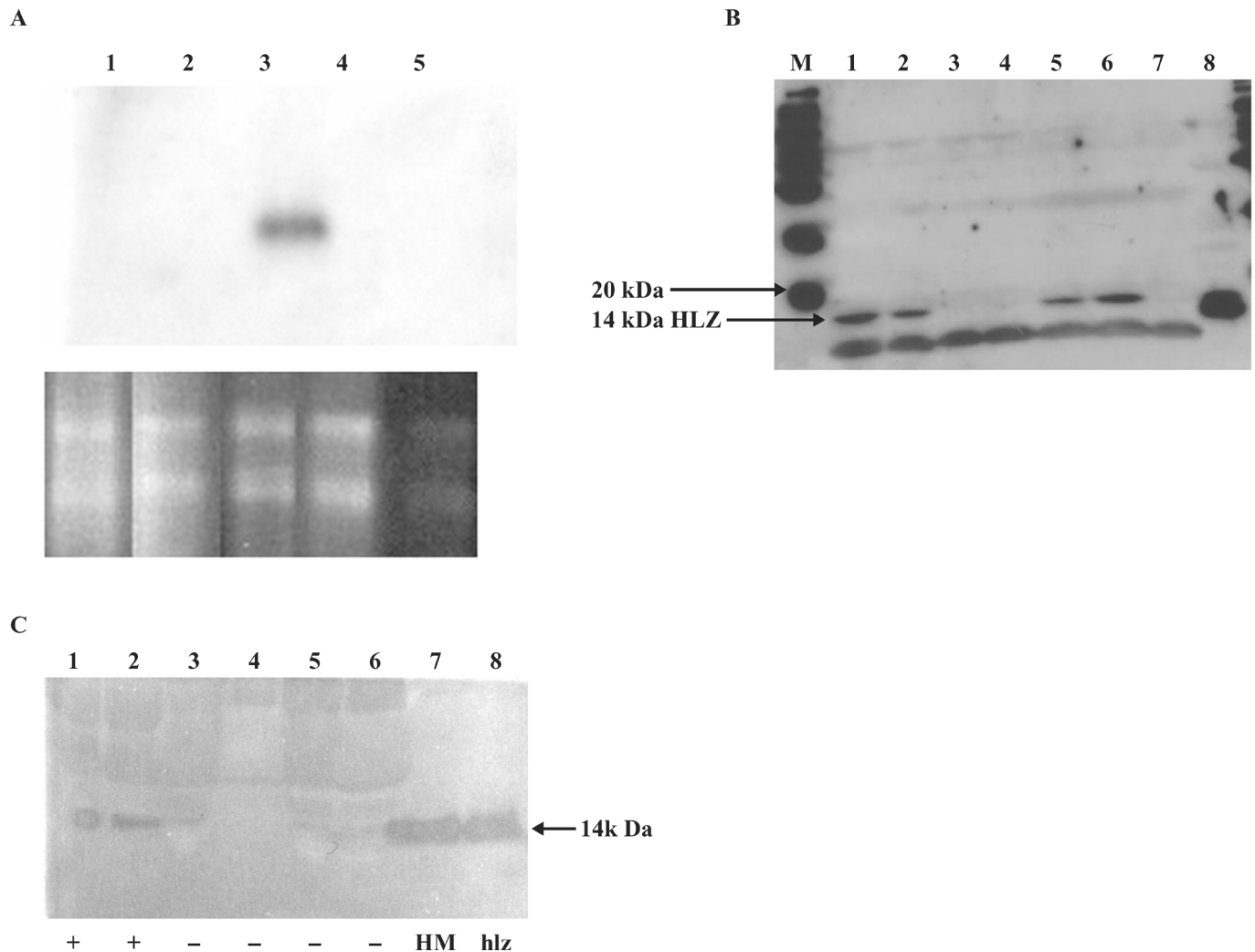


Figure 1. Expression of human lysozyme (HLZ) in transgenic goat milk. A) Northern blot analysis of total RNA isolated from the sloughed somatic cells of the milk from the expressing founder transgenic animal (lane 3) and nontransgenic control animals (lanes 1, 2, 4, and 5). Total RNA (28S and 18S bands) from each sample stained with ethidium bromide is shown in the lower panel. B) Western blot of transgenic and control goat milk. Milk was collected from transgenic and nontransgenic control goats at 1 mo of lactation. Standardized amounts (30 μ g total protein) of milk were subjected to standard SDS-PAGE and Western blotting with a polyclonal antibody specific to HLZ. Lanes 1 and 2 contain pooled milk from 4 lactating transgenic females before (lane 1) and after pasteurization (lane 2); lanes 3 and 4 contain pooled milk from nontransgenic control goats, before and after pasteurization, respectively; lanes 5 and 6 contain milk from individual transgenic does of the second generation and lane 7 milk from a contemporary, nontransgenic control; lane 8 contains HLZ standard; and lane M contains a molecular size marker. C) Milk samples were subjected to SDS-PAGE with HLZ substrate (*Micrococcus lysodeikticus*) incorporated into the gel. After electrophoresis, the gel was placed in renaturation buffer with HLZ activity indicated by the clearing of the incorporated bacteria at the molecular weight of HLZ (14 kDa). Lanes 1 and 2 contain milk from transgenic does and lanes 3 to 6 milk from nontransgenic controls; lane 7 contains human milk (HM) and lane 8 is 2 μ g of commercially purified HLZ.

demonstrated that the transgenic goats expressed the HLZ protein in their milk (Figure 1b) and activity assays demonstrated that the lysozyme was active (Figure 1c). Levels of HLZ protein in the milk of hemizygous does were estimated by Western blotting to be 270 ± 84 μ g/mL, approximately 68% of the level found in human milk. This technique allowed detection and quantification of HLZ only, because the human antibody used was not sensitive enough to detect endogenous goat

lysozyme. Expression of HLZ was consistent between individuals and generations (Figure 1b, lanes 5 and 6). On average, levels of HLZ in milk varied by 25% among individuals of the same parity, and by 14% between individuals in successive lactations. This variation is similar to reports in transgenic sheep expressing human α -1 antitrypsin in their milk (Carver et al., 1993). For instance, in one line, average variation in expression between lactations was 24% and expression levels

Table 2. Production parameters as determined by DHIA analysis for human lysozyme (HLZ) transgenic goats in their first (n = 5), second (n = 3), and third (n = 2) lactation compared with age- and parity-matched nontransgenic control goats (Herd; n = 13) raised in the same herd¹

Parity	Daily production (kg)		% Fat		% Protein		SCC/mL (×1000)	
	HLZ	Herd	HLZ	Herd	HLZ	Herd	HLZ	Herd
First	3.7 ± 0.9	3.4 ± 0.5	2.87 ± 0.75	3.25 ± 0.34	2.65 ± 0.36	2.72 ± 0.15	291 ± 130	351 ± 258
Second	4.2 ± 0.7	3.8 ± 0.5	2.91 ± 0.77	3.11 ± 0.37	2.57 ± 0.28	2.74 ± 0.15	208 ± 184	474 ± 261
Third	4.7 ± 0.3	4.3 ± 0.5	2.90 ± 0.71	3.12 ± 0.42	2.65 ± 0.33	2.55 ± 0.15	409 ± 140	609 ± 481
All	4.0 ± 0.8	3.8 ± 0.6	2.89 ± 0.72	3.17 ± 0.37	2.63 ± 0.32	2.67 ± 0.17	284 ± 157 ^a	458 ± 338 ^b

^{a,b}Means within a row with different superscripts differ ($P < 0.05$, $df = 44$).

¹Values are the means (± SD) at midlactation over a 4-yr period.

varied by up to 26% among individuals of the same line. The variation in HLZ expression is most likely due to genetic differences between animals that one would observe in any breeding herd. It should be noted that the line of goats used for study was hemizygous and produced 1,080 times more lysozyme than is normally found in goat milk. We anticipate that levels approaching that found in human milk will be obtained and the effects of HLZ expression enhanced when the animals are bred to homozygosity.

Production Parameters

Milk Components. The percentage of milk yield that represents total fat and protein for the transgenic line, as determined by testing through DHIA, fell in the same range as the means of our dairy goat herd for both fat and protein (Table 2). These results indicate that expression of the transgene did not disrupt the gross composition of the milk. This is similar to results of several studies with mammary-specific transgenic mice, which indicate that the expression of an additional protein in the mammary gland generally does not result in a significant increase in the total amount of protein being made (McClenaghan et al., 1995), thereby implying that the mammary gland has a certain limited capacity for protein production. Similar results have been reported in transgenic sheep and cattle (Wright et al., 1991; Wall et al., 2005). Western blotting with antibodies for β -lactoglobulin and total casein has not shown any discernible decrease in the amounts of these endogenous milk proteins (data not shown). In addition, daily milk production from the transgenic line was not different from that in the nontransgenic controls.

Udder Health. A measure of udder health was made by monitoring the SCC of milk taken from both halves of the udder. The SCC over a 4-yr period were significantly lower ($P < 0.05$) in the HLZ transgenic animals compared with our control herd over the same period (Table 2). The SCC of a population can be used as an indicator of udder health and milk quality (Schukken et al.,

2003), with lower SCC generally corresponding to a healthier udder and better quality milk. High SCC can be directly correlated with mastitis and a decline in milk quality due to associated chemical changes in the milk. Although the average SCC in both populations were not at levels considered unhealthy (750,000 to 1,000,000 cells/mL in goats), they were lower in milk from transgenic does, indicating a healthier udder with fewer sloughed mammary epithelial cells and leukocytes that are indicators of an intramammary bacterial infection. In fact, none of the 21 samples analyzed from transgenic animals had an SCC greater than 750,000 cells/mL, whereas 4 of the 25 samples analyzed from nontransgenic control does exceeded this level and were considered unhealthy. Additional studies are of great importance in evaluating the potential impact that milk of this type may have on the health of the lactating animal. Because levels of HLZ expressed in the mammary gland of our transgenic goats are over 1,000 times greater than is normally present in goat milk and are in the range of levels of immune compounds such as lysozyme and lactoferrin that are naturally present during infection (Chen et al., 2003), HLZ could offer a natural defense against mastitis and improve the health and well-being of lactating animals.

Processing Properties of HLZ Transgenic Milk

The presence of HLZ in transgenic milk affected several processing properties of milk. The rennet clotting time of HLZ milk was significantly lower ($P < 0.05$) than that of nontransgenic controls (Table 3). Milk from transgenic animals clotted on average 6 min faster than milk from control animals, equating to a 26% decrease in rennet clotting time. The strength of rennet-induced curd, as measured by G*, was significantly stronger ($P < 0.001$) when transgenic goat milk was used compared with nontransgenic controls of the same lactation (Table 3). The types of bonds present in curd from transgenic milk, as measured by the value $\tan\delta$, were not different from those formed with nontransgenic con-

Table 3. Physical properties of human lysozyme transgenic and control goat milk

Goat	Rennet clotting time ¹ (min)	Curd strength ² (Pa)	Degree of viscoelasticity ³
Transgenic	17 ± 6* (n = 5)	26.3 ± 4.0** (n = 4)	0.21 ± 0.42 (n = 4)
Control	23 ± 12 (n = 5)	20.7 ± 1.8 (n = 5)	0.29 ± 0.29 (n = 5)

¹Measure of the time for curd formation after the addition of rennet.

²Mean overall gel strength (G*) of rennet-induced curd over a range of 1 to 10 Pa of applied torque.

³Measure of the types of bonds formed during curd formation. Values are the mean tan δ readings over a range of 1 to 10 Pa of applied torque.

*Transgenic goats were different from age-, stage-, and parity-matched nontransgenic control goats ($P < 0.05$, df = 66).

**Transgenic goats were different from age-, stage-, and parity-matched nontransgenic control goats ($P < 0.001$, df = 38).

trol milk (Table 3). These results strongly paralleled those seen in our previous studies with transgenic mice (Maga et al., 1995).

It is likely that alteration of the processing properties of milk assessed here was a result of the positive charge carried by HLZ. The addition of polycations, including lysozyme, can promote the faster precipitation of casein micelles to form curd (Green and Marshall, 1977) and affect cheese yield and syneresis by binding to the caseins (Giangiacomo et al., 1992). This could account for the increase in gel strength by either charge—charge interactions of positively charged HLZ with the negatively charged micelles, or by lysozyme incorporation into the micelle itself during assembly. Interactions of this type could result in the tighter packing of the caseins during curd formation. This is supported by the fact that low tan δ values were obtained, indicating that the gels were more elastic than fluid and that HLZ may be altering the number, but not types, of bonds that are being formed. Again, if animals were bred to homozygosity to produce higher levels of HLZ, we would expect to see even greater differences in these processing parameters.

It should be noted that the levels of α_{S1} -casein protein in goat milk are variable and affect the coagulation properties of the milk (Clark and Sherbon, 2000). All samples, regardless of casein genotype, were considered and, although there was variability in the rennet clotting time between individual goats, a significant overall reduction was observed. This indicates that the effect on clotting of the milk resulted from the presence of HLZ and not from the casein genotype. The expression of HLZ in milk has the potential to influence the quality of soft and hard cheeses and allow for a wider range of products that can be produced, a benefit to both the producer and consumer. Due to the antimicrobial nature of HLZ, its presence in milk may affect the growth of starter cultures used for cheese production and thus influence ripening, flavor, and texture of the resulting product. The HLZ in milk may act to slow the growth

of bacterial contaminants in the milk and udder and thus affect the shelf life of dairy products and the disease state of the udder. These issues are currently under study.

CONCLUSIONS

Milk quality, food safety, and animal health are important issues for the consumer and the dairy industry alike. Results reported here characterizing the milk from transgenic goats expressing HLZ in the mammary gland indicate that the genetic engineering of dairy animals to express human milk components does have the potential to influence many of these issues. The presence of in vivo-produced HLZ can alter the functional and physical properties of the milk protein system, potentially leading to increased cheese yield or a wider range of potential products that can be manufactured. In addition, transgenic animals had lower SCC suggesting improved udder health. These transgenic goats represent one of the first genetically engineered livestock species with the potential to benefit the animal, producer, and consumer. Of course, for any of these benefits to be implemented into production systems, more data will need to be generated to determine if these animals would be of value to producers and accepted by consumers. This report is the first step in generating data that can then be used to debate the value of transgenic animals in agriculture.

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