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CHAPTER 5

Designer Milk

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Contents

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Abstract

Dairy biotechnology is fast gaining ground in the area of altering milk composition for processing and/or animal and human health by employing nutritional and genetic approaches. Modification of the primary structure of casein, alteration in the lipid profile, increased protein recovery, milk containing nutraceuticals, and replacement for infant formula offer several advantages in the area of processing. Less fat in milk, altered fatty acid profiles to include more healthy fatty acids such as CLA and ω-fats, improved amino acid profiles, more protein, less lactose, and absence of β-lactoglobulin (β-LG) are some opportunities of "designing" milk for human health benefits. Transgenic technology has also produced farm animals that secrete in their milk, human lactoferrin, lysozyme, and lipase so as to simulate human milk in terms of quality and quantity of these elements that are protective to infants. Cow milk allergenicity in children could be reduced by eliminating the β-LG gene from bovines. Animals that produce milk containing therapeutic agents such as insulin, plasma proteins, drugs, and vaccines for human health have been genetically engineered. In order to cater to animal health, transgenic animals that express in their mammary glands, various components that work against mastitis have been generated. The ultimate acceptability of the "designer" products will depend on ethical issues such as animal welfare and safety, besides better health benefits and increased profitability of products manufactured by the novel techniques.

I. INTRODUCTION

Reports of prolific and successful research in the areas of biotechnology and genetic engineering have unleashed potential ideas that were previously inconceivable in the subject of dairying. It is now firmly established that novel value-added products can be derived from milk and milk products with nutritional and biotechnological interventions. While until recently, breeding policies have aimed at producing more milk, attempts are now directed toward enhancing the value of milk and studying its health implications. This has found more support with clinically established epidemiological linkages between diet and chronic diseases that encourage search for new links between food and disease.

The extranutritional therapeutic attributes of milk and milk products have also been brought into this broad network of research. Milk composition can be altered by nutritional management or through the manipulation of naturally occurring genetic variation among cattle. The possible

channels of influencing milk composition to suit specific needs can be investigated with the help of a thorough comprehension of the biochemistry, genetic traits, and factors in the animal diet that affect milk synthesis and composition. By an intelligent combination of the two approaches—nutritional and genetic—a milk designed to suit consumer preferences can be developed. This ''designer milk'' may be rich in specific milk components that may have influence on well-being or on processing. This chapter examines the potential that exists in altering milk composition by nutritional and genetic approaches in order to achieve specific health benefits and/or processing opportunities.

II. MILK "DESIGNING": THE PROSPECTS

Man has been taming and manipulating other species for his own benefits for thousands of years. Several breeds of cattle that produce large quantities of milk exist today as a result of selective breeding adopted by farmers over centuries. The global appeal of milk as a healthy beverage that is good for adults as well as infants has prompted much investigation on the commodity.

Research on animal breeding, husbandry, and feeding conditions has always had a profound impact on the quality of milk, its constituents, and the subsequently manufactured products. Altering the composition of milk in a manner that suits health and processing needs forms the basis of the current research interests in the area. For example, a greater proportion of unsaturated fatty acids in milk fat, reduced lactose content in milk for lactose-intolerant people, and/or milk free from β-lactoglobulin (β-LG) would benefit human diet and health. From a technological point of view, there exist vast opportunities in altering the primary structure of casein to improve the technological properties of milk and producing milk high in protein content. Engineering milk that clots in less time leads to increased yield and/or more protein recovery during cheese manufacture. Milk that contains nutraceuticals and replacement ingredients for infant formula are other interesting avenues.

Genetic manipulation (GM) also offers the prospect of healthier animals with improved resistance to diseases such as mastitis or to the ticks that can infest cattle, thus reducing the need for antibiotics and pesticides. Medicines may be produced in the milk of cows. For example, GM cows could produce milk with a clotting factor for hemophiliacs, milk containing human serum albumin for blood transfusions, or milk with a hepatitis vaccine. Several of these medicines could be produced much more efficiently than with the technologies currently used. Some of the potential changes that can be brought about in milk are listed in Table 1.

TABLE 1 Selected reports on opportunities for "designing" milk

No.	Modification	Benefits	References[a]
A. Fat modification			
A.1	Remove/reduce fat	Low-fat milk and products, caters to the health-conscious consumers	Wall *et al.*, 1997
A.2	Alter the fatty acid chain length	Increased nutrition, better manufacturing properties, better product quality	CSIRO, 1999; O'Donnell, 1993; Mason, 2001
A.3	Increase CLA levels in milk	Anticarcinogenic and other therapeutic properties	Pszczola *et al.*, 2000; Stanton, 2000
A.4	Alter proportion of ω-6 to ω-3 fatty acids	Several health benefits	Dhiman *et al.*, 1999; Kao *et al.*, 2006
B. Carbohydrate modification			
B.1	Overexpress β-galactosidase enzyme	Better lactose digestibility, caters to the lactose-intolerant customers	Bremel *et al.*, 1989
B.2	Remove α-LA, produce lactase by transgenic technology	Reduced synthesis of lactose	Jost *et al.*, 1999; Karatzas and Turner, 1997
C. Protein modification			
C.1	Increase amino acids content, casein	Increased protein, better processing properties, better nutrition	www.agresearch.co.nz, 2001
C.2	Genetically engineer casein	Better manufacturing properties	Bleck *et al.*, 1998a,b; Brophy *et al.*, 2003
C.3	Remove β-LG	Less milk allergies, better processing properties	www.agresearch.co.nz, 2001
C.3	Modify bovine milk to simulate human milk	Better infant health, less mortality, less problems due to milk allergy	Lonnerdal, 1996; Maga *et al.*, 2006
C.4	Introduce human therapeutic proteins		AgResearch Now, 2005; Morgan, 2006; Pettus, 2006

TABLE 1 (*continued*)

No.	Modification	Benefits	References[a]
D. Miscellaneous			
D.1	Produce in milk antibodies, antimicrobials against pathogens	Safer food, prevention of mastitis and other diseases	Margawati, 2003; Wall *et al.*, 2005
D.2	Produce spider silk in milk	Industrial applications	Anonymous, 2002; Dove, 2000; Lazaris *et al.*, 2002

[a] An indicative and partial list.

III. MILK FAT MODIFICATION

The quantity of milk fat is a determinant of its value and hence a major indicator of the revenue accrued from milk. As a recent trend, advanced knowledge about the chemistry of milk fat and human physiology encourages product developers to modify the milk fat to counter the changing functional and nutritional challenges. Dairy products provide less than 15% of the total fat available in the diet (O'Donnell, 1993). Milk fat provides 25% of the saturated fat, which is still not as high as that in the two groups of fats and oils (29%) and meat, poultry, and fish (39%). The contribution of dairy products to the total cholesterol is 16%, much less than that in eggs (39%) and meat, poultry, and fish (43%).

Modifications of the composition and quality of fodder result in different milk fat compositions and influence the nutritional and technological value of fats. A sophisticated trend in the ''health market'' today is to modify the milk fat composition by either adopting suitable feeding strategies or by genetic modes. It is now almost possible to achieve the ideal composition of milk fat for human health and well-being recommended by O'Donnell (1989), after the Wisconsin Milk Board 1988 Milk Fat Roundtable. The combination suggested at the meeting was less than 10% polyunsaturated fatty acids (PUFA), less than 8% saturated fatty acids (SFA), and more than 82% monounsaturated fatty acids (MUFA).

A. Altering the fatty acid chain length and level of saturation in milk fat

The long-chain fatty acids of milk fat are derived from the diet via blood. The short-chain fatty acids (C10 and below) of milk fat are first synthesized in the mammary gland and then elongated to C12–C16. If the

mechanism for elongation is blocked by genetic technology, the ratio of medium-chain fatty acids (C12–C16) to short-chain fatty acids in milk fat should reduce. Since the C12–C16 fatty acids are generally regarded by nutritionists as less desirable, milk fat with reduced content of medium-length fatty acid chains would garner more value due to greater consumer demand.

There is ample experimental evidence to suggest that nutritional modifications can cause significant changes in milk fat composition. The degree of unsaturation of the serum lipids, tissue fat, and milk fat may be increased promptly by feeding unsaturated fats in an encapsulated or protected form to lactating animals (Ashes *et al*., 1997). It is established that MUFA (C18:1) content can be increased by 50–80% and may approach 50% of milk fatty acids by feeding lipids rich in 18-carbon fatty acids (Grummer, 1991). Feeding low-roughage diets increases the proportion of MUFA in milk fat, the effects of feeding low-roughage diets and lipid being additive. The SFA content (palmitic acid, C16:0) of milk fat can also be reduced by 20–40% unless the supplemented lipid is rich in palmitic acid. SFA particularly palmitic and other medium-chain fatty acids tend to increase levels of blood cholesterol (O'Donnell, 1993).

Feeding highly unsaturated oils (e.g., soybean oil) caused depression in milk fat, but increased the proportion of unsaturated fatty acids to SFA in milk (www.extension.iastate.edu). A study at the University of Alberta (Mason, 2001) revealed that feeding canola oil in the encapsulated form (to protect it from biohydrogenation by the rumen microorganisms) led to higher increases in linoleic (18:2) and linolenic (18:3) acids than while feeding unprotected oil seeds. As the melting point of milk fat containing unsaturated fatty acids is more, the spreadability of butter made from this milk improved tremendously. An Australian study involving the feeding of a special blend of canola and soybean meal in the protected form resulted in doubling the spreadability of butter (CSIRO, 1999). When taken out of a refrigerator at 5 °C, the butter was nearly as spreadable as margarine, without losing its special eating qualities. Clinical trials revealed that consumption of dairy products made from this milk led to decrease in low-density lipoprotein (LDL) levels in the blood of the consumers.

Chouinard *et al.* (1998) compared the results of feeding to Holstein cows, a control total mixed ration (TMR) with TMR supplemented with calcium salts of three fatty acids from oils with progressive degree of unsaturation—canola oil, soybean oil, or linseed oil. The digestibility of nutrients was higher for rations containing calcium salts than for the control ration. The milk yield increased in proportion to the degree of unsaturation in the feed supplement. The fat content in milk reduced in all the experimental diets as compared to the control. The addition of calcium salts to the ration decreased the proportions of SFA that contained C6–C16 and increased the proportions of C18:0, *cis*-9-C18:1, and *trans*-11-C18:1 in milk fat. These

findings were confirmed later by Aigster *et al.* (2000) who reported that feeding calcium salts of high-oleic sunflower oil (HOSO) containing more than 86% oleic acid at the rate of 7.5% of diet dry matter weight to Holstein cows increased the oleic acid content of milk fat from 26% to over 40% and decreased the cholesterol-raising saturates from 41% to 33%.

Reports by Lee *et al.* (2004) elucidated the feeding of goats with different kinds of protein-oil supplements to alter the milk fat composition. Combinations of HOSO with keratin (KN), casein (CN), and dry casein (DCN) were fed to lactating goats. Oleic acid levels increased to 19.0% on the KN-oil supplement diet, 19.2% on the CN-oil supplement diet, and 25.2% on DCN-oil supplements diet compared to 12.5% in milk fat from goats on normal diet. Feeding the protein-oil supplements also decreased solid content in milk fat at 10 °C from 34.8% to 23.0% for DCN, to 26.5% for CN, and to 29.6% for KN. It was suggested that the DCN-oil supplement diet might increase the spreadability of butter at refrigerated temperatures.

Studies at the University of California (Davis) are focused on the desaturase gene to produce milk with decreased levels of SFA (CDRF, 2004). The researchers targeted the stearoyl-CoA desaturase enzyme that converts specific medium- and long-chain SFA to their monounsaturated forms. The overall fatty acid composition of the milk of a transgenic line of goats that expressed a bovine β-LG promoter-rat stearoyl-CoA desaturase gene tilted in favor of a less saturated and more MUFA profile at the seventh day of lactation (Reh *et al.*, 2004). Efforts are under way to determine if genetic differences among breeds and individual animals are translated into ratios of SFA and unsaturated fatty acids.

B. Increasing CLA levels in milk fat

Milk fat is a good source of the putative anticancer agent, conjugated linoleic acid (CLA), a product synthesized in the rumen during the biohydrogenation of linoleic acid (LA). Table 2 lists the CLA content in selected dairy products. Research has shown that it is possible to influence the extent of ruminal biohydrogenation and the concentration of CLA absorbed and incorporated into milk fat. There is evidence that the concentration of CLA in milk influences its pharmaceutical properties (Kelly and Bauman, 1996). The level of CLA could, therefore, influence the value of the milk as a commodity, although it is not at present a criterion for deciding the price of milk.

CLAs reportedly suppress carcinogens, inhibiting proliferation of leukemia and cancers of the colon, prostate, ovaries, and breast. They are the only natural fatty acids accepted by the National Academy of Sciences of United States as exhibiting consistent antitumor properties at levels as low as 0.25–1.0% of total fats (Eynard and Lopez, 2003). The other reported

TABLE 2 CLA content in selected dairy products[a]

Dairy product	Total CLA (mg/g fat)
Buffalo milk	6.1
Cow Milk	5.5
Homogenized milk	4.5
Butter	6.0
Cultured buttermilk	5.4
Ice cream	3.6
Yoghurt	
Low fat	4.4
Nonfat	1.7
Plain	4.8
Cheese	
American processed	5.0
Cottage	4.5
Mozzarella	4.9
Ricotta	5.6
Sharp Cheddar	3.6
Romano	2.9

[a] Compiled from Muller and Delahoy (1988), National Dairy Council (2000), and Tyagi *et al.* (2004).

beneficial health effects of CLA as supported by biomedical studies with animal models are antiatherogenic effect, altered nutrient partitioning, improved lipid metabolism, antidiabetic action (type II diabetes), immunity enhancement, and improved bone mineralization (Bauman *et al.*, 2001; Bell and Kennelly, 2001).

Reports suggest that feeding lipid sources rich in linoleic and linolenic acids either as seeds or free oil increases the CLA content of milk when oil is accessible to the rumen microorganisms for biohydrogenation (Dhiman *et al.*, 2000). The scientists found that supplementing the dietary dry matter with 2% or 4% soybean resulted in a 237% or 314% increase in CLA content of milk compared with the control.

Stanton (2000) and her team worked on the supplementation of cow's diet with ingredients such as full fat rapeseed, full fat soybean, and pulp-n-brew (by-product of brewers' grains rich in LA) to study their effect on the CLA levels in milk. When diets of pasture-fed cows were supplemented with full fat rapeseed and full fat soybean, the CLA levels in milk fat increased by 53% and 34%, respectively, after 18 days of feeding when compared to the unsupplemented group of cows on pasture which served as control. The yield and proximate composition of milk were unaffected by the supplementation.

Milk from a grass-fed cow can have five times as much CLA as milk from a grain-fed animal (Robinson, 2003). An experiment supplementing either silage, autumn grass, or spring grass over three periods with pulp-n-brew revealed that the CLA levels increased in case of supplementation of silage and autumn grass, but was less effective in the case of spring grass (Stanton, 2000). Spring grass feeding led to a 2.1-fold increase in CLA content of milk. The CLA-enriched milk fat exhibited cytotoxicity toward mammary and colon cancer cells.

Incorporating CLA along with soy oil in the diet of cows increased the CLA levels, simultaneously decreasing the SFA in milk fat (Pszczola *et al*., 2000). In an attempt to increase the CLA content in milk via the cow's diet, Bell and Kennelly (2001) divided 28 Holstein cows into 4 groups and fed them different diets—control diet (CTD), low-fat diet (LFD), high-fat diet 1 (HF1), and high-fat diet 2 (HF2). The animals were kept on CTD for 8 days before starting the different diet regimen. All experimental diets resulted in lower fat percentage in the milk when compared to CTD, whereas other parameters such as milk yield, protein, and lactose were unaffected. The CLA concentration in milk fat was 0.49%, 0.56%, 3.7%, and 5.63% in the group fed CTD, LFD, HF1, and HF2, respectively. Thus, increasing the fat content in the diet increased the CLA content up to 9–12 times, despite lower total fat content.

AbuGhazaleh *et al*. (2003) found that feeding lactating dairy cows a blend of fish oil and MUFA and PUFA resulted in an increase in the concentrations and yields of CLA in milk, the greatest increase being with a blend of a high LA source (e.g., regular sunflower seeds). Beaulieu and Drackley (2004) reported similar results where a diet rich in LA led to increasing the CLA levels in milk fat twofold. Supplementing nonluminous green fodder with mustard cake in the feed of buffaloes resulted in 6.18-mg CLA per gram of fat as compared to 6.05 mg/g when the supplement was groundnut cake (Tyagi *et al*., 2004). The total CLA in buffalo milk and milk products increased significantly when the animals were fed berseem and wheat straw in the ratio 87:13.

Tsiplakou *et al*. (2006) examined the CLA content in the milk fat of sheep and goat milk segregated into two groups. Animals in Group 1 were totally on pasture from April onward with supplementary feeding during winter, whereas animals in Group 2 served as the control group and were kept indoors without grazing. The study revealed that the CLA content in milk fat of Group 1 increased in April and May, during the availability of early grass and declined thereafter, whereas that in Group 2 remained more or less constant. The CLA content (*cis*-9, *trans*-11) in sheep milk was 2% of the total fatty acids fat content and was much higher than that in goat milk (0.62%).

Animal variation is also a major source of differences in the CLA content of milk fat. Bauman and Perfield (2002) discovered that the 9,11

isomer of CLA in milk fat is synthesized by the cow and not rumen bacteria as had earlier been reported. Synthesis involves a mammary enzyme, delta-9 desaturase, which acts on a *trans*-fatty (vaccenic) acid produced by rumen bacteria. Several genetic factors that regulate the expression of the delta-9 desaturase gene have been identified. In a line of transgenic goats that contained a rat stearoyl-CoA desaturase gene targeted at converting medium- and long-chain SFA to their monounsaturated forms, Reh *et al*. (2004) found that the desaturase enzyme also converted the rumen-derived MUFA C18:1 *trans*-11 to the C18:2 *cis*-9 *trans*-11 isomer (CLA) in the milk fat of one of these animals.

C. The omega fatty acids

Omega-6 and omega-3 are essential fatty acids, but the body requires them in a ratio that is not normally achieved by the typical diet of today's developed nations. It is reported that the current average intakes of essential fatty acids expressed as ratios of ω-6 to ω-3 fatty acids are 8:1 in United Kingdom, 10:1 in United States, and 12:1 in Australia (www.omega-3info.com). Health bulletins indicate that the proportion of ω-6 to ω-3 fatty acids should be equal or close to 5 for cardiovascular health (Simopoulos, 1999).

At present, the average PUFA content in modern diets (nearly 30% of calories) is too high. It is suggested that our PUFA intake should not be much greater than 4% of the caloric total, in approximate proportions of 2% ω-3 linolenic acid and 2% ω-6 linoleic acid (Fallon and Enig, 2000). The intake of total ω-3 fatty acids in the United States is ~1.6 g/day (Kris-Etherton *et al*., 2002). Of this, α-linolenic acid (ALA) accounts for 1.4 g/day, whereas eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) together account only for 0.1–0.2 g/day. DHA is required by the brain and nerve cells and is essential for normal visual and neurological development in infants (Tomlinson, 2003). The major food sources of ALA are vegetable oils, principally canola and soybean oils. Oily fish are the richest source of EPA and DHA. EPA and DHA can be made by the body from ALA, but sometimes this capacity is impaired, so oily fish remains the best source. The recommendations for intake of ω-3 fatty acids range from 0.5 to 2 g/day. ISSFAL (International Society for the Study of Fatty Acids and Lipids) recommend 0.65-g EPA and DHA per day (Willumsen, 2006). Of this, the content of each should be at least 0.22 g. Omega-6 is the essential fatty acid that is in ample supply in oils, nuts, and seeds. Table 3 lists the ω-6 and ω-3 fatty acid contents in commonly available food ingredients.

Too much ω-6 in the diet creates an imbalance that can disrupt the production of prostaglandins leading to increased tendency to form blood clots, inflammation, high blood pressure, irritation of the digestive tract,

TABLE 3 The ω-3 and ω-6 fatty acids content in some common food ingredients[a]

Food ingredient	ω-3 FA	ω-6 FA
Vegetable oils (g/100g)		
Almond oil	0	17
Canola/rapeseed oil	9	20
Corn oil	0.7	58
Flax/linseed oil	58	14
Grapeseed oil	–	68
Olive oil	0.60	7.90
Palm oil	0.2	9
Safflower oil	–	74
Sesame oil	0.3	41
Soybean oil	7	51
Sunflower oil	–	63
Walnut oil	11.5	58
Wheat germ oil	7	55
Fish oils (g/100g)		
Cod-liver oil	20.5	1.9
Salmon oil	36	4.5
Sardine oil	26	5
Nuts (g/100g edible portion)		
Almonds	Trace	10
Brazilnuts	Trace	23
Cashew nuts	Trace	8
Hazelnuts	Trace	4
Peanuts	Trace	16
Pine nuts	1	25
Pistachios	0.254	13
Walnuts	9	37
Seeds (g/100g edible portion)		
Flax/linseeds	15–25	6
Pumpkin seeds	7–10	20
Safflower seeds	0.111	28
Sesame seeds	Trace	25
Sunflower seeds	Trace	30
Meat and fish (EPA + DHA g/100g edible portion)		
Poultry	0.05	–
Oily fish	1.8–1.9	–
Bacon and ham	0.008–0.009	–

[a] Compiled from: www.annecollins.com, www.longevinst.org, www.nutraingredients.com

depressed immune function, sterility, cell proliferation, cancer, water retention, and weight gain. On the other hand, deficiency in ω-3 is associated with asthma, heart disease, and learning deficiencies. It is established that ω-3 fatty acids have a hypolipidaemic action in human, reducing harmful cholesterol levels, particularly plasma triglycerides (Tomlinson, 2003). It also has an anti-inflammatory action and helps to reduce platelet aggregation. Essential fatty acids have proven to be effective in the treatment of several other ailments including eczema, rheumatoid arthritis, asthma, Alzheimer's disease, and Attention Deficit Hyperactivity Disorder (ADHD). There are reports that approximately equal amounts of these two fats in the diet will result in lower risk of cancer, cardiovascular disease, autoimmune disorders, allergies, obesity, diabetes, dementia, and some mental disorders (www.flax.com/newlibrary/ESSENT.html).

Dietary manipulation in cows is a practical way to maintain a desired ratio of ω-6 to ω-3 fatty acids in milk. Milk from pastured cows contains an ideal ratio of essential fatty acids. Dhiman *et al*. (1999) reported equal quantities of the omega fatty acids (16.5 mg/g fat) in the milk of cows entirely on pasture. Reducing the proportion of grass to two-third of the ration increased the ω-6 fatty acids to 31.4 and decreased ω-3 fatty acids to 13.5 mg/g milk fat. Further reduction in the dietary proportion of grass to one-third resulted in 42.7 and 8.2 mg/g fat of ω-6 and ω-3 fatty acids, respectively. There are reports that organic milk contains almost 70% more ω-3 fatty acid than nonorganic milk (Cheek, 2006).

Mammals are dependent on dietary sources of essential fatty acids as they lack the desaturase enzymes necessary to synthesize them. Kao *et al*. (2006) engineered transgenic mice expressing the ω-3 fatty acid desaturase enzyme from the nematode *Caenorhabditis elegans*, which synthesizes a wide range of PUFA and possesses the only known example of an ω-3 desaturase enzyme in the animal kingdom. The milk from these mice had more ω-3 and less ω-6 PUFA, and hence had showed an overall decrease in the ω-6:ω-3 PUFA ratio in the milk. The milk phospholipids from the transgenic mice had an ω-6:ω-3 ratio of 1.78 as compared to 9.82 in the control animals. The authors anticipate that this may be a suitable method to improve the nutritional profile of dairy-based diets.

D. Reducing fat content in milk

It has long been recognized that the yield of milk fat can be altered through nutritional interventions. Several workers have reported that supplementing normal diet with fats in different forms and concentrations decreases the yield of fat in milk (Baumgard *et al*., 2000; Bell and Kennelly, 2001; Chouinard *et al*., 1999; Peterson *et al*., 2002). Genetic studies also pointed to the power of hereditary traits in influencing the quality of milk. Genetic markers for milk quality of dairy cattle were

discovered and reported by the Iowa State University in the United States in 1996 (www.biotech.iastate.edu/biotech_update). Laboratory experiments with the marker revealed that animals with the ability to produce low-fat milk could be accurately identified. Such genetic testing was aimed at improving dairy herd performance by identifying animals with the potential to produce low-fat milk. A herd producing low-fat milk was seen as a means to reduce milk-processing costs as the low-fat milk eliminated the necessity to separate fat from milk.

As a variation to altering the fat composition, Wall *et al*. (1997) suggested that modifying the cow's genetic makeup to enable it to produce milk with 2% fat would reduce the cost of feed per kilogram milk by 22%. In changing the fat composition, targeting enzymes that influence the synthesis of fat is important. As an example, reduction of acetyl-CoA carboxylase that regulates the rate of fat synthesis within the mammary gland would translate to a drastic reduction in the fat content of milk and reduce the energy required by the animal to produce milk (Ntambi *et al*., 1999).

E. Type of fatty acids versus product quality

The type of fatty acids present in milk fat can influence the flavor and physical properties of dairy products. There are reports that butter produced from cows fed high-oleic sunflower seeds and regular sunflower seeds were equal or superior in flavors to the control butter (Middaugh *et al*., 1988). The experimental butter was softer, more unsaturated and exhibited acceptable flavor, manufacturing, and storage characteristics. Other workers (CSIRO, 1999; Mason, 2001) have also reported the increase in the unsaturated fatty acids content in milk fat, leading to an improvement in the spreadability of butter even at refrigerated temperatures (Section III.A).

Extruded soybean and sunflower diets yielded a Cheddar cheese that had higher concentrations of unsaturated fatty acids while maintaining flavor, manufacturing, and storage characteristics similar to that of control cheese (Lightfield *et al*., 1993). It is also beneficial from a safety point of view as the accumulation of fatty acids, namely C12, C14, C18:1, and C18:2, enhanced the safety of cheeses against *Listeria monocytogenes* and *Salmonella typhimurium* (Schaffer *et al*., 1995).

Increasing the oleic acid content of milk fat from 26% to over 40% by feeding calcium salts of HOSO containing more than 86% oleic acid at the rate of 7.5% of diet dry matter weight to Holstein cows did not affect the sensory and physicochemical properties of Latin American white cheese (Queso Blanco). There was also no difference (as a result of the modified and improved fatty acid profile) between the firmness of the product from modified milk and that made from normal milk (Aigster *et al*., 2000).

IV. MILK SUGAR (LACTOSE) MODIFICATION

Lactose, the major milk sugar, is also responsible for the osmotic regulation of lactation, thus causing the movement of water into milk. This carbohydrate is synthesized in the secretory vesicles of the mammary glands by the lactose synthase complex. As lactose cannot diffuse out of the vesicles, it draws water into the vesicles by osmosis. Thus, the volume of milk produced is directly dependent on the amount of lactose synthesized.

Lactose cannot be transported to the bloodstream directly. It can be absorbed only after its enzymatic hydrolysis to the monosaccharides glucose and galactose by intestinal lactase (β-galactosidase). For many human beings, the level of β-galactosidase declines early in life to the point of virtual absence in adulthood, making them lactose intolerant. It is reported that more than 75% of the human adult population suffers from deficiency of β-galactosidase (Vilotte, 2002). When such individuals ingest milk or milk products, the lactose remains undigested and malabsorbed in the gut, where it causes retention of water by its osmotic action. This water retention coupled with the bacterial production of large volumes of carbon dioxide leads to intestinal upset and dehydration (Vesa, 1999).

One management tactic suggested for such patients is the avoidance of dairy products. However, as milk is a major component in the human diet, this deprives them from the use of a valuable nutritional source. In addition, since milk can provide much of the required calcium for maintaining bone health, lactose intolerance can also be associated with osteopaenia in old people (Corazza *et al*., 1995). A report suggests that by 2020, half of all American citizens older than 50 will have low bone mass and be at risk for fractures from osteoporosis if appropriate dietary and other precautions are not followed (Carmona, 2006). Therefore, excluding milk from diet has adverse effects on health.

The consequences of lactose intolerance can also be limited through the use of β-galactosidase-replacement (preharvest) or hydrolyzed low-lactose (postharvest) products. Besides the obvious nutritional advantage, a reduction in milk lactose content could also benefit agricultural and industrial purposes with less volume to transport, better milk coagulation, and less effluent production. The complete removal of lactose from milk creates milk that is extremely viscous, containing very little water. It is extremely difficult to extract this milk from the mammary gland, making the milking process difficult and painful for the animal. However, research has shown that with controlled reduction in the lactose content of milk, it is possible to decrease the water, increase the percentage of total solids, and reduce the lactose yield of the milk while keeping fluidity intact.

A. Preharvest methods of lactose reduction

α-Lactalbumin (α-LA) is one of the major milk proteins present in almost all mammalian milks. It interacts with β-1,4-UDP-galactosyl transferase (UDP-gal) to modify substrate specificity of this enzyme, virtually creating a unique binding site for glucose and leading to the synthesis of lactose (Vilotte, 2002). The preharvest methodologies of reducing lactose involve either the introduction of β-galactosidase enzyme into milk via mammary gland-specific expression or the removal of α-LA and gene ''knockout'' methodologies. Although these successful approaches provide valuable tools to address milk physiology, they reduce the overall sugar content of the milk, resulting in highly viscous milk. Studies on mice have revealed that reduction of lactose via α-LA deletion was inappropriate because it impaired milk volume regulation. The milk of such mice was highly viscous with very high protein (88%) and fat (60%), no α-LA and no lactose (Karatzas and Turner, 1997). Knocking out the UDP-gal gene in mice also produced milk with no lactose but very high viscosity (Vilotte, 2002).

An alternative to produce low-lactose milk is overexpression of β-galactosidase in milk. However, the monosachharides produced within the formed milk increases the osmotic pressure within the alveolar lumen, thereby drawing more water and resulting in further dilution of other milk components (Bremel *et al*., 1989).

Jost *et al*. (1999) explained an *in vivo* technique for low-lactose milk production. They generated transgenic mice that selectively produced a biologically active β-galactosidase in their milk. In these transgenic mice, the lactose content of the milk is at least halved, even though the β-galactosidase expression levels were relatively low. The authors claim that it is likely that at least twofold greater levels of lactose reduction could be achieved. In contrast to the previous studies by Bremel *et al*. (1989) and Karatzas and Turner (1997), these experiments led to reduction in the lactose content while retaining most of the monosaccharide content of the milk. β-galactosidase synthesis in the mammary gland caused a significant decrease in milk lactose (50–85%) without obvious changes in fat and protein concentrations. It thus helped to maintain a balanced nutrient supply as reflected in the similar growth curve reared on transgenic or control milk. It is likely that transgenic low-lactose milk production could offer a more balanced approach to managing lactose intolerance than postharvest or lactose-replacement products. It is also technically feasible to produce transgenic livestock carrying this transgene and probably similar or better expression levels could be achieved. However, more detailed analysis on several aspects such as the effect of splitting the lactose into glucose and galactose on the osmotic balance in the milk in the gut after ingestion and also the economic viability of the technology need to be investigated (Whitelaw, 1999).

V. MILK PROTEIN MODIFICATION

One of the major products of the mammary glands being protein, exciting opportunities in research and technology extend the benefits of better protein supplementation. One of the most obvious changes in milk is the selective increase of a component that is already present. For example, an increase in one of the casein components in milk might provide a method of increasing the value of milk for the production of cheese. It was estimated that an increase of 20% in the $\alpha s1$-casein ($\alpha s1$-CN) would increase the revenue of the cheese industry by almost $200 million annually (Hennighausen *et al.*, 1990). Similarly, improvement in the amino acid profiles by increasing the amounts of L-taurine, L-leucine, and L-phenylalanine offers additional nutritional benefits.

Protein modification in milk started with experiments on laboratory animals two decades ago. The success of these experiments on small animals prompted researchers to extend the work on cattle and other farm animals with considerable success. The Dairy Cooperative Research Centre (Dairy CRC) in Australia reported the cloning of 14 calves with an extra copy of the cow's own gene for casein besides the 4 normal ones found in cattle (CRC Factsheet, 2006). This increased the quantity of protein secreted in the milk, thus increasing the nutritional quality and the value of milk.

A. Modifying the major milk proteins

The four bovine casein genes lie within a single, multigene locus of ~200 kb in length. Zuelke (1998) worked on the hypothesis that this multigene locus contains all of the DNA sequences required to regulate the coordinated expression of all four individual casein genes. A bacterial artificial chromosome (BAC) library of genomic DNA from elite dairy cattle was prepared in his laboratory and tested in mice with the hope that transgenic calves that possess this BAC casein construct could be produced.

Jeng *et al.* (1997) characterized and partially purified bovine β-casein (β-CN) from the milk of transgenic mice. The approximate expression of the protein was 3.0 mg/ml of milk. The workers reported that phosphorylation of the bovine β-CN in the milk of transgenic mice was the same as that of native bovine β-CN. If the modification and/or enhancement in the β-CN could be extrapolated into farm animals, there would be several other potential advantages to processing. The extra β-CN would increase the cheese yield besides improving the curd strength.

Bleck and Wheeler (1998) also reported the generation of β-CN in the milk of transgenic mice. While murine milk normally contains about 30% total solids, the experimental animals produced milk that had 40–50%

total solids (10–20% higher). This increase in total solids obviously caused a decrease in the amount of water in the milk and was accompanied by the concomitant decrease in total volume of milk. Thus, the milk was very viscous, lacked fluidity, and could not be removed easily from the mammary gland. There has also been an attempt to form a glycosylated β-CN in milk (Bleck *et al.*, 1998a). This has the potential to increase the solubility of β-CN and modify other functional properties such as viscosity, water-holding capacity, foaming, and emulsification.

κ-Casein (κ-CN) is responsible for micelle formation and establishes micelle size and function, thus influencing many of the physical characteristics of milk. Gutierrez-Adan *et al.* (1996) generated transgenic mice bearing the bovine κ-CN gene. They found that the milk from transgenic mice with high bovine κ-CN had a significantly smaller micelle size than did control milk. Although there was no effect on the rennet coagulation time, the milk of transgenic lines had stronger curd in gels produced by rennet.

Brophy *et al.* (2003) introduced additional copies of the genes encoding bovine β-CN and κ-CN into female bovine fibroblasts. The transgenic cows secreted elevated levels of β-CN (8–20%) and κ-CN (twofold) and had a considerably modified κ-CN to total casein ratio. β-CN, which is the most abundant milk protein, is involved in binding calcium phosphate and thus controlling milk calcium levels. Higher κ-CN content in milk is linked to smaller micelles, better heat stability, and improved cheese-making properties. In the transgenic animals engineered by Brophy *et al.* (2003), the total milk protein increased by 13–20% and total milk casein by 17–35% compared to nontransgenic control cows. This has obviously a positive influence on the cheese yield and also the casein and milk protein concentrate industry. Edible casein is used in vitamin tablets, instant drinks, and infant formulas, whereas acid casein is used for paper coatings, cosmetics, button making, paints, and textile fabrics (Karatzas, 2003).

There was a measurable variation in the concentration of both β-CN and κ-CN among the eight transgenic genetic clones generated by Brophy *et al.* (2003). In addition to embryonic cell-derived nuclear DNA, the transgenic animals contained oocyte-derived mitochondrial DNA. St. John (2002) postulated that this mitochondrial DNA may also have been derived from donor cell. As several metabolic reactions responsible for lactation are located in the mitochondria, it is possible that differences in the source of the genetic bloodline of the mitochondria in these transgenic animals may account for differences in the physiology of lactation and ultimately milk production.

A2 Milk™ from commercial dairy herds is being marketed in New Zealand and Australia at a small premium over regular or A1 milk. A2 Corporation scientists claim that as A2 Milk™ has only negligible amounts of the A1 β-CN in it, the perceived risks associated with the

consumption of this type of casein (such as autism or Asperger's syndrome, child diabetes, schizophrenia, and coronary heart disease) are effectively removed (Lacefield, 2003). They further maintain that A2 β-CN was the original β-CN gene, whereas subsequent genetic mutation generated A1 β-CN. Hence, all milk produced by cattle thousands of years ago, before the large-scale domestication of cows, was A2 Milk™ (www.a2corporation.com). The animals producing A2 Milk™ are not genetically modified. They have been selected with the help of genetic markers that indicate cows that naturally produce in their milk, the original form of casein protein (A2β) rather than the altered A1 β-form (Goel, 2005).

B. Modifying the minor milk proteins

The first attempts at modification of milk proteins through genetic engineering techniques started with the minor milk proteins. Simons *et al.* (1987) generated transgenic mice carrying the sheep β-LG gene. The β-LG was specifically and plentifully expressed in the mammary gland of mice during lactation, though the protein is not naturally present in rodent milk.

Bleck and Bremel (1994) produced transgenic mice to study the production of bovine α-LA in their milk. Milk of multiple mice from the second, third, and fourth generation from each of the three transgenic lines was analyzed for the presence of bovine α-LA. The protein was present at concentrations up to 1.5 mg/ml of mouse milk.

Bovine α-LA from the milk of transgenic mice was characterized, partially purified, and quantified as 1.0 mg/ml by Jeng *et al.* (1997). The N-terminal amino acid sequence of HPLC-purified bovine α-LA from mouse milk was identical to native bovine α-LA. In addition, the calcium-binding properties of this protein were also similar to the native protein. More details on the modification of minor milk protein fractions are enumerated in other segments (Section VI.A–C) of this chapter.

C. Targeting the proteinase-cleavage sites

Caseins, particularly the β-, α_{s1}-, and α_{s2}-caseins being easily digestible, are quite sensitive to plasmin, a serine protease occurring naturally in milk along with plasminogen. Plasmin activity leads to limited proteolysis in milk. This offers a dual disadvantage of decreasing the curd yield, besides causing bitterness in cheese and inducing organoleptic defects and gelation in ultra high temperature-treated milk. Milk augmented with specific inhibitor of either plasmin or plasminogen activator would therefore be a boon for the process industry (Murthy and Kanawjia, 2002). Bleck *et al.* (1998a) modified β-CN to remove the plasmin-cleavage site. They also report the removal of the chymosin-cleavage site from β-CN, thus positively influencing the cheese yield.

VI. DESIGNER MILK FOR INFANT HEALTH

It is said that breast milk is the ultimate designer food for babies. Nature has designed human milk for optimal nourishment and growth during infancy and also for supplying certain bioprotective factors that afford protection against commonly occurring infections. However, in certain situations such as lactation failure, insufficient milk secretion, and where mothers suffer from transmittable diseases, human milk substitutes serve as precious lifesavers during vulnerable stages of infancy. Then it becomes imperative to have infant formulas, which closely imitate human milk so as to provide comparable nutritional and health benefits. The composition of these formulas could be greatly improved to suit the needs of the infant by incorporating ingredients that resemble those of human milk, thereby ''humanizing'' the bovine milk.

A. Lactoferrin

Lactoferrin (LF) is a single-chain, metal-binding glycoprotein of 77 kDa and is a component of the intrinsic host defense of mammals. It has antibacterial, antifungal, anti-endotoxin, and antiviral activities. It is an iron-binding protein and may also mediate some effects of inflammation and have a role in regulating various components of the immune system. LF in milk might play a role in iron absorption and/or excretion in newborns, as well as in promotion of intestinal cell growth. Its level in human milk is about 1 g/liter and in human colostrums, about 7 g/liter. As the levels of LF in cow milk is only about one-tenth of that in human milk, this has caught the attention of those involved in designing human milk replacement formulas.

Oral feeding of bovine LF (1 mg/ml) led to an increase in the probiotic species bifidobacteria in infant gut (Roberts *et al.*, 1992). Several such infant formulas are marketed in Japan under brand names such as Hagukumi, Chilmil Ayumi, Non-Lact, E-Akachan, GP-P, and New-NA-20-Morinaga. The consumption of such formulas may result in anti-infection, improvement of orogastrointestinal microflora, immunomodulation, anti-inflammation, and antioxidation (Wakabayashi *et al.*, 2006).

Researchers (Nuijens *et al.*, 1997) at the Leiden University (the Netherlands) in collaboration with Pharming, NV (Leiden, the Netherlands) compared recombinant human lactoferrin (rhLF) expressed in the milk of transgenic mice with natural human milk-derived lactoferrin (hLF). They concluded that the unsaturated rhLF and natural hLF had comparable properties, indicating that hLF produced in bovine milk will exert similar, if not identical, antibacterial and anti-inflammatory activities *in vivo*. Pharming also developed the first transgenic bull in the late

1980s and a line of transgenic cows to produce several proteins including hLF (Subramanian, 2004). The company believes that as receptors in the human gut have better affinity to a human protein than a bovine one, the ingredient would be more effective in boosting gut health.

Four lines of transgenic cows that harbor the rhLF were developed (van Berkel *et al.*, 2002). The milk of these animals had 0.4, 0.8, 2, and 3 g/liter of the rhLF in their milk. These levels of expression remained constant throughout the lactation period of 280 days. The milk volume, cell counts, and proximate composition were not altered by the genetic transformation. The recombinant protein was structurally and functionally comparable to natural hLF and had similar iron binding and release and antibacterial activities. The authors further postulate that with such expression levels and an assumed milk yield of 8000 liters of milk per cow annually, one cow can produce about 24-kg rhLF in a year. Thus, a herd of a few hundred animals could produce enormous quantities of this biological protein in a year.

B. Lysozyme

Lysozyme (LZ) is an enzyme that is abundantly present in the mucosal membranes that line the human nasal cavity and tear ducts. It can also be found in high concentration in egg white. LZ destroys bacterial cell walls by hydrolyzing the polysaccharide component of the cell wall. Human milk contains 0.4 g/liter of LZ, an enzyme that contributes to antibacterial activity in human milk.

Active human lysozyme (hLZ) has been produced in the milk of transgenic mice at the concentrations of 0.78 g/liter (Maga *et al.*, 1995). Milk from these transgenic lines had the same antibacterial activity as human milk LZ. The researchers found a zone of clearance in the gel containing the test organism *Micrococcus lysodeikticus* and the recombinant protein indicating that the hLZ in the mouse milk was active. On the processing front, the expression of LZ in milk results in the reduction of rennet clotting time and greater gel strength in the clot. In the transgenic line of mice generated by Maga *et al.* (1995), the milk exhibited a 35% decrease in rennet clotting time, a smaller casein micelle size (157 nm as against 172 nm in the nontransgenic animals) and a 2.5- to 3-fold greater gel strength than control milk. A group of researchers in China also developed two lines of transgenic mice that expressed fully active recombinant hLZ in the mammary gland (Yu *et al.*, 2006). The antibacterial activity of the LZ from the transgenic lines (480.4 and 301.6 U/ml) was 18 and 11 times greater than that of the nontransgenic mice (25.9 U/ml).

Maga *et al.* (2006) designed a line of transgenic goats that expressed hLZ in the mammary gland. On characterizing the milk from five transgenic goats of this line, they found that the hLZ content in the milk was

270 μg/ml or 68% of the level found in human milk. Milk from these transgenic animals had a lower somatic cell count, which may influence udder health positively. It also had a shorter rennet clotting time and increased curd strength (17 min and 26.3 Pa, respectively, in the experimental samples as against 20 min and 20.7 Pa in control samples). The aim now is to produce cows that will produce LZ in their milk. Such LZ-fortified milk has the potential to reduce udder infections in dairy cows and intestinal ailments in humans who drink milk (Bailey, 2001). A double transgenic cow that coexpresses both hLF and hLZ in milk may also reduce the incidence of intramammary infection or mastitis.

Feeding young goats and pigs with this LZ-enriched milk produced by transgenic goats altered their intestinal bacterial profile (www.eurekalert.org, 2006). Pigs were chosen owing to the similarity of their digestive system to that of humans. The choice of goats extended the study to ruminant models. The young pigs fed the LZ-rich milk from transgenic goats had lower levels of coliform bacteria in the small intestine, including fewer *Escherichia coli*, than did the control group. In contrast, the kid goats fed LZ-rich goat's milk had higher levels of coliform bacteria and roughly the same level of *E. coli*, compared to control group. The researchers attributed this variation to the difference in the respective digestive systems and the bacterial profile of the systems. Despite the difference, both animal groups were healthy and exhibited normal growth patterns. The researchers anticipate that these results will pave the way for protection of infants and children against diarrheal illnesses through milk-feeding programs.

C. Cow milk allergy

An allergic reaction to cow milk is a complex disorder involving an abnormal immunological response to one or more of milk's proteins and more than one immunological mechanism. Both casein and whey proteins are reported to be responsible for these allergic responses. Although the reasons for cow milk allergy are not well understood, genetic and environmental factors and their interaction are thought to be responsible (Crittenden and Bennett, 2005; Halken, 2004; Wal, 2002). Comparatively few infants develop cow milk protein allergy. Usually, infants and young children (~2%) suffer from this ailment and outgrow it by the age of five (Host, 2002). It is rare in adults (0.1–0.5%). Cow milk protein allergy can be diagnosed by one or more of cutaneous (e.g., eczema, rashes), gastrointestinal (e.g., nausea, vomiting, diarrhea), or respiratory (e.g., asthma, rhinitis, wheezing) symptoms. Severe symptoms would need special prescription medications such as antihistamines and epinephrine (Nuble, 2006). The only effective management strategy

for cow milk protein allergy is avoidance of cow milk and its products, which in turn negatively influences nutritional management through diet.

Cow milk allergenicity in children is often caused by the presence of β-LG, which is absent in human milk. Although β-LG has been implicated most often in allergic reactions to cow milk, the caseins, α-LA, serum albumin, and immunoglobulins and digests of these proteins are also allergenic in infants and children. Elimination of β-LG by knocking out its gene from cow milk is unlikely to have any detrimental effects on either cow or human formula and might actually overcome many of the major allergy problems associated with cow milk. AgResearch (New Zealand) is field-testing dairy cattle that have been genetically modified to eliminate the β-LG gene (www.agresearch.co.nz, 2001).

Further, as milk protein allergenicity studies demonstrate that all food proteins are potential allergens and that allergenic structures are widely spread throughout the protein molecule, milk is a good model in the search for means of characterizing allergenic structures in food (Wal, 1998). Therefore, while developing strategies for the identification and evaluation of potential allergenicity in novel foods, many of the technological practices used in the assessment of milk protein allergenicity can be adapted.

D. Lactose intolerance

Lactose intolerance is a distinct entity from cow milk protein sensitivity and causes abdominal pain, diarrhea, nausea, flatulence, and/or bloating. While avoidance of milk and other dairy products will bring relief in children suffering from lactose intolerance, it may cause problems in optimal bone mineralization owing to lack of calcium in diet. Several lactose-free and lactose-reduced milks are now available in markets to cater to such infants. The scope of transgenic technology to reduce the lactose content in the milk of small animals has been reviewed elsewhere in this chapter (Section IV.A). The extension of this technique to include farm animals is targeted in the future.

Inability to digest milk is not exclusively due to lactose intolerance. From a study involving African-Americans between the ages 12 and 40 years, Johnson *et al*. (1993) concluded that cause of milk intolerance in as many as one-third of the subjects claiming symptoms after ingestion of a moderate amount of milk was not its lactose content. Many infants, especially those born before term, have low lipase activity. One potential application of transgenic technology could be to produce the human lipase, which is stimulated by bile salt in the milk of bovines. The lipase thus produced could be used as a constituent of infant formulas to increase the digestibility of milk lipids, particularly in premature infants who have low lipase activity (Lonnerdal, 1996).

VII. MILK WITH HUMAN THERAPEUTIC PROTEINS

The preparation of high-value, low-volume therapeutic proteins in the milk of domestic animals through transgenic technology is becoming a reality. Several high-affinity and high-specificity monoclonal antibodies for *in vivo* therapy in human beings using transgenic mice have been reported earlier (Gorman and Clark, 1990; Little *et al.*, 2000; Thomas, 2001; Yang *et al.*, 2001). Antibodies are used for a number of human clinical applications such as treatment of infectious diseases, cancer, transplanted organ rejection, autoimmune diseases and also as antitoxins. Statistics suggest that at least 33 different drugs in clinical testing and in pivotal trials contain variable regions encoded by human sequences from transgenic mice (Lonberg, 2005). Progress in research may make it possible to extend this technology to use transgenic farm animals to directly generate and produce human proteins.

There were several bottlenecks envisaged in realizing this hypothesis a decade earlier. First, the unpredictability of the expression level of the genes of interest associated with milk protein gene control regions was recognized as a challenge. Then, the recombinant proteins secreted in milk are not always in a satisfactory biochemical form. It was also observed that cleavage and glycosylation are not always carried out correctly. The problem of the possible presence of agents pathogenic for humans in proteins extracted from milk was also a major worry (Houdebine, 1995).

Despite these disadvantages, the major benefit of transgenic technology offers a means to produce proteins at a very low cost. Mammalian cell culture systems are often used for expression of recombinant human proteins (rHP), as the latter can only be obtained in a biologically active conformation when produced in such cells. However, this approach has limited production capacity and is expensive. In contrast, the production of rHP in milk of transgenic cattle is a safe and less-expensive alternative with the advantage of better protein output (Brink *et al.*, 2000).

Ebert *et al.* (1991) reported the generation of two transgenic goats that expressed a variant of human tissue plasminogen activator (htPA). The milk from one of these contained enzymatically active longer acting tissue plasminogen activator (LAtPA) at a concentration of 3 μg/ml. Economic comparison of production costs of htPA through bacterial fermentation, mammalian cell culture, and cow transgenic technology estimates the cost per gram of htPA to be 20,000, 10,000, and 10 US$, respectively (Karatzas and Turner, 1997).

Shani *et al.* (1992) tested the feasibility of producing large quantities of human serum albumin, which is used in blood transfusions, in the milk of transgenic livestock by generating transgenic mice as a model system. Charlie and George were two calves created from fetal cells in the US in 1998 to produce the human serum albumin in their milk (Johnston, 2006).

The first report on expression of recombinant human fibrinogen (rHF) to the mammary gland of transgenic mice appeared a decade ago (Prunkard *et al.*, 1996). Fibrinogen is a complex plasma protein composed of two each of three different polypeptide chains. The workers coinjected three expression cassettes, each containing the genomic sequence for one of the three human fibrinogen chains controlled by sheep whey protein β-LG promoter sequences into fertile mouse eggs. Analysis by PAGE revealed that the milk from the highest producing founder animal contained human fibrinogen subunits at concentrations of 2000 μg/ml. Incubation of the transgenic milk with thrombin and factor XIII produced a cross-linked fibrin clot, demonstrating that a major portion of the secreted fibrinogen was functional. Coleman (1996) reported that the concentration of rHF produced in mouse milk was more than 2 g/liter, whereas the amount generated by the cell culture method was only 0.002 g/liter.

Because α-LA has a well-balanced amino acid composition, increasing the amount of α-LA in milk at the expense of β-LG may, besides lowering the risk of cow milk allergies, improve the nutritional quality of the milk. The technique of custom-designing amino acids in a protein to obtain special foods with therapeutic properties offers a ray of hope for patients of phenylketonuria (PKU). This is a congenital disease occurring in those without the enzyme that metabolizes phenylalanine. Although products are now available with no or low phenylalanine content, they are unappetizing. The knowledge that α-LA contains only four phenylalanine residues in its amino acid makeup and their position can be determined easily makes this whey protein a possible target for treatment of PKU (Coleman, 1996). Replacing these four phenylalanine residues with other amino acids by site-directed mutagenesis followed by the subsequent expression of the modified protein in milk and its purification would offer a logical sequence to the preparation of an improved dietary formula for PKU patients.

Transgenic animals can also secrete proteins such as blood clotting factors needed by human hemophilia sufferers in their milk (Suraokar and Bradley, 2000). On these lines, Polly, a genetically altered sheep was created at the Roslin Institute in Scotland to produce milk that contained the protein used to treat human hemophilia (Pettus, 2006).

Wright *et al.* (1991) described the generation of five transgenic sheep (four female and one male) for a fusion of the ovine β-LG gene promoter to the human α-1 antitrypsin (AAT) genomic sequences. AAT is a single-chain glycoprotein secreted by the liver and its main function is the inhibition of the enzyme elastase. The absence of active AAT in the system leads to emphysema (loss of elasticity of the lung tissue) and/or other lung-related ailments such as cystic fibrosis and adult respiratory distress syndrome. Milk of three of the ewes generated by Wright *et al.* (1991)

expressed human AAT (hAAT) at levels greater than 1 g/liter. In one case, initial levels of hAAT exceeded 60 g/liter and stabilized at ~35 g/liter as lactation progressed. hAAT purified from the milk of these animals was fully *N*-glycosylated and had a biological activity indistinguishable from human plasma-derived material. These animals and their progeny exhibited stable transmission of the transgene. The founder animals yielded very similar levels of hAAT protein in milk continuously over several lactations. A flock of seven first generation ewes (derived from a founder male) yielded comparable levels of hAAT protein in first and second lactation milk. Two second generation ewes of this line also produced equivalent quantities of the human protein (Carver *et al.*, 1993). Several animals from this group are reported to secrete milk containing 35–47 g/liter without affecting the production of other proteins in milk (Coleman, 1996).

With successful advances in research, several other recombinant proteins of pharmaceutical interest have been developed from the milk of transgenic animals. In this context, some human proteins have already been expressed with success. Products such as insulin and growth hormone have also been obtained from the milk of transgenic cows, sheep, or goats (Margawati, 2003).

GTC Biotherapeutics (Framingham, MA) uses both goats and cows to produce more than 60 therapeutic proteins, including plasma proteins, monoclonal antibodies, and vaccines. A recombinant human antithrombin III—an anticoagulant protein found in blood—which was produced in goat milk and was in the last stage of testing a couple of years ago (Subramanian, 2004) is now almost ready to be marketed. The company claims that the antithrombin (ATryn®) has been recommended for market authorization for the prophylaxis of venous thromboembolism in surgery of patients with congenital antithrombin deficiency (www.transgenics.com/news.html). Besides being the first antithrombin product approved by the European Medicines Agency for use in all 25 countries of the European Union, ATryn® will also be the only available antithrombin product that is produced by recombinant biotechnology and is not derived from the human blood supply. This drug is envisaged to be a boon for those suffering from a deficiency in antithrombin, a condition which becomes dangerous during surgery or childbirth, when they cannot take conventional blood-thinning pills.

The Scientific American (www.sciam.com, 2001) reported a study at the National Institute of Allergy and Infectious Disease (United States) in which two mouse strains were genetically engineered to produce large quantities of a malarial parasite surface protein from *Plasmodium falciparum*. The malaria vaccine secreted in their milk was able to contain the disease in monkeys vaccinated with the same. This study has now been extrapolated to target livestock as the source animals. GTC is currently

working on a project to develop the malaria vaccine from goat milk. It is understood that a liter of goat milk can contain up to 9 g of the transgenic protein and that eight goats can produce enough vaccine to inoculate 20 million people. The cost to produce a transgenic protein in goat milk can thus be 3–30 times cheaper than the current method using mammalian cell culture. In addition to these, GTC is also working on the development of a recombinant hAAT, a recombinant human albumin, and a CD137 antibody to stimulate the immune system as a potential treatment for solid tumors.

PPL Therapeutics (Edinburgh, United Kingdom, and Blacksburg, VA) is working with rabbits and sheep to produce AAT, fibrinogen, and a lipase to treat pancreatic insufficiency in digesting dietary lipids. They are also attempting to engineer sheep to produce in milk a protein that reduces lung damage, thus providing hope to cystic fibrosis sufferers (Morgan, 2006).

The Environment Risk Management Authority (ERMA) of New Zealand has admitted an application from AgResearch, New Zealand, for field testing of some cattle which are genetically modified with copies of genes or nucleic acids derived from humans or cattle (www.ermanz.govt.nz, 2001). The first of these involves inserting additional copies of two cattle milk casein genes to increase the protein content of milk (www.agresearch.co.nz, 2001). The second one is to disrupt the β-LG in order to decrease causes of milk allergies. The field tests also involve the insertion of a copy of the human myelin basic protein (MBP) gene in cattle. The protein, when secreted in their milk, may be purified and tested for its efficacy in the treatment of multiple sclerosis. Multiple sclerosis is a chronic demyelinating disease of the human central nervous system connected with clinical neurological signs of paralysis and histopathological changes. It is expected that secretion of MBP in cattle milk will allow the generation of large amounts of human MBP and will ultimately facilitate as a drug for the treatment of multiple sclerosis.

Scientists at AgResearch have also patented a technology to produce bovine milk with enhanced quantities of immunoglobulin (IgA) antibodies (AgResearch Now, 2005). IgA is the dominant immunoglobulin in human milk and provides infants with essential protection against pathogens. IgA also contributes to adult health by helping to protect human mucosal surfaces like the stomach, intestinal tract, lungs, nose, ears, and eyes. One application of the technology being pursued is the prevention of human fungal infections including thrush, caused mostly by *Candida albicans.* These antibodies can also be tailored to protect against specific gut or oral diseases.

In this context, the milk is undergoing trials at Otago's school of dentistry to assess its potential for protecting teeth against decay-causing bacteria (New Zealand Herald, 2006). It is reported that about 10,000 cows

immunized to protect against fungal infections would be sufficient to provide a protein powder for a mouthwash or lozenge that would form a barrier on the teeth, tongue, and cells lining the mouth and hence repel oral thrush.

Pharming, NV (Leiden, the Netherlands) has obtained a US Patent for the production and composition of pharmaceuticals containing human α-glucosidase (Biotech Patent News, 2000). They have developed transgenic cattle capable of producing this enzyme in their milk. The company claims that this can help in treating Pompe's disease, a hereditary, lethal muscle disorder that annually affects almost 5000–10,000 people living in the West. Genzyme Transgenics (Framingham, MA) have also succeeded in producing human α-glucosidase from transgenic animals (Dove, 2000).

The Pharming Group maintains a transgenic dairy herd in the Dane County town of Vienna and claims that over the next decade their 13-strength herd will procreate 200 or more Holstein and Brown Swiss cows. The cattle will produce milk from which medicinal proteins can be turned into drugs that fight human illnesses such as hemophilia, hereditary angiodema, and gastrointestinal infections besides Pompe's disease (Millard, 2000).

Nexia, a US company, has engineered a herd of goats with milk containing an antidote for the nerve agents sarin and VX (Morgan, 2006). Experiments are under way at Hematech, South Dakota to create a transgenic cow (Transchromic, Tc cow) that will have an immune system that is half human and half bovine. Its cells will contain an additional artificial chromosome, with the genes for human antibodies (Morgan, 2006). The firm claims that when the Tc cow is immunized with an infectious agent, such as inactivated botulinum toxin, it will produce human polyclonal antibodies. These can then be purified from the cow's blood and given to patients who cannot fight these infections owing to defects in their own immune systems. Hematech has already created Tc calves, which carry the human chromosome. The remaining work is to knock out the equivalent bovine antibody genes, so the Tc cow produces purely human antibodies in its blood. The firm anticipates that the technology will be more efficient and less expensive than present methods for generating human antibodies—cell culture—which can make only monoclonal (single variant) antibodies.

VIII. DESIGNER MILK FOR ANIMAL GROWTH AND HEALTH

Vaccines, antibiotics, and the natural immune system of the animal have been used to cure diseases in the cow till recently. The use of gene transfer technologies to produce dairy cows that resist several infections and diseases is a novel development in biotechnology.

The volume of water drawn into milk by osmotic forces is related to lactose synthesis and therefore, α-LA is a deciding factor in the ultimate milk volume. Noble *et al*. (2002) used transgenic gilts expressing bovine α-LA in their milk to study whether the presence of the transgene influences lactation. They reported that lactose concentrations and milk production increased in the experimental group and that the piglets reared on the transgenic animals exhibited enhanced growth rates.

In the area of pig husbandry, the trend of reduced lactation lengths increases the number of pigs born per sow annually, but also creates the need for sows that produce more milk in early lactation, to obtain maximal pig growth during the short lactation period. This becomes difficult in pigs as the maximum milk production does not occur until between the 21st and 28th days of lactation. Also, the higher number of pigs born per litter increases the demand for milk production. With a view to provide better energy intake through higher lactose levels in sow milk, Bleck *et al.* (1998b) attempted overexpression of bovine α-LA in porcine milk. The two lines of transgenic pigs so produced had 3.8% lactose in their milk as compared to 2.6% in the milk of control animals.

The tracheal mucosa of the cow is the source of tracheal antimicrobial peptide (TAP), a member of the *β*-defensin family of antibiotic peptides. TAP protects the upper airway of bovines from infection. The limited availability of bovine TAP (bTAP) prompted researchers to create transgenic mice expressing bTAP (Yarus *et al.*, 1996) in their milk. They purified bTAP from milk by acid precipitation, reverse-phase HPLC, and ion-exchange chromatography. This milk-derived bTAP had antimicrobial activity against *E. coli*. The work may herald the expression of the bTAP in bovine milk and its evaluation or use as an antibiotic in agriculture and medicine.

Mastitis, an inflammatory reaction of the mammary gland, usually resulting from a microbial infection, is a widespread disease seen in cattle throughout the world. The major bacterial species that are responsible for bovine mastitis are *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *E. coli*. Of these, the first three cause a contagious route of transmission, whereas *Streptococcus uberis* and *E. coli* are considered to be environmental agents. While stringent disease control plans have eradicated *Streptococcus dysgalactiae* and *Streptococcus agalactiae* from many herds (Kerr and Wellnitz, 2003), controlling *S. aureus* has been difficult.

Transgenic technology to control mastitis would involve the production of antibacterial enzymes by the mammary epithelial cells. These being degraded along with other milk proteins would not pose a health risk to the consumer like regular antibiotics. Initial studies on the application of transgenic technology involved the generation of mice that produced milk containing hLZ (Maga *et al.*, 1994) and human lactoferrin

(Platenburg *et al.*, 1994). While the former has limited effect on mastitis-causing organisms, no conclusive study on the efficacy of the latter is reported.

Lysostaphin is a potent peptidoglycan hydrolase naturally secreted by *S. simulans*. This antimicrobial protein has a potent antistaphylococcal activity and its secretion into milk offers considerable resistance to infection caused by *S. aureus*. Kerr *et al.* (2001) developed three lines of transgenic mice that produced varying levels of lysostaphin (100 μg/ml in two and 1 mg/ml in the third) in their milk. Transgenic as well as control mice were challenged with intramammary infusion of a strain of *S. aureus*. None of these glands from the transgenic animals were visibly infected. These glands contained less than 10% of the bacterial load observed in the heavily infected controls.

Wall *et al.* (2005) engineered and introduced into Jersey cows, a transgene that includes the genetic code for producing lysostaphin. The gene for secreting lysostaphin was introduced from a nonpathogenic species of *Staphylococcus* that uses the protein to deter the pathogenic counterpart, *S. aureus*. The lysostaphin was secreted into milk of the modified animals to the order of 0.9–14 mg/ml. The milk destroyed the causative organisms *in vitro*. Ten nontransgenic cows that were given intramammary infusions of *S. aureus* showed positive signs of mastitis infection, whereas three transgenic cows under the same treatment remained unaffected. A small dose of 3 mg/ml of lysostaphin in milk was sufficient to provide protection against *S. aureus*. Although milk containing other natural antimicrobial proteins such as lactoferrin and LZ has been approved for human consumption, milk containing lysostaphin would need permission from regulatory agencies (Bliss, 2005).

Coliforms account for about 40–50% of mastitis cases in the United States. One-tenth of the affected animals become useless for milk production and several die from shock induced by the bacterial toxin, or endotoxin, causing an estimated loss of $1.4 billion annually for farmers in terms of medical expenses and cost of milk that cannot be sold (McBride, 2002). Vaccines, while having limited success in reducing clinical symptoms, do not remove the organisms. Wang *et al.* (2002) report the identification and characterization of the gene for soluble CD14, which binds and neutralizes endotoxins responsible for mastitis. This soluble protein which can be found set in the membranes of white blood cells in cows was also discovered in cow milk. Just as the protein increases during coliform infections in humans and laboratory animals, it was shown to tone down the severe reaction of the bovines to coliform endotoxin as well as initiate a suitable response to the infiltrating bacteria. The gene was cloned and the recombinant bovine CD14 protein (rb-CD14) produced was evaluated. Intraperitoneal injection of rb-CD14 together with endotoxin reduced fatality in mice. Preliminary trials showed that intramammary injection

of soluble rb-CD14 is 100% effective in preventing mastitis by *E. coli* in lactating dairy cows. The workers have filed a patent application on rb-CD14 which promises both effective treatment for infected cows and prevention in future cows genetically engineered with the gene. They claim that the gene for CD14 can be designed and inserted into the modified animals so that they can produce the protein only in their mammary cells.

IX. ASSORTED ADVANTAGES

The use of molecular biology to reduce the presence of pathogenic organisms in milk is a potentially advantageous prospect and has been reviewed in the previous section (Section VIII). It is clear that it might now be possible to produce specific antibodies in the mammary gland that are capable of preventing mastitis infection or those that aid in preventing human diseases. Thus, one can foresee antibodies against *Salmonella*, *Listeria*, or other pathogens that will produce safer milk products. Active recombinant immunoglobulin capable of neutralizing *Coronavirus* was produced in mouse milk (Castilla *et al*., 1998). Increasing the concentration of IgA receptors in mammary cell may potentially lead to the accumulation of the protective antibodies in milk (De Groot *et al*., 1999). While recombinant immunoglobulins have been expressed in mammalian transgenic milk (Gavilondo *et al*., 2000), a calf with a gene that promotes the growth of red cells in humans has been produced by transgenesis (www.publicscan.fi). Research is also under way to manufacture milk through transgenesis for treatment of diseases such as PKU, hereditary emphysema, and cystic fibrosis (Margawati, 2003).

In an interesting application of transgenic technology combining sericulture and dairying, goats that produce spider silk in milk have been engineered (Dove, 2000). Spider silk is made of protein-based polymer filaments or threads secreted by specialized epithelial cells. These fibers are flexible and lightweight and have extraordinary strength and toughness comparable to those of synthetic high-performance fibers. Spider silk is, thus, one of the strongest and most versatile naturally occurring materials in nature (Samson, 2004). Although it appears delicate and is one-tenth the width of a human hair, it is stronger than steel and stretches more than nylon (www.sciam.com, 2002). Several attempts have been made to synthesize spider silk for industrial and medical applications. Unfortunately, the high shearing forces in conventional fermentations cause the spidroin protein to aggregate, making it useless for manufacturing fibers. So, these spider genes were introduced into the cells of lactating goats and they secreted silk in tiny strands along with their milk. These polymer strands could be woven into threads after extracting them

from the milk and used for applications such as military uniforms, medical microsutures, and tennis racket strings (Anonymous, 2002). Nexia, a US company, has developed a strain of fast-maturing Breed Early Lactate Early (BELE) transgenic goats that secretes the spider silk in milk (Dove, 2000).

Nexia is already working with the US army to develop bulletproof vests and surgical suture material from spider silk. The team produced soluble recombinant spider silk proteins with molecular masses of 60–140 kDa (Lazaris *et al.*, 2002). They were able to wet spin the silk monofilaments derived from a concentrated aqueous solution of soluble recombinant spider silk protein under conditions of low shear and coagulation. The spun fibers were water-insoluble with diameters ranging from 10 to 40 μm and exhibited toughness and modulus values comparable to those of native dragline silks but had lower tenacity. They anticipate that the manufacturing processes for these products would be more environmental friendly than the production of conventional plastics.

X. THE FUTURE

Novel research in genetic engineering attempts to alter and control the genetic makeup of animals in several ways. Some of these attempts may target only individual animals and not generations together. One example is that of somatic cell therapy, wherein specific cells of an individual animal are modified to produce desired characteristics without changing its genetic characters. On the other hand, transgenesis involves the modification of the genetic line wherein the altered traits can be inherited by the progeny. Both techniques are useful in the enhancement of animal productivity, faster growth, improved feed conversion, better quality of animal products, and improved resistance to diseases.

However, the technology presents several hurdles to its ultimate development. The most daunting challenge in producing transgenic animals is producing healthy adults. Cattle, for example, often suffer at birth from immature respiratory systems. They are also frequently born late, leading to problems with delivery. The survival rate in cloning attempts also is low. Generally only 1–5% of embryos typically survive to term (Kling, 2001). Dolly, the sheep and the first animal to be cloned from an adult cell in 1996, was the lone success out of the 277 attempts (Taylor, 2006).

Successful transgenic studies have generated mice that produce milk with 33% more total solids (40–50% TS) and 17% less lactose than normal mouse milk. As the increase in the total solids is associated with a decrease in total milk volume, the net result is the same quantity of fat and protein being produced in a lesser total milk volume. If this technology could be extrapolated in dairy animals, milk that contains

6.5% protein, 7% fat, 2.5% lactose, and 50% less water is not an improbable accomplishment. The advantages in terms of animal health would include less stress on the cow and on her udder since she would be producing one-half her normal volume of milk and decrease in mastitis owing to less lactose availability for the causative organisms. The processing industry would gain in terms of (1) skim milk with twice protein content and half the lactose content of normal milk, (2) easier to produce low lactose or lactose-free dairy products, (3) better product yields due to concentration, (4) reduction in total whey output because of low milk volume and lactose content, and (5) direct economic benefits in terms of 50% reduction in the cost of milk transportation owing to reduced volumes.

The future of biotechnologically derived foods is, however, uncertain even after three decades of positive results. Improved and sophisticated equipment for milk processing may be more acceptable to consumers than genetic and transgenic technology for altering milk composition. Consumer acceptance will always contribute to and guide decisions about biotechnological manipulations aimed at increasing milk production or altering milk composition. Various ethical, legal, and social aspects of biotechnological research would need to be addressed in the current economic and social climate before designer transgenic herds thrive just as their counterparts in organic herds do. Animal welfare, demonstrable and sustained safety of the product, improved health properties of the product, and enhanced profitability as compared with conventional practices would be the key factors that would eventually decide the future of designer foods.

The natural human tendency to resist change, especially those that trouble their feeling and instincts, is an important point to consider. As all consequences of biological research involving animal studies could be classified under this category, there is bound to be tremendous resistance to topics such as transgenic technology. The perception among various groups of human beings is also different. While farmers interpret animal welfare in terms of health and production, consumers interpret it in terms of freedom to move and fulfill natural desires. That a human being controls these natural desires of animals itself is a reason for dissent.

The topics of genetic engineering and transgenic technology have been under debate ever since the idea was first conceived. In this context, animal bio- and moral ethics are new keywords that have found entry into the dictionary of animal agriculture. The activists argue that the transformation of animals according to human needs smudges the clear demarcation between man and animal in ethical, moral, and biological perspectives. Thus, the moral principles, which were behind the treatment of animals in the past, are no more valid or adequate. Researchers while considering human requirements also need to find new methods of dealing with animals with their interests, suffering, and welfare in mind.

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