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Milk: Microbes' Heaven Making Human's Paradise

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ABSTRACT

Milk is one of the most complete nutrient sources evolved in nature to enable the successful development of mammalian offspring. Given its particular richness, it is not surprising the fact that, almost as ancient as the origin of milk, so is its colonization by microorganisms. Different types of bacteria, yeast, and moulds, have adapted their lifestyles to favor their continued survival on this ecological niche. Success of such a diverse community largely depends upon the establishment of tight symbiotic relationships. Milk microorganisms have learned to survive on milk by relying on each other's metabolisms. Certain microbes have the ability to metabolize the relatively complex milk carbon source lactose, releasing simpler carbon compounds as byproducts that can then be assimilated by other species. Other microorganisms have, instead, strong lipolytic and proteolytic activities that help the rest of the community to assimilate the fat and proteins present on milk. But no relationship is idyllic, for the members of this community have also developed defensive mechanisms that strictly select their neighbors while keeping potential enemies at bay. As a result of this complex microbial interaction, important changes occur in the structure and organoleptic properties of milk. And there is yet, a third organism, which importantly contributes to the maintenance of an equilibrium within this particular ecosystem and this is precisely our own kind, which for thousands of years, and long before noticing the existence of these microscopic world, has learned to take further nutritional advantage of milk by favoring its microbial communities to thrive on it while generating what we most commonly know as dairy products. This review reexamines the historical origin and types of dairy products derived from the interaction of different groups of microbes able to survive on milk, with a particular focus on the milk yeasts populations and the main physiological characteristics that have allowed their exceptional adaptation to life in a nutritious but rather complex medium. An ultimate emphasis is also placed in the latest scientific advances that are allowing further improvements in many desirable characteristics of milk and dairy products such as their beneficial effect on health, by means of technologies involving the metabolic engineering of dairy microbes.

Keywords: cheese, dairy products, galactose, kefir, lactose, lactic acid bacteria, metabolic engineering, whey, yeasts, yogurt
Abbreviations: BOD, biological oxygen demand; **GOS**, galactooligosaccharides; **GRAS**, generally regarded as safe; **LAB**, lactic acid bacteria; **Lac$^+$**, lactose-positive; **UHT**, ultra-high temperature

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INTRODUCTION

Mammals stand out in the animal kingdom because of the development of a unique organ feature: mammary glands. These organs are designed so that pregnant/recent mother females have the ability to secrete a fluid containing essential nutrients and components of the mothers' immune system. The latter range from blood proteins, such as enzymes

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Invited Review

Table 1 Composition of milk in humans compared to the most common milk-producer ruminants[1].

	Protein (%)[2]	Fat (%)	Carbohydrate (%) (Lactose)	Energy (Kcal)
Human	1.1	4.2	7.0	72
Cow	3.2-4.0	3.5-5.0	4.6-4.9	66
Goat	2.9-3.1	3.5-3.8	4.4-4.7	60-67
Sheep	5.4	6.0	5.1	95
Water buffalo	4.1-4.5	8.0-9.0	4.8-4.9	110-118

[1] The data shown in this table are taken from Webb *et al*. 1974; and from the web site: http://www.reference.com/browse/wiki/Milk
[2] % values are expressed as grams (or kilocalories, kcal) per 100 grams of total fresh milk.

and antibodies, to whole cells, such as leukocytes. Both components, nutrients and immune factors, will help the newborn to survive and develop a primary barrier of defense against pathogens before further development allows the digestion of alternative foods. In late pregnancy and the few days after giving birth, the first fluid that is produced is called colostrum (Thapa 2005; Cox 2006). This fluid delivers nutrients in a concentrated low-volume form to help the newborn's digestive system to adjust to oral feedings. Later on, colostrum is replaced for what is most commonly known as milk.

Milk is a slightly acidic fluid (pH between 6.5 and 6.7) composed, in relative percentages depending on the mammal being considered, of: protein (from 1 up to 10% g/v), fat (from 0.6 up to 49%), and carbohydrates (from 0.1 to 7%) (**Table 1**). Apart from water-soluble proteins, the soluble part of milk contains additional nutrient elements: non-protein nitrogen sources, lactic and citric acid, salts and minerals (phosphorus, calcium, potassium sodium, iron, copper, zinc and manganese), vitamin and pro-vitamin components such as vitamins A and C, thiamin, pyridoxin, riboflavin, calcium panthotenate, biotin, and cobalamin.

In spite of being a nutritious food, each mammal has adapted the relative composition of milk to the specific needs of their own kind. For instance, the highest levels of protein and fat and lowest levels of carbohydrates are mostly observed in species adapted to cold (e.g. marine spe-

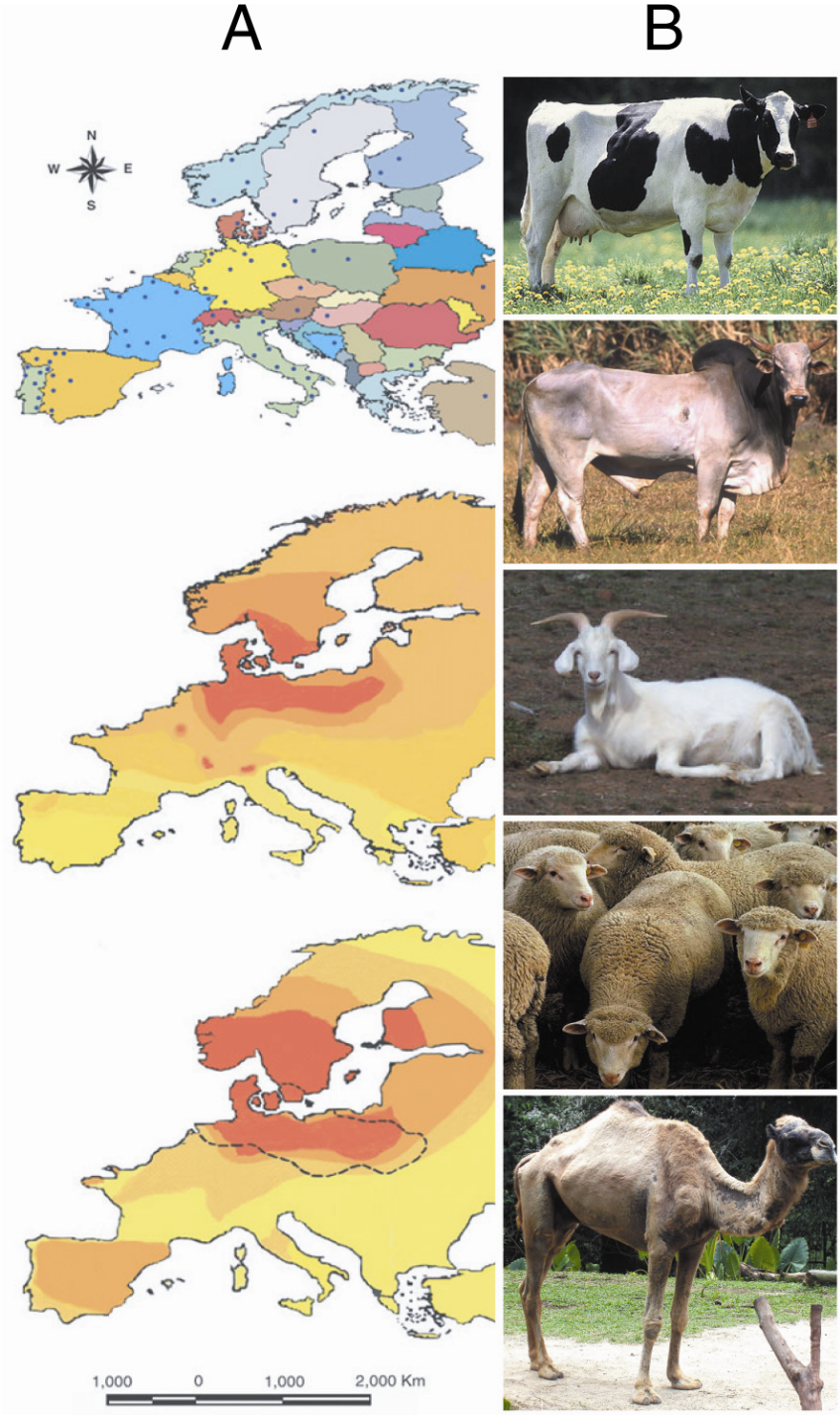

Fig. 1 Neolithic cattle farming regions in North Central Europe and main ruminant species used for milk production. (**A**) Top; geographic distribution of the 70 cattle breeds (dark dots) sampled across Europe and Turkey; middle: in darker color, regions in which the greatest milk gene uniqueness and allelic diversity in cattle has been found; bottom: in darker color, geographic distribution of the lactase persistence allele in contemporary Europeans. Reprinted from Beja-Pereira *et al*. (2003) *Nature Genetics* **35**, 311-313, ©2003 with permission from Macmillan Publishers Ltd. (**B**) Ruminants for milk production (images released into the public domain by Wikipedia, the free encyclopedia http://en.wikipedia.org). From top to bottom: European milk-cow, *Bos Taurus* (image by Keith Weller, from USDA Agricultural Research Service Image Gallery); zebu, *Bos Taurus indicus* (image by Scott Bauer, from USDA Agricultural Re-search Service Image Gallery); male goat, *Capra ae-gagrus hircus* (released by Wikipedia under GNU Free Documentation License); sheep, *Ovis aries* (file from Wikipedia Commons, USDA Agricultural Re-search Service Image Gallery); camel, *Camelus dromedaries* (file from Wikipedia Commons).

cies), which are in inverse correlation to the milk of humans and most animals domesticated for the production of milk (milk; http://en.wikipedia.org/). Different livestock also have differences in their relative levels of protein, sugar, and fat, not only among each other but also when compared to humans (**Table 1**). By the middle of the 20th century, analysis of the contents of animal and human milk revealed that the milk of animal origin does not contain enough iron and unsaturated fats, and that instead contains higher levels of proteins that may trigger allergies in human infants (Wal 2002). Ever since, specific formula with content-levels closer to those of human breast milk has been developed to replace milk in the feeding of humans younger than one year.

As I will discuss in the next sections, lactose-intolerance is another important issue derived from milk consumption. Lactose is the main carbohydrate present on milk and is a disaccharide composed of glucose and galactose (*O*-β-D-galactopyranosyl-(1-4)-β-D-glucose). It is a relatively rare sugar in nature, whose assimilation has required the development of particular enzymes known as β-galactosidases (EC 3.2.1.23). These enzymes break the sugar into the more readily assimilating monosaccharide components, glucose and galactose. Once most mammals are weaned they stop being exposed to lactose, phenomenon that usually leads to a reduction in the levels of expression of this particular enzyme. In fact, lactose consumption in adulthood is almost exclusive of humans and lactose-tolerant adults are a distinct minority on the planet, mostly descendents from people of certain regions of Europe that, at some point in evolution, underwent a genetic change that allowed them to produce lactose throughout life. This ability co-evolved with the heavy reliance of these populations on milk and dairy products as staple of their diets (**Fig. 1A**; Beja-Pereira *et al*. 2003; Hollox 2005; Gibbons *et al*. 2006). For the remaining population, lactose-intolerance results from lactose reaching the intestines intact, where it is used by bacteria, leading to bloating and diarrhea. Paradoxically, some of the most effective solutions for this common problem rely in the consumption of milk in the form of dairy products containing populations of a different type of lactose-consuming microorganisms – probiotics –, which are not only able to produce enzymes that hydrolyze lactose but have also the beneficial property to reach the intestine when consumed, overriding growth of malignant flora within the gut. In the early 1900's, Metchnikov was the first to propose that lactic acid bacteria (LAB) in fermented milk help to eliminate toxic microbes in our digestive system that would, otherwise, contribute to shorten our lives (Metchnikoff 2004). A particular type of LAB, Bifidobacteria, was discovered that is first passed to the newborn's gut through the consumption of breast milk, and that colonizes the intestinal walls, helping digestion, producing beneficial by-products, and providing a first-aid for the immune system in the fight against pathogens (Leahy *et al*. 2005). After weaning, Bifidobacteria are usually replaced by other, sometimes less friendly species of bacteria, such as Streptococci, Staphylococci, *Escherichia coli*, and yeasts. Luckily for us, some of the bacteria and yeasts present in dairy products seem to also be able to surpass the digestive barriers and produce similar beneficial effects as those of Bifidobacteria (Borriello *et al*. 2003; Kumura *et al*. 2004). They help to shield our intestinal walls against pathogens and parasites, boosting the body's immune system, helping to reduce cholesterol levels, and the generation of carcinogens derived from the catabolism of ingested food. In this review we will revisit the main components of the microbial ecosystems present in the most popular dairy products, the methods that have helped to taxonomically classify them, and the particular physiologies that help them to survive in this particular ecological niche as part of a complex community of microorganisms.

Lactose is not only problematic from the consumer's point of view but also because it is the most energetic component present in milk. In fact, lactose contributes as much as a 40% to the total whole cow milk calories content (milk; http://en.wikipedia.org/). The presence of this sugar, soluble in high levels in the liquid fraction of milk, thus, poses another major problem because of its solubility in the whey-fraction discarded by cheese-manufacturing industries. This industrial by-product has, because of its lactose contents, an elevated biological oxygen demand (BOD: a way to measure the rate of oxygen uptake by microorganisms in a liquid sample that is used to infer the concentration of biodegradable matter present on it) and is considered as a major cause of environmental pollution. As we will also see below, growth of lactose-assimilating microorganisms on cheese-whey is providing industries with interesting alternatives to tackle this problem.

INTERESTING FACTS ABOUT MILK AND THE ORIGIN AND TYPES OF DAIRY PRODUCTS

Structure of milk

In terms of physicochemical structure, milk is basically an emulsion of fat globules within a water-based fluid (see **Fig. 2A-C**, and McGee 2004). The major lipid components of milk are tri-, di-, and mono-glycerides, cholesterol, phospholipids, free saturated or polyunsaturated fatty acids, cerebrosides, and gangliosides (Christie 1995). The fat portion of the milk is very important since it also contains the fat-soluble vitamins A, D, E, and K. Milk fat is stored within fat globules, which are basically fat droplets protected by surrounding membranes composed of phospholipids and proteins that act as emulsifiers preventing clustering of the fat within, and protecting it from fat-digesting enzymatic activities (lipases) present in the fluid portion of the milk. Milk fat globules are synthesized as vesicles derived from the secretory pathway of the mammary gland cells (Jensen *et al*. 1991; Mather and Keenan 1998; Mather 2000; Keenan 2001; Heid and Keenan 2005). More than half of the enzymes present in the fat globule membrane are, predominantly, glycoproteins acting as hydrolases, oxidoreductases, or transferases (Kennan and Dylewski 1994).

The fluid portion of the milk contains two main classes of proteins: proteins forming part of micelles (curd proteins) and proteins that are fluid-soluble (whey proteins), (Swaisgood 1993; Wong *et al*. 1996; Robinson 2002). Protein micelles are, basically, aggregates of several thousand protein-molecules bonded by calcium phosphate (hence the rich contents of calcium present in milk). These roughly spherical micelles have, in average, a diameter of about 150 nm (approximately 1/50 the size of a fat globule) and constitute around 1/10 of the total milk volume (**Fig. 2D**). Around 80% of the protein in milk is present in the micelles and belongs to four different groups of caseins (Ginger and Grigor 1999). Caseins are all phosphorylated proteins with their phosphate group sterified to serine residues in the protein. These phosphate groups bind large amounts of calcium, being responsible for the formation of micelles. One special type of casein, κ-casein, caps the micelles and has a negatively charged tail that controls the size and aggregation of micelles because of repulsion between identical charges (Creamer *et al*. 1998). Once ingested, casein is hydrolyzed by digestive enzymes into smaller peptides that are thought to have beneficial hormone-like effects in metabolism, e.g. by reducing heart rate, triggering levels of insulin in blood, and stimulating white blood cells (Meisel and Bockelmann 1999; Kilara and Panyam 2003).

The remaining 20% of milk proteins are water-soluble and remain in the liquid portion of milk obtained after the coagulation of caseins into curds. The whey proteins also have important roles not only as amino acid suppliers but also as defensive proteins, molecules transporting other nutrients (e.g. trace elements), and as enzymes. The most abundant proteins in cow's whey fraction are β-lactoglobulin (~50%), followed by bovine serum albumin, α-lactalbumin, and immunoglobulins (basically, antibodies), (Robinson, 2002). β-Lactoglobulin belongs to the family of lipocalins,

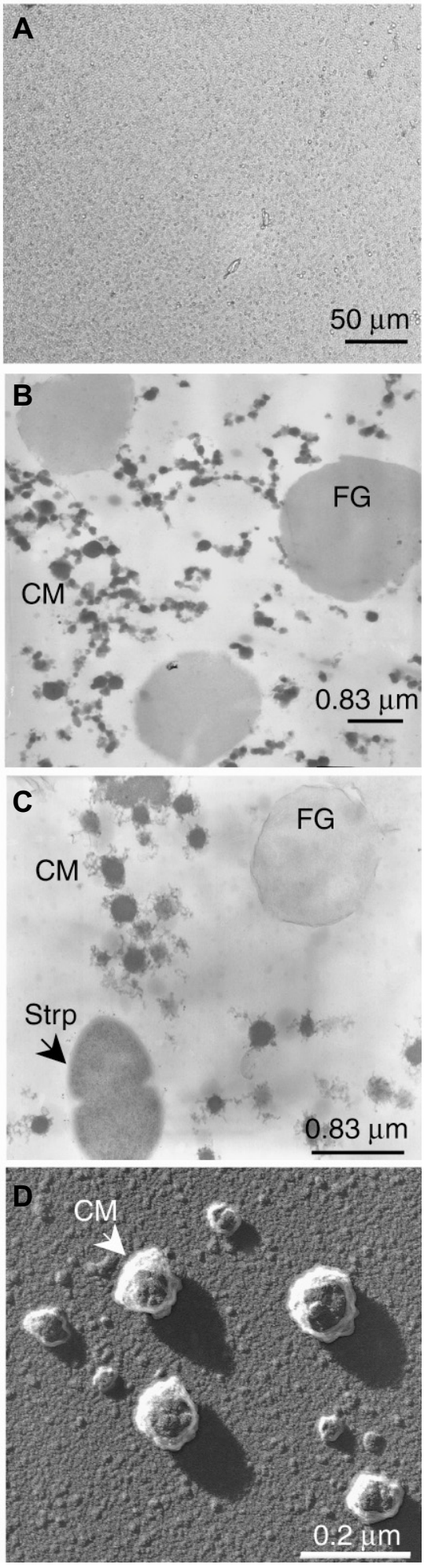

Fig. 2 Molecular structure of milk. (**A**) Optic microscopy image of homogenized milk, at an original magnification of 10X; average diameter of fat globules is 0.4-2 µm (by courtesy of Dr. Christian H. W. Holtze); (**B**) Transmission electron microscopy (TEM) micrograph showing fat globules (FG) and surrounding casein micelles (CM) in liquid kefir, at an original magnification of 13,500X; (**C**) TEM micrograph with Streptococci (Strp), fat globules (FG) and casein micelles (CM) in liquid yogurt, at an original magnification of 24,000X. (Both **B**) and **C**) were provided by courtesy of Diane Montpetit, Agriculture and Agri-Food Canada.); (**D**) TEM of casein micelles undirectionally shadowed with Pt and **C**, courtesy of Miloslav Kalab (Agriculture and Agri-Food Canada).

characterized by binding small hydrophobic ligands, which indicates their potential role as ligand-transporters (Kontopidis *et al*. 2003). Sequence comparisons reveal its close resemblance to glycodelin, a protein found in the human endometrium during early pregnancy. Both β-lactoglobulin and glycodelin seem to have effects in the immune system. β-Lactoglobulin is also considered, like the rest of whey proteins, an important source of amino acids for the offspring of animals that produce it.

The fat globules tend to cluster and, because of their particular interaction with water-soluble proteins and their lower density than water, fresh standing raw milk has a natural tendency to separate into a high-fat cream layer on top of a larger, low-fat milk layer. To prevent this separation, milk is subjected to homogenization, a process that consists in pumping hot milk through very small nozzles at high pressure in order to reduce the size of the fat globules. The smaller fat globules occupy a larger surface area, which favors the interaction of casein particles with their surfaces. The protein coating formed over the smaller globules counteracts their initially low density and interfere with their aggregation, allowing the fat to remain evenly dispersed throughout the milk. This phenomenon is different from that caused by prolonged heating of raw milk. The fat globules are usually quite resistant to heating, but continuous heating denature milk proteins, which in this case associate to the fat globules in a way that promotes clustering and their lifting onto the top layer as cream. In fact, most whey proteins contain disulfide bonds that, upon heating, undergo denaturation and generation of intermolecular bonds, influencing positively to the process of separation of cream. Fat globules are quite sensitive to the opposite process: freezing. The formation of crystals pierces them, so that in thawed milk small butter grains are released that can lead to oil puddles when the milk is later heated (McGee 2004).

What do the separated components of milk, 'curd' and 'whey', in fact, mean? Curd is the product obtained by 'curdling' (coagulating) milk proteins and then draining off the liquid portion known as whey. Curdling is naturally induced by the action of *rennet*, a natural complex of enzymes produced in young mammalian stomachs to digest the mothers' milk, mostly composed of proteases like rennin or chymosin (EC 3.4.23.4), pepsins, lipases, and other enzymes (Garg and Johri 1994). These enzymes are initially synthesized in an inactive perform that is activated by the stomach's acids. The crude *rennet* extract, therefore, needs the addition of acids, in order to become active. Acidification of milk for curdling can be achieved through the use of edible acids like lemon juice or vinegar. In several cases, LAB are added to milk precisely for this purpose. These bacteria produce lactic acid, which decreases pH. In an acidic environment, casein micelles fall a part and the proteins start interacting in much smaller aggregates, forming a continuous meshwork that traps the whey and fat globules in small pockets, turning the fluid milk into a fragile solid. In the case of using *rennet*, chymosin cuts the capping casein tail in milk micelles, which also facilitates a similar process of clumping (McGee 2004). As we shall see in the next sections, curdling is important in the generation of dairy products like yogurt and cheese.

The taste of milk is influenced by lactose, which provides sweetness, salts, and a slightly acidic pH. Milk's aro-

ma is mostly caused by volatile short-chain fatty acids and esters. Flavor is dependent on animal's feed: for instance, dry hay and silage are responsible for a mildly cheesy flavor whereas lush pasturage gives a sweeter, fruity flavor. Prolonged heating drives off certain flavors whereas adding sulfur-like flavors, both phenomena resulting from protein denaturation. At high temperatures, Maillard reactions of sugars may lead to butterscotch-like flavor. These phenomenon, also known as 'caramelization', results from the condensation of sugars with free amino groups of proteins, that lead to subsequent rearrangements and degradations ending up in the generation of a variety of brown-colored compounds. Milk off-flavors are usually caused by oxygen, light, and/or bacterial contamination (discussed in more detail in the next sections). For more details about the types of aromas and flavors present in milk and dairy products see further sections, and Bendall (2001), Karagul-Yuceer *et al*. (2002), van Aardt *et al*. (2005) and van Boekel (2006).

And why is milk so white? In fact, the whiteness of milk is due to the scattering of light by the colloidal particles of the milk emulsion. However, certain components may have an effect on its color. For example, certain cattle breeds convert more or less carotenes, proceeding from their own food, into vitamin A (Noziere *et al*. 2006). Contents of carotenes in milk may add yellowish-orange tonalities. Riboflavin (greenish) can also affect the color of skim milk and whey draining from curdled products like yogurt, in special when exposed to light (Lee *et al*. 1998).

Ancient origins of the use of animal milk for human consumption

Neolithic ("New Stone Age") started in the 10th millennium BC and is the term that describes the era in which humans started domesticating crops (agriculture), and animals (herding, and farming) (Richards 2002; Salamini *et al*. 2002; Bruford *et al*. 2003). At some point within this period, which dates as far back as 9000-8000 BC, in certain parts of the Middle East (the "Fertile Crescent"; Diamond 1997; Salamini *et al*. 2002), certain human populations realized that extracting milk from animals (dairying) was a far more efficient way to get nutrients out of poor uncultivated land than their slaughtering. Only a few species of mammals were suitable, all falling within the group of ruminants, which are characterized for their ability to extract nutrients out of high-fiber, poor-quality plants (**Fig. 1B**). Different species of ruminants thrive both in wet and in dry grasslands, which gave humans living in poor environments the possibility to adapt to seasonal changes by surviving on livestock. The first animals to be domesticated for farming were goats and sheep (between 9000-8000 BC). These were animals adapted to semidesertic and mountainous regions of Central and South West Asia (currently, Iran and Iraq). Around 8000 BC, first in the Middle East and later expanded towards the Western lands of Europe, the European race of dairy cow was domesticated (Troy *et al*. 2001). The different breeds regionally developed were strongly selected over many centuries for the production of milk, to the point that modern dairy breeds put most of their energy onto the production of milk rather than in bone and muscle. The domestication of the European race happened more or less at the same time as that of the African variant (Check 2007), and the humped central Asian race, or zebu. However, in tropical Asia the most popular bovine is the water buffalo, which was initially domesticated as a draft animal around 3000 BC. By 700 AD, water buffaloes were introduced by Arabs into the Middle East and European regions. Meanwhile, in the cold and inhospitable regions of the Tibetan plateau and mountains of Central Asia, yaks (hairy cousins of the common cow) were domesticated around the same time as lowland cattle. Camels were domesticated around 2500 BC in Central Asia and their milk is still a staple food in modern Northeast Africa (Lister 1997). Within Eurasia, China was about the only region that did not dedicate to farming (probably because of unsuitable grassland crops), although nomads introduced dairy products to some extent. Similarly, dairy was also largely unknown by the New World populations until Columbus started the import of dairy livestock.

Milk was initially collected in clay pots or, most commonly, in animal skins or hives (McGee 2004). It must have been after leaving milk standing for a while in those containers that humans accidentally discovered the separation of the cream fraction that, when agitated, becomes butter, and the acidification of the remaining milk into thick yogurt that could be separated into solid curd and liquid whey. Climate in different regions also played a role in the methods chosen by different populations to preserve milk and derivates. For example, salting of the curd fraction was a first step in the generation of cheese. Sun drying, covering with olive oil, churning into butter, boiling and long dehydration in the presence of sugar, were also early alternatives ideated to preserve milk-derived products. The relative quality of milk also had an influence in the type of product obtained. For example, milks richer in fat and protein were the base to produce mozzarella cheese (water buffalo), yogurt, feta, Roquefort and Pecorino (sheep), or butter (yak). In other climates, milk was left to sour and ferment by microbial activities, which led to the origin of *kefir* or *koumiss* (the latter, a semialcoholic beverage made by Tartars through fermentation of mare's milk). For more information about this section see Tannahill (1988), Toussant-Samat (1994) and Brothwell and Brothwell (1997).

Most common processing of milk and types of dairy products

Milk is a very rich nutrient, therefore, highly susceptible to microbial contamination. Methods involving the heating of milk were soon found as the most effective to reduce the presence of pathogenic microorganisms and spoilage of milk. In the 19th century, methods to heat milk at high temperatures for short periods of time were developed. Pasteurization involves heating at 72-77ºC for 15 seconds and this allows milk to be fresh for 10 to 18 days. Ultra-high temperature (UHT) involves heating at 130-150ºC for 1-3 seconds and this allows milk to be fresh for months. These methods involve further storage of the milk at low temperatures. Sterilized milk is obtained by treatment at 110-121ºC for 8-30 minutes, which allows milk its storage at room temperature almost indefinitely. These three methods obviously imply the use of pre-sterilized packages in order to keep the milk preserved from further contamination. The milder procedures are intended at the preservation of milk nutrients and qualities as intact as possible since long heating at high temperatures causes undesirable changes, such as degradation of nutrients, also affecting color and flavors (Robinson 2002).

However, the presence of certain microorganisms is essential in the making of several dairy products (McGee 2004; Henning *et al*. 2006; dairy products; http//en.wikipedia.org). Milk products can be separated in two groups depending on whether they require microbial fermentation (fermented dairy products) or not (non-fermented dairy products). Most, if not all, of the products belonging to the first category were discovered by accidental contamination of milk and non-fermented dairy products by environmental microbes. Among the most popular fermented dairy products we can include *kefir* (and related fermented milks), yogurt, sour cream, fermented butter and buttermilk, and cheese. Within the second category we can consider condensed milk, dried milk, milk foam, cream, ice cream, and non-fermented butter and buttermilk. In this section we will briefly revisit how the non-fermented dairy products are made, and the origin and characteristics of fermented products will be described in more detail in the next section.

Using variants of the process of homogenization it is possible to generate milk with different fat contents. Whole-milk contains ~3.5% in fat, low-fat milks contain 2 or 1%, and skim milk, between 0.1 and 0.5%. Unlike ho-

mogenization, methods of centrifugation have been developed that accelerate and improve the process of separation of cream from milk (Robinson 2002). By combining both methods cream can be manufactured with different consistencies and fat levels, from light to heavy cream (Sodini *et al*. 2006). Sour cream is a dairy product rich in fat, obtained by fermenting regular cream with LAB (*Streptococcus lactis* and *Leuconostoc citrovorum*; sour cream; http//:en.wikipedia.org). It contains 20% of fat milk but also enough protein so that the texture of the final product is partially the result of churning and protein curdling. A particular variant, *crème fraîche*, results from heavy cream only slightly souered with the bacterial culture (it is usually not as thick and sour as sour cream). Addition of salts to sugared/flavored cream lowers its freezing point, which is key for the characteristic soft texture of ice cream.

Condensed milk is obtained by prolonged heating of milk for its dehydration (Bienvenue *et al*. 2003). One of the many traditional methods ideated in the past to preserve milk, involved condensation in the presence of sugars, which led to the production of sweetened condensed milk. The high concentration of sugars and low levels of water difficult further microbial growth in this medium, so that this milk can also be preserved by relative long periods of time in sterile packages. The high temperatures cause 'caramelization' of sugars that is responsible for the yellowish to brownish color of condensed milk. Condensation is also an intermediate step to obtain dry milk, which results from evaporating milk to a 90% of its volume and then placing the remaining 10% in a stray drier. There the concentrated milk is misted into a chamber of hot air, where the milk droplets quickly dry into tiny particles of solid milk. Dried powdered milk is also very resistant to microbial contamination because of its lack of water and can be stored at room temperature for many months. Milk foam results from mixing hot milk and air together in a steaming machine, and is possible to generate it because, under such conditions, whey proteins coagulated by heat form a stable web containing air bubbles.

Butter is an intermediate product between the non-fermented and fermented categories because, in some cases, it is subjected to microbial fermentation (McGee 2004). Butter is a very ancient product from milk, which results from churning – agitation – of cream until the fat globules are destroyed and their fat contents released and free to form clusters. Usually cream is first heated, and later cooled to help water crystals break the fat globules. Then the cream is churned which generates small butter grains that are drained off from the liquid portion (buttermilk, containing free globule membranes and a 0.5% of fat). Once freed off the buttermilk, further mixing finally consolidates the fat into a solid phase. The yellow color of butter is usually derived from the carotenes previously present in milk. Pre-industrial butter was made from raw milk, which had been previously soured by the action of LAB, hence the appearance of a fermented butter version. As we will comment in more detail in the next section, LAB not only produce lactic acid but also aromas, such as diacetyl, that intensify the flavor of butter and other dairy products. Nowadays, the method involves previous addition of a starter culture to cream prior to churning, or addition of pure lactic acid and flavoring compounds in the case of artificially flavored, non-cultured, butter.

MICROBIAL COMMUNITIES COEXISTING IN DAIRY PRODUCTS

As we shall see below, the main microbial communities coexisting in milk and responsible for the synthesis of dairy products include multiple species of bacteria, yeasts, and fungi. Nowadays, methods involved in the identification of these microbial populations combine classical taxonomic procedures, such as the study of morphological characteristics (e.g. cell and colonies size and shape; vegetative or sexual reproduction; formation of spores and filaments, etc.), physiological characteristics (e.g. ability to ferment and/or assimilate different carbon and nitrogen sources; production of metabolic by-products such as ethanol, lactic, or acetic acid; growth in the presence of different stresses: high/low temperatures, high osmotic pressure, etc.), and biochemical characteristics (e.g. reaction of cell dyes with the plasma membrane/cell wall; resistance to drugs, vitamin require-ments, etc.), with several new technologies for the analysis of genomic DNA (mostly based on methods of amplifica-tion by polymerase-chain reaction (PCR), sequencing, and map restriction analysis). Advances in taxonomic analysis for the determination of microbial communities present in different environments are reviewed in great detail in Diaz Ruiz and Wacher Rodarte (2003), Tringe and Rubin (2005) and Collado and Hernandez (2007). Next we will review the most popular dairy products according to the participation, on their making, of bacteria, bacteria and yeasts, or bacteria, yeasts, and fungi.

Dairy products produced by bacteria (yogurt, soured cream, butter, and buttermilk)

Within this group the most popular product is, indeed, yogurt. This product was originated in the warm climates of Southwest Asia and Middle East, and its accidental discovery probably was the result of people storing milk in animal stomachs and skins (similarly to kefir, see below). Yogurt (Turkish word coming from 'thick') results from the symbiotic metabolism of two main thermophilic bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* (**Fig. 3A; Table 2**). The optimum growth temperature is 45ºC for *L. delbrueckii*, and 37ºC for *Strp. thermophilus* (although the latter can also grow at higher temperatures). Both bacteria are LAB, meaning that they are able to use lactose as the carbon source producing lactic acid as the main by-product. This acid causes a particular type of curdling of the proteins in milk, which gives yogurt its semi-solid consistency. *Strp. thermophilus* is usually the faster organism at the beginning of the fermentation, but is more sensitive to low pH than *L. delbrueckii*, which tends to predominate at the end of the process. Each bacterium stimulates the growth of the other, so that their combination acidifies the medium much faster than either partner on their own. The basis of the synergistic association of both bacteria relies in their particular abilities to use the nitrogen sources present in milk. *L. delbrueckii* has better proteolytic activities than *Strp. thermophilus*, releasing peptides and, to a lessened extent, free amino acids that *Strp. thermophilus* then uses as nitrogen source. However, the peptidases that *Strp. thermophilus* is able to produce at the beginning of the fermentation also release amino acids that help boosting growth of *L. delbrueckii* (Christensen *et al*. 1999; Robinson 2002).

To prepare yogurt, milk is usually subjected to a prolonged boiling first to denature the whey protein lactoglobulin, which provokes its clustering on the surface of the casein particles. This limits the ability of casein particles to interact with each other, in such way that, instead of gathering in clusters, during the fermentation they form a fine matrix of chains that retains the whey liquid giving yogurt its semi-solid consistency (**Fig. 3B**). The relative high temperature at which the fermentation is carried out (40-45ºC) once the starter culture is added (usually at ratio 1:1 of each species of bacteria), also has a strong influence in the particular gelling of proteins by formation of lactic acid. The total process at this temperature is of just 2 to 3 hours. Lower temperatures, like 30ºC take up to 18 hours for the product to set. Slow gelling, however, causes a matrix of finer consistency, with a better ability to retain the whey.

The distinct flavor profile of yogurt is the result of the combined metabolisms of sugars, proteins, and lipids present in milk. Sugar fermentation leads to the generation of by-products like acetaldehyde, acetone, acetoin, and diacetyl. Proteolysis produces smaller peptides and amino acids, whose catabolism into α-keto acids, ketones, aldehydes, or-

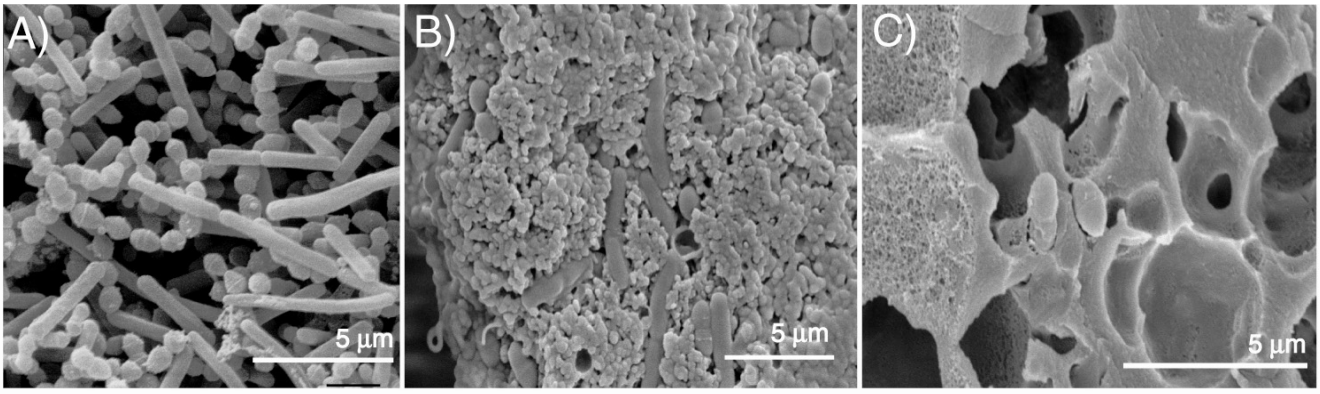

Fig. 3 LAB in dairy food products. (**A**) TEM micrograph of a mixed culture of *Lactobacillus bulgaricus* (rod-shaped) and *Streptococcus thermophilus* (round-shaped); (**B**) SEM micrograph showing bacteria embedded in a yogurt matrix, taken at an original magnification of 6,000X; (**C**) Scanning electron microscopy (SEM) micrograph of bacteria in Cheddar-like cheese, at original magnification of 10,000X. (**A**) was provided courtesy of Miloslav Kalab, and (**B**), (**C**) courtesy of Diane Montpetit, Agriculture and Agri-Food Canada.

ganic acids, alcohols, and esters, have significant contribution to flavor. A certain level of lipolysis, particularly acting over short-chain triglycerides, also helps to produce similar volatile compounds (Robinson 2002; Marilley and Casey 2004; Smit *et al*. 2005). Since these bacteria need certain vitamins for growth, on average, an increase in niacin and folic acid, but a decrease in vitamin B_{12}, thiamine, riboflavin, and panthotenic acid, are observed after fermentation. The bacteria also contribute to the thicker texture of the final product through the production of exo-, glucan-like, polysaccharides. These provide additional viscosity of the product and protection from physical shocks by maintaining the semisolid structure of the product (sometimes the manufacturer also adds gelatin, starch, or other stabilizers with similar aims; Robinson 2002; McGee 2004).

As mentioned in the previous section, other typical products being the result of bacterial fermentation are those resulting from the separation of the cream fraction of milk (i.e. sour cream, buttermilk, butter, and related products). Traditionally, the separation of milk in two phases was let to occur without the modern centrifugation techniques and during that period of time, bacteria would start to grow that would provide the cream, and the butter and buttermilk made from it, with characteristic aromas. Cream cultures mainly consist of mesophilic bacteria of the genera *Lactococcus* and *Leuconostoc*. These bacteria are milder producers of acetic acid, so that the products obtained through their fermentations are never as sour as yogurt. Certain strains have the ability to convert citrate into diacetyl, one of the major flavoring components in butter, sour cream, and buttermilk. Nowadays, the manufacturers favor this process by adding citrate to the milk or cream and fermenting in cool conditions to favor the production of diacetyl. In particular, *Lactocccus lactis* subsps. *lactis* and *cremoris*, are the main lactic acid producers, whereas *Lactocccus lactis* subsps. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*, are the main diacetyl producers (**Table 2**).

Additional examples of dairy products obtained by fermentation using only bacteria are Bulgarian buttermilk and "bio"-fermented milk products. These fermentations are carried out at temperatures ranging from 37 to 45ºC, using thermophilic species of the genera *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* in variable combinations, depending on the product (**Table 2**). For Bulgarian buttermilk, *Lactobacillus delbrueckii* subsp. *bulgaricus* is the only species usually cultured onto buttermilk (Robinson 2002). Ever since the discovery by Metchnikov of certain bacteria from milk and dairy products acting as 'probiotics', modern dairy companies have been exploring the utilization of starter cultures based on Bifidobacteria (see references in **Table 2**; Robinson 2002). Probiotic dairy products range from yogurts, yogurt-type fermented milks, and dried powder products, and the studies of their effects in human health and constant improvement are currently the focus of much research attention worldwide (for a more detailed review on the subject, see Adolfsson *et al*. 2004; Leahy *et al*. 2005; Parvez *et al*. 2006).

Table 2 Most common bacterial species isolated from dairy products[1,2].

Lactobacillus acidophilus
bifermentans
brevis
casei subsp. *casei*
casei subsp. *plantarum*
delbrueckii
fermentum
helveticus
kefiri
kefiranofaciens subsp. *kefiranofaciens*
kefiranofaciens subsp. *kefiranogranum*
parakefiri
Lactococcus lactis subsp. *cremoris*
lactis subsp. *diacetylactis*
lactis subsp. *lactis*
Leuconostoc mesenteroides subsp. *cremoris*
mesenteroides subsp. *dextranicum*
mesenteroides subsp. *mesenteroides*
Streptococcus thermophilus
Enterococcus durans
Acetobacter aceti
pasterianus

[1] Fermented milks, kefir, yogurt, and/or cheese
[2] For citations see: Narvhus and Gadaga 2003; Farnworth 2005; and Lopitz-Otsoa *et al*. 2006

Dairy products produced by bacteria and yeast (kefir and related milk beverages)

Within this category we can consider a series of fermented, mildly alcoholic, milk beverages from which kefir is the most commonly known in the modern Western world (for more information about products similar to kefir, see Robinson 2002; Narvhus and Gadaga 2003; Farnworth 2005). Kefir (word thought to come from Turk and Caucasian roots meaning 'pleasant', 'well-being', and 'best quality') is, perhaps, one of the most ancient products resulting from microbial fermentation of milk, being accidentally discovered by humans. This fermented-milk beverage is produced by what is known as kefir grains. Kefir has its origins in villages located at great altitude in the northern Caucasian mountains. The particular climatologic conditions (cold, relative dryness, and altitude) are thought to have played an important role in the evolutionary selection of mesophilic microorganisms adapted to growth on milk. As mentioned before, by that time, milk from cows, goats, and sheep, used to be kept in oak vats or bags made of goat hides. Some theories point to the origin of the kefir grains in the manufacturing of a related fermented milk beverage, Ayran, which was pro-

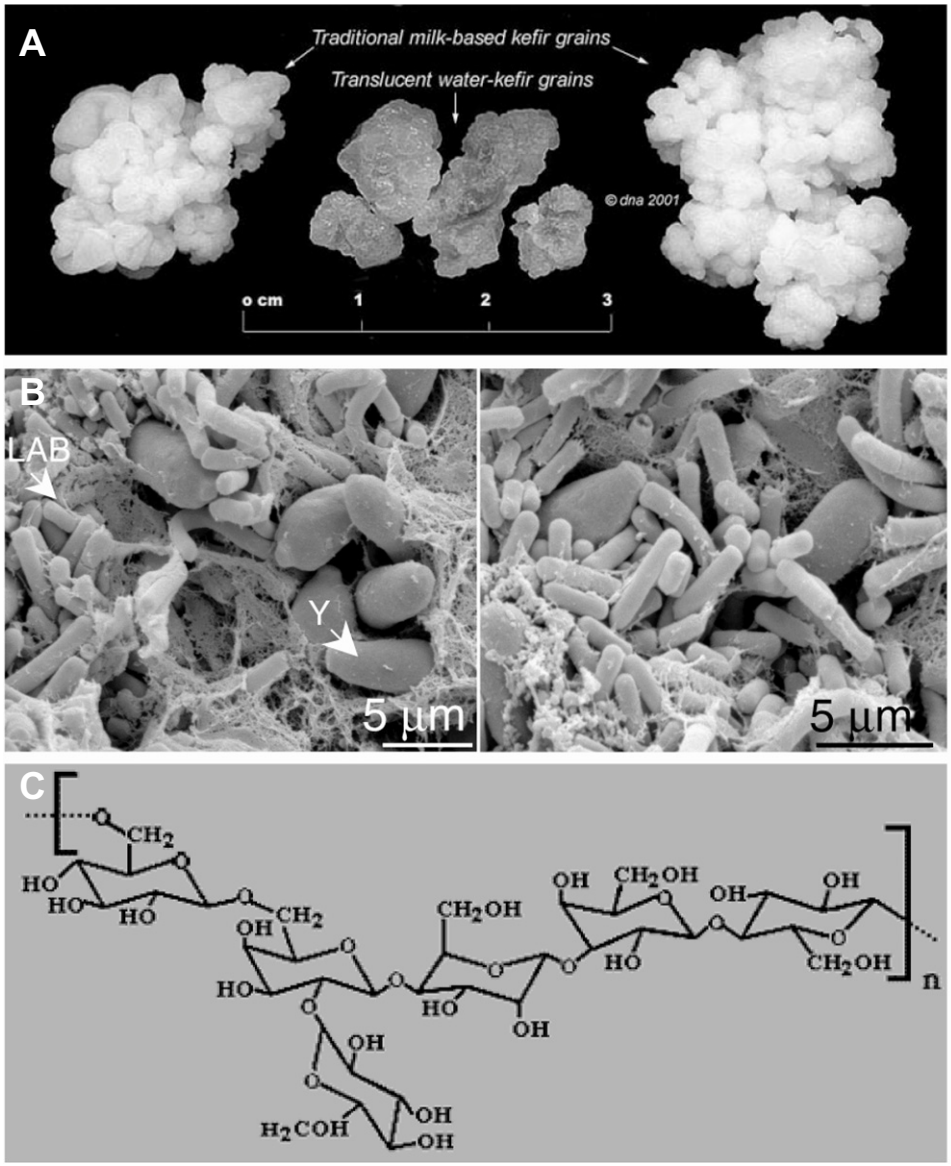

Fig. 4 Structure of kefir grains. (**A**) Normal appearance of milk kefir grains (left and right) compared to that of kefir grains cultured in alternative media like soy, seed, nut, or coconut milk (center). (**B**) EM micrographs showing bacteria (LAB) and yeasts (Y) growing in the surface of a kefir grain, and embedded in kefiran. (**C**) Molecular structure of kefiran, polysaccharide composed by branched hexa- or hepta-saccharide repeating units. (**B**) is provided courtesy of Miloslav Kalab (Agriculture and Agri-Food Canada). (**A**) and (**C**) are provided courtesy of Dominic N. Anfiteatro, Dom's Kefir In-Site, http://users.chariot.net.au/~dna/kefirpage.html.

duced by mixing fresh milk with pieces of calf's or camel's stomach within these 'organic bags' (Fröhlich-Wyder 2003). Thus, the ancestors of kefir grains are believed to have been collected from the walls of these containers and later propagated in fresh milk, which then gave rise to kefir and many other related products (Farnworth 2005).

At a first glance, kefir grains are soft, gelatinous, cauliflower-like living masses of biological material, of 1 to several cm in diameter (**Fig. 4A**). They are in fact composed of a matrix of protein, lipids, and a hot water-soluble/cold water-insoluble polysaccharide, denominated kefiran (**Fig. 4C**). This particular glucogalactan is secreted by certain members of the microbial community residing within the kefir grains, to protect and hold together the two main classes of microorganisms present on this unique ecosystem, bacteria and yeast (**Fig. 4B**). The bacteria group is mostly composed of lactic acid *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., and acetic acid bacteria (*Acetobacter* spp.) (**Table 2**). Bacteria present in kefir are, in fact, the same or closely related to bacteria present in the fermentation of other dairy products. Thus, they are characterized for their ability to use lactose as carbon source and to produce lactic, citric, and acetic acids, as main metabolic by-products. Their overall metabolism, therefore, reduces the pH until a point in which the production of more acid is inhibited and the metabolism is then deviated to the production of other compounds (e.g. acetaldehyde, acetoin), which provide many of the characteristic aromas and flavors present in fermented milk. Reduction of pH and the secretion by certain species of bacteriocins (peptide antibiotics of anti-microbial activity, as shown by Flôres and Alegre 2001; Guinane *et al*. 2005) and other toxic compounds (Caplice and Fitzgerald 1999) help the whole community to fight the growth of many other bacteria (e.g. *Salmonella*, *Shigella*, *Helicobacter*, *E. coli*, *Enterobacter aerogenes*, *Bacillus subtilis*, *Streptococcus aureus* and *pyogenes*, *Listeria monocytogenes*, etc; Mufandaedza *et al*. 2006; Topisirovic *et al*. 2006), certain yeast (e.g. *Candida*), and fungi (Batish *et al*. 1997), which just happen to be from moderately opportunistic to acutely pathogenic for humans and other animals. These and the probiotic properties commented above explain the healthiness of kefir and other dairy products in which these bacteria are present (Lopitz-Otsoa *et al*. 2006).

The yeast group can be separated into two subgroups, yeasts able to ferment/assimilate lactose (e.g. *Kluyveromyces marxianus*, *K. lactis*) and yeasts unable to ferment/assimilate this sugar (e.g. *Saccharomyces cerevisiae*, *Yarrowia lipolytica*), (**Table 3**). Yeast and bacteria (such as Lactobacilli) able to use lactose are usually found associated to the surface of the kefir grains, whereas the remaining species, which basically rely on the metabolic by-products released by these Lac^+ microorganisms, are more often found in the interior of the grains (Fröhlich-Wyder 2003). In fact, very few yeast isolates from kefir are Lac^+. Most of the yeasts found are instead able to use galactose, lactate, acetate, and/or citrate (**Table 3**), which are ensured by-products from the metabolic activities of the Lac^+ microorganisms. The particular spatial arrangement that seems to occur for the two different groups of yeast may, at least partially, help us to understand why *Kluyveromyces* are yeasts predomi-

Table 3 Most common yeast species isolated from dairy products[1,2].

Yeast species[3] (teleomorph)[5]	Synonyms (anamorph)[5]	Lactose[4]	Galactose	Lactic acid	Acetic acid
C. catenulata		N	A	A	A
C. friedrichii		N	A	N	A
C. inconspicua		N	N	A	A
C. intermedia		A	F/A	N	A
C. tenuis		A	A	N	A
Cry. albidus		N	N	N	N
Cry. humicola		A	A	A	A
Cry. laurentii		A	A	N	N
Deb. hansenii var. *hansenii*	*C. famata*	A	A	A	A
Deb. occidentalis var. *occidentalis*	*Schw. occidentalis*	A	A	A	N
Dek. anomala	*Bret. anomalus*	F/A	A	A	N
Gal. geotrichum	*Geo. candidum*	N	A	A	N
Iss. orientalis	*C. krusei*	N	N	A	A
K. marxianus var. *marxianus*	*C. kefyr*	F/A	F/A	A	N
	C. pseudotropicalis				
	K. bulgaricus				
	K. fragilis				
	K. marxianus				
	S. fragilis				
	S. kefir				
	Tp. kefir				
K. lactis var. *lactis*	*C. sphaerica*	F/A	F/A	A	N
	S. lactis				
	To. sphaerica				
K. lodderae		N	F/A	A	N
S. cerevisiae	*C. robusta*	N	F/A	N	N
	S. carlsbergensis				
	S. italicus				
	S. pastorianus				
S. unisporus		N	F/A	N	N
S. delbrueckii	*Tp. delbrueckii*	N	A	A	N
S. exiguus	*C. holmii*	N	F/A	N	N
	To. holmii				
S. florentinus	*Zy. florentinus*	N	F/A	N	N
P. fermentans var. *fermentans*	*C. firmetaria*	N	N	A	A
	C. lambica				
P. membranifaciens	*C. valida*	N	N	A	N
P. jadinii	*C. utilis*	N	N	A	A
Rho. glutinis var. *glutinis*		N	N	N	N
Rho. minuta var. *minuta*		A	A	A	A
Rho. mucilaginosa var. *mucilaginosa*	*Rho. rubra*	N	A	A	A
Trp. cutaneum var. *cutaneum*		A	A	A	A
Ya. lipolytica	*C. lipolytica*	N	A	A	A
Zy. rouxii	*C. mogii*	N	A	N	N

[1] Fermented milks, kefir, yogurt, and/or cheese
[2] For citations see: Fröhlich-Wyder 2003; Narvhus and Gadaga 2003; Farnworth 2005; and Lopitz-Otsoa *et al*. 2006
[3] Nomenclature based on the CBS (http://www.cbs.knaw.nl) and NCYC (http://www.ncyc.co.uk/) Yeast Databases, and Deak and Beauchat 1996. Abbreviations: *Bret = Bretanomyces*; *C = Candida*; *Cry = Cryptococcus*; *Deb = Debaryomyces*; *Dek = Dekkera*; *Geo = Geotrichum*; *Gal = Galactomyces*; *Iss = Issatchenkia*; *P= Pichia*; *Rho = Rhodotorula*; *S = Saccharomyces*; *Schw = Schwanniomyces*; *Tp = Torulaspora*; *To = Torulopsis*; *Trp = Trichosporon*; *Ya = Yarrowia*; *Zy = Zygosaccharomyces*
[4] Ability to use lactose as a carbon source: by fermentation (F), by respiration and fermentation (F/A), only by respiration (A), or unable by any means (N).
[5] Teleomorph: sexually-reproducing form. Anamorph; asexually-reproducing form.

nantly adapted to the consumption of the sugar lactose through respiratory metabolism, since in their usual location they probably have much better access to oxygen. Lac^- yeasts like *S. cerevisiae*, able to use glucose and galactose derived from lactose hydrolysis in an oxygen-limited environment through the fermentative pathway, are instead able to thrive in the interior of the grains. The release of intracellular β-galactosidases by partial cell lysis of Lac^+ yeasts and LAB, also helps to increase the levels of extracellular galactose that can, subsequently, be used as carbon source by many of the Lac^- yeasts. As I will comment later on, yeasts habitually found in kefir are usually the same species that cause spoilage of milk products and, because of this fact, there is controversy as of whether certain yeasts are actively participating in the production of kefir or are instead mere contaminants present in this product. It has been proposed that only true Lac^+ yeasts should be considered as participants in the fermentative process. However, there is further evidence that the metabolism of Lac^- yeasts is also helping the bacteria present in the kefir grains. For example, the protective polysaccharide matrix, kefiran, is produced by *Lactobacillus kefir* (formerly, *Lactobacillus brevis*), and other related Lactobacilli, such as *Lactobacillus kefiranofaciens* (La Rivière 1967; Arihara *et al*. 1990). It has been shown that the growth of this particular flora is largely dependent on the presence of yeasts (La Rivière 1969; Linossier and Dousset 1994; Cheirsilp *et al*. 2003). For instance, Mitsue *et al*. (1999) showed that when combined with the yeast *Torulaspora delbrueckii*, *Lactobacillus kefiranofaciens* produces the highest levels of kefiran. Some of the Lac^- yeasts have strong lipolytic and proteolytic activities that not only help the neighboring microorganisms by releasing more easily-assimilating peptides and amino acids but that, through their quick respiratory metabolism of the organic acids produced by the lactic and acetic bacteria, are also able to locally re-increase the pH, which then allows restoration of bacterial growth.

Kefir grains are, in fact, among the most complex symbiotic communities involved in the fermentation of food, to the point that *bona fide* kefir can only be reproduced by

using kefir grains as the starter culture. Obviously, heterogeneity among kefir grains from different origins and different conditions for the fermentation process also affect the delicate interaction of the multiple members of this complex ecosystem, leading to important variations in the organoleptic characteristics of the final product. Use of kefir grains as starter culture on an industrial scale is, often, quite tricky and for such reason, ever since the main microbial species were identified in the kefir grains, multiple attempts have been made to produce a similar starter culture by artificial combination of isolates from the main component species (Lopitz-Otsoa *et al*. 2006).

Manufacturing of kefir using kefir grains involves two phases, fermentation and ripening. For the fermentative phase, using low temperatures favor yeast growth whereas higher temperatures favor lactic acid bacteria growth. The use of intermediate temperatures (18-22ºC) for less than 1 day, therefore, helps to control the relative levels of ethanol produced by yeast and the acidification produced by bacteria. The significant drop in pH attained after this period still favors growth of Lactobacilli but causes a decline in the *Leuconostoc* and Streptococci populations. After separating the grains from the kefir, ripening at a temperature of 8-10ºC is let to happen for 1-3 days. During this period, ethanol and other flavoring/texturing components are accumulated in the product mainly because of the fermentative activity of yeasts (Fröhlich-Wyder 2003). The increase in pH caused by yeast finally provokes a switch so that growth of LAB is, again, favored over that of yeasts and acetic acid bacteria (Farnworth 2005).

Kefir at an industrial level is more often produced by artificial starter cultures. In general, levels of LAB are naturally greater in the kefir grains than that of yeasts and acetic acid bacteria. Keeping this ratio is important, among other reasons, to prevent excessive production of CO_2 by yeasts, which could result in blowing of the packages, or the excessive appearance of vinegary flavor caused by the acetic bacteria. In some cases, bacteria are first used for the fermentation process and yeasts are added afterwards, for the ripening part. Control of the starter culture composition at all times is crucial since in some cases the relative ratios of the microbial populations are switched, leading to predominance of Streptococci over yeasts and Lactobacilli (Fröhlich-Wyder 2003). The organoleptic properties of the product obtained from starter cultures tend to differ from those obtained from kefir grains, reason for which improvement of the starter culture is still a step to overcome. Addition of sweetening and flavoring supplements has, in the meantime, significantly helped to please the palate of the consumers (Farnworth 2005).

Dairy products produced by bacteria, yeast, and moulds (cheese)

The word 'cheese' originally derives from the Latin word *caseus* that, in turn, derives from a Proto-Indo-European word meaning 'to become sour'. Cheese is the dairy product showing both the highest complexity in its fermenting flora (composed of bacteria, yeast, and moulds; **Fig. 3C**) and diversity in flavors, textures, and processing conditions. The earliest evidence found for cheese making is dated as far back as 2,300 BC. This dairy product evolved in regions of Center Asia and the Middle East, as an alternative form to preserve milk for long periods of time (McGee 2004). Similarly to the other products, it was probably discovered by accident when milk was stored within containers made of animal skins and stomachs. The presence of pieces of stomach of unweaned calves, lambs, and goats had the effect to curdle milk. Later on, a brine extract was obtained and kept for further uses – *rennet* –, which contained the semi-purified enzymes responsible for curdling (mostly, chymosin). Bacteria present in milk and in these containers had the ability to sour milk, which helped in the process of curdling. Romans also used acids derived from plants in order to help this process. Now we know that the importance of using *rennet* relies on the specificity of chymosin for κ-casein. Acid alone just causes a dispersion of the casein micelles and a lot more acidification is needed in order to cause curdling which would inactivate some of the useful flavor-producing milk enzymes. By attacking specifically κ-casein, chymosin causes casein particles to interact and cluster in curdles so that the use of *rennet* prevents the need for a high level of acidification (Garg *et al*. 1994).

Going back to ancient times, at some point after finding the phenomenon of curdling, humans soon discovered that, by draining the curds off the liquid whey fraction and further salting them, the corresponding milk product could be stored for much longer periods of time than milk and other forms of soured-milk products. The cheese was let to 'age' or ripen in particular conditions of temperature and humidity which let microorganisms already present, as well as further colonizers, to grow on the product producing alterations on its flavor and final texture. Cheese diversity comes, in fact, from the multiplicity and variability of parameters affecting its production, from the type of animal producing the milk, the type of feeding, the seasonal climate conditioning feeding and later ripening conditions, to the type of local flora participating in the ripening process. Milk from animals feeding in pastures from high altitudes is often richer in flavors because of the higher abundance and diversity of flora present in the feeding pastures (Leiber *et al*. 2005a, 2005b). The production of certain cheeses was consequently adapted to the variability of the milk being caused by seasonal changes in the feeding pastures (Nudda *et al*. 2005). For instance, as mentioned previously, fresh pastures are particularly rich in plants and flowers containing carotenoids in the spring (Britton 1998). When fresh pasture plants are consumed by animals, the damaged plants activate their lipoxygenase systems which break down carotenoids and lipids into a wide range of volatile and nonvolatile aroma compounds which are passed onto the milk collected from animals fed on them during the spring/summer seasons (Galliard and Chan 1980; Carpino *et al*. 2004). Unfortunately, modern animals are often annually fed with an invariable mixture (corn and alfalfa silage) so that the additional richness that used to be provided to cheese through animal feeding has been lost with the exception of local regions of the world in which processing still follows the most traditional ways. Similarly, the once complex microbial flora of each cheese maker's dairy is now substituted by pure microbial cultures, and *rennet* (chymosin) is produced by engineered microorganisms, purified, and then added to the milk for curdling. In other cases, the processing of cheese involves mixing aged and fresh cheeses by blending with emulsifiers and repasteurization. Pasteurization of milk eliminates part of the important flora and milk enzymes needed for the production of cheese, reason for which pasteurization of milk for cheese production is actually forbidden and substituted by close monitoring of the quality of milk and producer animals in several European countries.

By order of action, the first type of microorganisms acting in the manufacturing of cheese are, as for other dairy products, LAB which are let to moderately acidify milk to facilitate curdling. Even when their numbers are, in later stages, reduced, they still participate in the ripening (which is considered the stage at which microbes and milk enzymes transform the salty curd into flavored cheese) through many of their still active enzymatic activities. These activities are known to act during ripening of hard and semi-hard cheeses (e.g. Cheddar, Gouda, and Parmesan). Usually, the main LAB involved are mesophilic Lactococci. However, in cases in which acidification for cheese making involves a cooking step (e.g. in mozzarella making), thermophilic Lactobacilli/Streptococci are selected because they are the only ones to survive the heating process.

Another type of bacteria growing in cheese out of the lactic acid produced by the previous group is Propionibacteria, in particular *Propionibacteria freudenreichii* ssp. *shermanii* (Thierry *et al*. 2005). The original habitat of these bacteria is probably animal skin, so they grow in the rela-

tively unfamiliar environment of cheese quite slowly, needing warm temperatures. For such reason, processes in which its action wants to be favored, involve ripening at 24ºC for weeks. These bacteria release important levels of propionic and acetic acids, diacetyl, and CO_2 (the latter characteristic being important for the generation of internal air bubbles, 'holes', in cheeses like Emmental).

Smear-ripened cheeses are characterized by the presence of another group of bacteria usually found in high-salt environments (e.g. seashore, human skin/sweat). These belong to the species *Brevibacterium linens*, characterized by high-salt tolerance, acid-sensitivity, and need for oxygen, which favors its growth only in cheese surfaces and after a certain period of ripening. *Brevibacterium linens* has the ability to break down proteins to amino acids, and these to biogenic amines (e.g. trimethylamine, putrescine, ammonia), isovaleric acid, and sulfurs. All of these volatile compounds are able to diffuse to the interior of the cheese, affecting flavor and texture, being responsible for the fishy, garlicky aromas of cheeses like Camembert, Gruyère, Münster, Epoisses, or Limburger (Arfi *et al*. 2003).

Moulds are very popular components in the ripening process of many types of cheeses. They are, in general, characterized for their requirement of oxygen, the ability to tolerate drier conditions than bacteria, and their production of strong proteases and lipases. Moulds involved in cheese ripening usually belong to the genus *Penicillium*. Blue moulds like *Penicillium roqueforti* grow better in lower concentrations of oxygen, being able to colonize small fissures within the cheese. This mould is used in combination with certain enzymes to produce Pecorino and Provolone cheese. In particular, is able to break up fat to short-chain fatty acids, ketones, and alcohols, all of them responsible for the peppery flavor and aromas typical of blue cheese (Kinsella and Hwang 1976). *Penicillium glaucum* is characteristic of Gorgonzola, and Stilton cheese. The white mould *Penicillium camemberti* is found, as it name indicates, in Camembert and Bries, and its ability to break down proteins is responsible for the creamy texture, and the mushroom/garlicky/ammonia flavors of these types of cheeses. In general, moulds make cheese less acid and that, especially in hard cheeses where there is low moisture, may provoke the formation of crystals of calcium lactate or tyrosine (e.g. in aged Cheddar, Parmesan, Gruyère, and Gouda cheese; Agarwal *et al*. 2006).

Yeasts are also found within the microflora of many types of cheese (Fleet 1990). They have particular properties that make them easily adapted to life in this medium, for instance, resistance to low pH and moisture, low storage temperatures, and high salt concentrations. In general, the highest numbers of yeast counts are usually found in soft and blue-veined cheeses (Roostita and Fleet 1996). The most frequent species found are *Kluyveromyces lacti*, *K. marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Yarrowia lipolitica*, *Trichosporon cutaneum*, *Rhodotorula mucilaginosa*, *Geotrichum candidum* and *Torulaspora delbrueckii* (**Fig. 5**; Fröhlich-Wyder 2003). These species have been frequently isolated from cheeses such as Cheddar, Gouda, Roquefort, Gruyère, Camembert, and others. In the earliest stages of processing Lac^+ species predominate, whereas later stages of ripening are more characterized by the presence of yeasts like *Yarrowia lipolytica*, *Debaryomyces hansenii*, and *Geotrichum candidum*. As mentioned for kefir, one of the main actions of yeasts consists in metabolizing lactic acid, increasing the pH, which then favors growth and further contribution to ripening of acid-sensitive bacteria (Corsetti *et al*. 2001). Fermentation of the milk sugars by certain yeasts contributes to the production of CO_2 and, consequently, of cavities in cheese, although the strong ability of yeasts to produce gas has to be closely monitored to impair blowing of cheese. Several yeasts participate in a more important manner to the production of volatile flavors, aromas, and textures, because of their strong proteolytic and lipolytic activities (properties that will be revisited in more detail in a further section). Moreover, yeasts not only promote growth of LAB by increasing local pH, but also because species like *Deb. hansenii* are also able to supply bacteria with important growth factors, such as vitamins (lactoflavone, thiamine, pantothenic acid, nicotinic acid, folic acid, and biotin; La Rivière 1969; Lenoir 1984; Schlegel 1993).

Spoilage: when 'microbe's heaven' becomes 'human's hell'

Microbes able to populate milk and dairy products are not always benign in their action. Many of them are pathogenic for humans and animals. Undesirable mixtures of microbial communities may also lead to spoilage of a particular dairy product. In fact, many of the microbial species present in the normal development of one particular dairy product, may instead act as contaminants that deteriorate the quality of another dairy product (e.g. the presence of yeasts is desirable in kefir but undesirable in yogurt). In general, fresh dairy products, with the higher levels of moisture (e.g. milk, yogurt, soft cheeses, creams, and even ice cream), are the most susceptible to moulds, yeasts, and bacterial contaminations (Massa *et al*. 1992; Deak and Beauchat 1996; Valdes-Stauber *et al*. 1997; Mounier *et al*. 2005).

Potentially harmful contaminations start to happen at the level of raw milk, reason for which methods of heating, as explained above, have been developed that are efficient

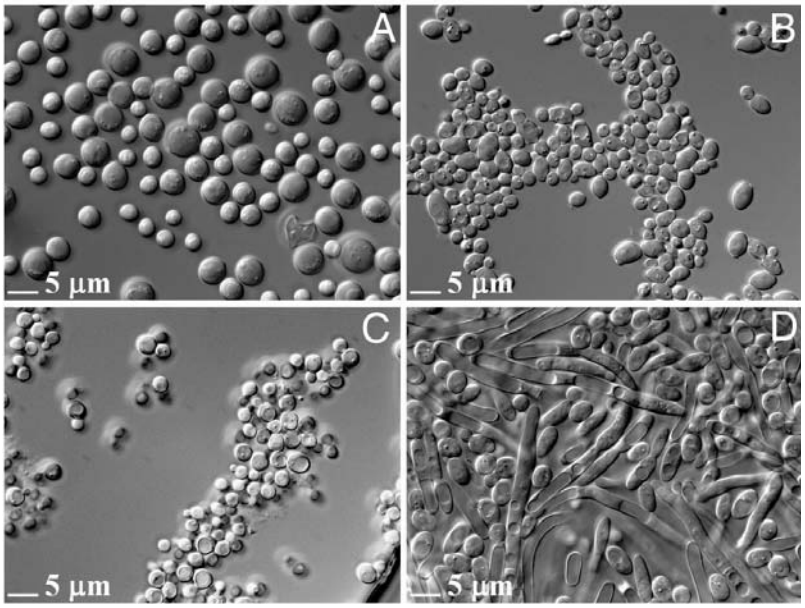

Fig. 5 Yeasts most commonly found in dairy products. (**A**) *Saccharomyces cerevisiae*; (**B**) *Kluyveromyces lactis*; (**C**) *Debaryomyces hansenii*; (**D**) *Yarrowia lipolytica*. All of the pictures were made in normal DIC optics at an original magnification of 100X.

enough to kill most of the pathogenic microorganisms. Sources of potential contamination of raw milk are mainly the producer animal, and the environment in which the product is manufactured. Most of the bacteria found in the raw milk belong to the genera *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Corynebacterium*, *Arthrobacter*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Enterobacter*, *Klebsiella*, *Aerobacter*, *Escherichia*, *Serratia*, *Bacillus*, *Clostridium*, *Micrococcus*, *Microbacterium*, and *Alcaligenes* (Franz *et al*. 1999; Robinson 2002). Many of these genera include pathogenic bacteria, and the latter five are thermoduric genera, meaning that they are potentially able to survive a short heating procedure like pasteurization. *Bacillus* and *Clostridium* species are particularly dangerous because of their ability to form spores that can later germinate in packaged milk. Others, like *Pseudomonas*, *Aerobacter*, and *Klebsiella*, are psychrotrophic microflora able to thrive in milk stored under refrigeration (Persson *et al*. 1980; Dogan and Boor 2003). Even when some of them can be destroyed by pasteurization, their lipolytic and caseinolytic enzymes may remain active contributing to the development of rancid flavors. The presence of coliforms belonging to the genera *Enterobacter* and *E. coli* are sometimes indicators of contamination of fecal origin (e.g. *Salmonella*, *Campylobacter*) and their growth in milk at ambient temperature also causes spoilage of milk. These genera include opportunistic and pathogenic species, for example, *Enterobacter sakazakii*, which contaminates infant formula prepared from powdered milk, representing a significant risk for the health of neonates (Drudy *et al*. 2006). In general, dairy industries are subjected to strong regulations in which, the health of the producer animals, the sanitary conditions followed by milk handlers, and the safety of the milking equipment in the processing plants, are continuously monitored for the presence and potentially harmful levels of this type of microorganisms.

Almost equally dangerous is the appearance of certain types of moulds able to synthesize micotoxins, which can be lethal at very low concentrations. Micotoxins may be produced by certain species of the genera *Aspergillus* and *Penicillium* (Coulombe 1993). Although these contaminations are relatively rare, the potential presence of dangerous mycotoxins in some foods is always an important cause for concern. Sometimes, cheeses held in storage for longer time than their expiration date suffer contamination with some of these air-borne toxin-producing foreign moulds (e.g. *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. versicolor*, *A. ochraceus*, *Penicillium viridicatum*, *P. cyclopium*, *P. griseofulvum*), reason for which is advisable to discard cheeses that appear overgrown with unusual moulds. Moulds of *Aspergillus*, *Penicillium*, *Fusarium*, and *Byssochlamis* genera, also cause food spoilage through discoloration, production of off-flavors, and allergens (Filtenborg *et al*. 1996). Lipases, proteases, and carbohydrases of fungal origin are usually responsible for this problem. For instance they may give raise to volatiles such as dimethyldisulfide, geosmin, and 2-methyl-isoborneol that produce musty odors and affect the quality of foods even when produced in very small amounts (Schnurer *et al*. 1999).

Raw milk or opened packages, held at refrigerated temperatures and contaminated after their opening, will support the growth of most of the yeast species mentioned as participants in the making of certain dairy products (**Table 3**). The commercial development of mixtures of yogurt with fruits, fruit flavors, and sugars, has also given raise to secondary contamination of yogurt with diverse yeast species inhabiting these other media (Suriyarachchi and Fleet 1981; Bleve *et al*. 2003; Mayoral *et al*. 2005). The same species participating in the ripening of cheese may, in this case, cause spoilage by producing blowing of the packages and gassy, fruity, bitter, rancid, or yeasty off-flavors. In certain cases, yeasts like *Yarrowia lipolytica* and *Deb. hansenii* produce discoloration in cheeses due to oxidation of tyrosine to melanine by tyrosinase (EC 1.14.18.1) (Nichol *et al*. 1996; Carreira *et al*. 1998; Fröhlich-Wyder 2003). Under poor hygienic conditions, growth of the yeast pathogen, *Candida albicans*, and related species may also occur in cheese. Species such as *Sporobolomyces roseus* and *Trichosporon pullulans* are also responsible for the development of surface biofilms in this medium (Deak and Beauchat 1996).

PHYSIOLOGICAL CHARACTERISTICS OF YEASTS ADAPTED TO LIFE IN DAIRY PRODUCTS

Respiratory and fermentative metabolisms and their effect in dairy products

Yeasts and other organisms have the ability to use carbohydrates as a source of carbon and energy through respiration and/or by fermentation (**Fig. 6**; Fraenkel 1982; Wills 1990; van Dijken *et al*. 1993). On each case, the carbon source is first converted into a glycolytic intermediate (usually into glucose or a close derivate) so that the first steps of these two pathways are common until the level of action of the pyruvate kinase, which produces pyruvate from the glycolytic intermediate phosphoenolpyruvate. After this step, in the presence of oxygen, aerobic organisms target pyruvate to the mitochondria where it is fully oxidized to carbon dioxide and water by pyruvate dehydrogenase (EC 1.2.4.1) and the set of enzymes of the TCA (tricarboxylic acids) cycle (Barnett 2003). The products of pyruvate are sequentially dehydrogenated as they pass through the cycle, powering the reduction of NAD^+ to NADH. The NADH is, in turn, oxidized by an electron transport chain using oxygen as final electron acceptor to produce ATP via the action of the ATP synthase complex, a process known as oxidative phosphorylation. Part of the ATP is also produced in steps of the glycolytic pathway and by substrate-level phosphorylation during the TCA cycle so that, overall, a total of 36 molecules of ATP are produced for each molecule of glucose that is consumed by respiration (Pronk *et al*. 1996).

In the presence of low levels or absence of oxygen, certain organisms (facultative anaerobs) have devised alternative ways to survive, still obtaining energy from glycolysis but by coupling this pathway to alternative outcomes that do not involve the TCA cycle and the electron transport chain. For yeasts, the most common way of anaerobic respiration is alcoholic fermentation, which consists in the decarboxylation of pyruvate by pyruvate decarboxylase (4.1.1.1) into acetaldehyde which is then converted to ethanol by alcohol dehydrogenases. This process only produces 2 molecules of ATP per molecule of glucose but it allows the regeneration of NAD^+, which keeps the glycolytic pathway ongoing (van Dijken *et al.* 1993). LAB use a similar mechanism but, in their case, acetaldehyde is reduced to lactic acid with regeneration of NAD^+. Homofermentative bacteria only produce lactate whereas heterofermentative bacteria are able to produce both lactate and ethanol through this alternative pathway. Some of these bacteria are also able to produce more energy (one more molecule of ATP) by producing acetate from pyruvate, lactate, or acetaldehyde (Teusink and Smid 2005).

These alternatives are, at first sight, a much less efficient way to obtain energy. However, yeasts like *S. cerevisiae* have learnt to take a special advantage out of the fermentative pathway, even in the presence of oxygen. In particular, the production of ethanol through this pathway causes the inhibition of growth of many other competing microorganisms so that the first thing this yeast does is to produce ethanol to get rid of the competitors. Once all the carbohydrates present in the medium have been converted to ethanol, *S. cerevisiae* then uses ethanol through a reversible mechanism, also carried out by the alcohol dehydrogenase, by which ethanol ends up being reconverted into pyruvate, which can, this time, be used through the respiratory pathway. In order to be able to achieve this switch, *S. cerevisiae* first inhibits the expression of genes for the use of carbon sources alternative to glucose as well as the expression of respiratory genes while in the presence of glucose. Glucose

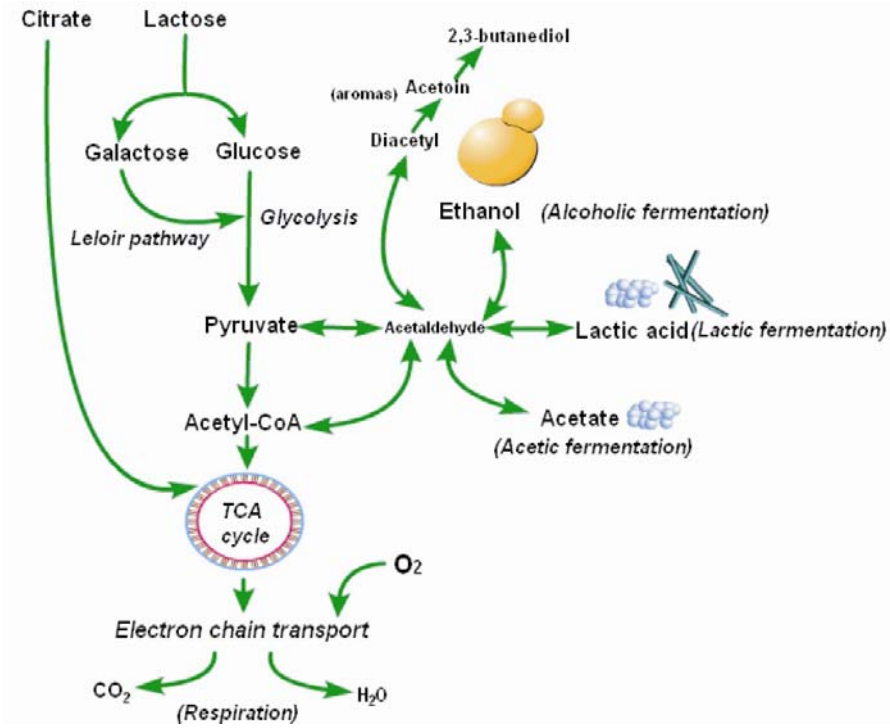

Fig. 6 Microbial metabolization of milk carbon sources. Microorganisms able to use lactose hydrolyze it into its monosaccharide components, glucose and galactose. Glucose enters glycolysis directly, and galactose is converted into glycolytic intermediate via the Leloir pathway. In aerobic organisms, pyruvate is transformed by pyruvate dehydrogenase into acetyl-CoA, which enters the TCA cycle, producing reducing equivalents that can be converted into energy by the mitochondrial electron transport chain, which uses O_2 and releases H_20 and CO_2 in the process (respiration). Citrate from milk enters the pathway at the TCA cycle level (oxalacetate synthesis). Facultative anaerobes produce acetaldehyde from pyruvate by the action of a pyruvate decarboxylase. Acetaldehyde may be reversibly converted into ethanol (alcoholic fermentation, prevalent in certain yeasts), lactic acid (e.g. by lactic acid bacteria), or acetate (e.g. by acetic acid bacteria). Fermentation acts as an alternative pathway independent from oxygen from which cells can obtain energy and reducing equivalents to sustain growth. Yeasts and other microorganisms present on milk have the ability to convert acetaldehyde into diacetyl, acetoin, and 2,3-butanediol, which confer aroma and flavor to dairy products.

repression in this yeast is so strong that, when levels of glucose superior to 0.2% are present in the medium, this inhibitory phenomenon is turned on and causes what is known as Crabtree effect, or deviation of glycolysis towards the production of ethanol regardless of the presence of oxygen. *Saccharomyces* is, therefore, a Crabtree (+) yeast. However, this phenomenon is more the exception than the rule in the world of yeasts. Most yeasts, including those found in dairy products, range from strictly aerobic (e.g. *Cryptococcus*, *Yarrowia lipolytica*, *Schwaniomyces occidentalis*, *Trichosporon*, *Geotrichum*; **Table 3**), to only moderately anaerobic facultatives (*Kluyveromyces*) and have their metabolisms adapted to the utilization of carbohydrates via respiration (Wolf 1996; Barth and Gaillardin 1997; Flores *et al*. 2000; Boutrou and Guéguen 2005).

The type of carbon assimilation mechanism evolved by each yeast greatly depends on the rate of the glycolytic flux, which is, in turn, dependent on the type of sugar encountered in the yeast's normal environment and on its particular ability to transform the sugar into a glycolytic intermediate (van Dijken *et al*. 1993). In general, low glycolytic fluxes are associated to respiration, and are influenced by the utilization of sugars, such as disaccharides, which require previous transport and/or break down mechanisms to obtain the monosaccharide components, and/or by the need of additional catalytic steps to convert these monosaccharides into glycolytic intermediates. The expression of all of these activites and their relative catalytic strength greatly influence the rate of glycolysis. When a sugar is quickly transported inside the cell and converted into glycolytic intermediate (e.g. in the case of glucose transport in *Saccharomyces*, which has up to 20 genes encoding for glucose/hexose transporters; Özcan and Johnston 1999) a high flux through the glycolytic pathway happens that saturates the activity of pyruvate dehydrogenase, leading part of the pyruvate generated towards the production of ethanol (Pronk *et al*. 1996). In yeasts in which the conversion of a sugar is not done at a fast rate, this switch is not possible, and then they experience Kluyver effect, which is described as the inability to grow on certain sugars in the absence of respiratory pathway (Fukuhara 2003).

When comparing two of the most characterized yeasts found in dairy products, *S. cerevisiae* and *K. lactis*, it is possible to appreciate how the former has been adapted to fermentation and the latter to respiration/assimilation of carbon sources. Yeasts from these two genera where separated in evolution prior to the whole-genome duplication event suffered by the closest ancestor of *S. cerevisiae* (Dujon *et al*. 2004). The occurrence of such a phenomenon has provided *S. cerevisiae* with a great amount of genetic redundancy. In particular, *S. cerevisiae* can rely in glycolysis as the main pathway to catabolize carbon sources because it has two or more genes expressing enzymes for each of the glycolytic steps. However, *K. lactis* only has one copy, in most cases, for each of these essential functions so that this yeast has to rely in a different mechanism for back up. In *K. lactis*, the pentose phosphate pathway is particularly active in a way that is able to by-pass several of the glycolytic steps. But, in order to maintain this back-up system, *K. lactis* is forced to keep a respiratory lifestyle, because this pathway needs reoxidized NADPH, and the main pathway for NADPH reoxidization depends on mitochondrial external alternative dehydrogenases that catalyze the transfer of electrons to the ubiquinone in the respiratory chain (Overkamp *et al*. 2002). For this reason, *K. lactis* has a pyruvate dehydrogenase branch twice as active and a pyruvate decarboxylase branch half as active as in *S. cerevisiae* (Zeeman *et al*. 2000), only initiating alcoholic fermentation in conditions of very low oxygen, such as when the population reaches high cell density (Kiers *et al*. 1998). Other reason thought to be forcing *K. lactis* to keep a predominantly respiratory metabolism seems to be the lack of sterol uptake systems. The biosynthesis of sterols requires oxygen, but under anaerobiosis, *S. cerevisiae* can import sterols from the environment (Wilcox *et al*. 2002). Genes involved in this process have not been found in *K. lactis* (Snoek and Steensma 2006).

All of these phenomena are important in terms of the type of carbohydrates and conditions in which each yeast will be able to grow while they are in the milk environment. As we will see next, some yeasts will be directly able to take up lactose as a carbon and energy source whereas others will have to rely in the metabolic by-products released by the lactose-consuming yeast and bacteria in order to be able to survive in milk and dairy products. The relative ability of yeasts to grow aerobically, anaerobically or both, and to use certain carbon sources via respiration or fermentation will also condition their particular location in the dairy product and their temporary prevalence during different stages of the fermentation process.

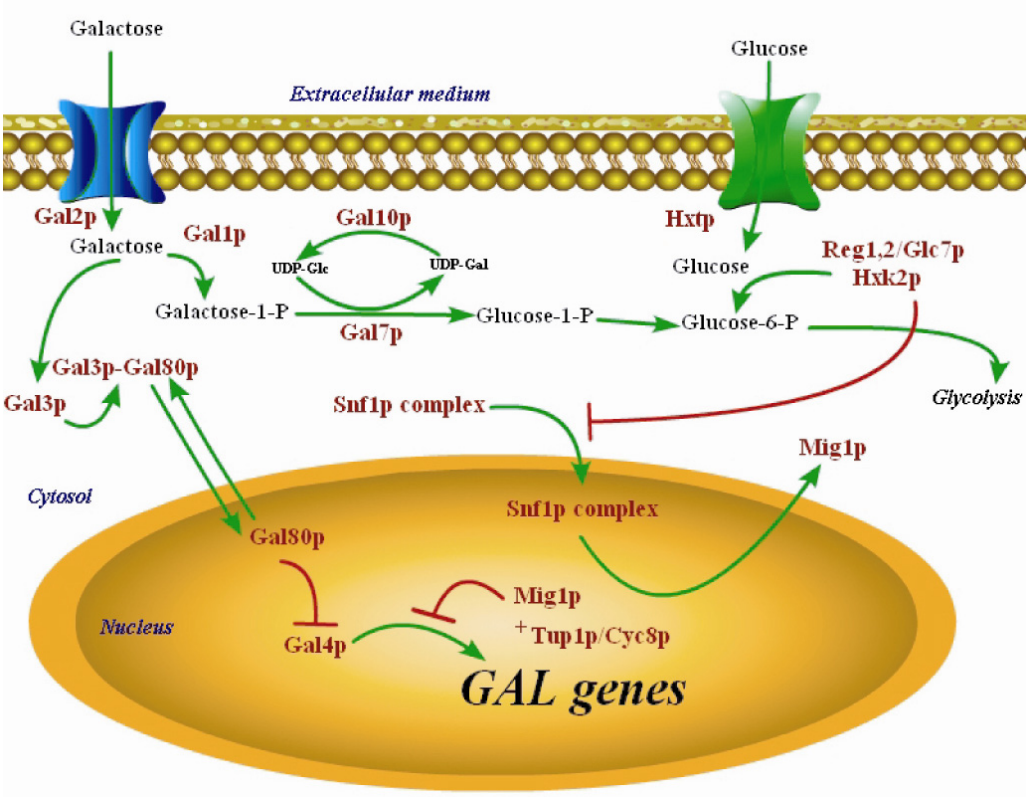

Fig. 7 Galactose utilization pathway from *Saccharomyces cerevisiae*. Galactose is internalized through the action of a galactose permease, Gal2p. In the cytosol Gal3p binds to galactose and ATP, which allows it to bind to Gal80p with more efficiency. This shifts the equilibrium of Gal80p molecules towards the cytosol, which releases the transcriptional activator Gal4p from Gal80p repression. Gal4p is then able to recruit the transcriptional machinery and activate the expression of GAL genes. This increases the levels of enzymes from the Leloir pathway (Gal1, Gal7, and Gal10), which convert galactose in a glycolytic intermediate to allow its metabolization. Glucose enters cells through the action of a multiple set of hexokinase transporters (Hxt) and, once inside the cell, proteins such as Reg1,2 and Glc7, trigger a series of phosphorylation/dephosphorylation events that inactivate the Snf1 kinase complex, impairing its translocation to the nucleus. This allows the glucose repressor Mig1p to recruit the corepressor complex Tup1-Cyc8 to *GAL* and other genes, therefore, inhibiting their expression. When the levels of glucose are low, or the yeasts are in the presence of non-fermented carbon sources, this event is reversed so that Snf1 kinase can travel to the nucleus where it phosphorylates the glucose repressor Mig1p. This favors its export back to the cytosol, which releases *GAL* and other genes from glucose repression.

Yeasts with the ability to metabolize lactose and galactose

As we can observe in **Table 3**, in spite of the many species of yeasts that can be found in dairy products, only a few of them are actually able to ferment and/or assimilate lactose and galactose, which are the main sugars present in milk. The remaining yeasts take instead profit of the metabolism of Lac^+/Gal^+ bacteria and yeast living on milk, using their metabolic by-products (lactic acid, citrate, succinate, etc.) as carbon sources. Interestingly, recent studies have shown that a common ancestor of many of the modern yeasts seemed to have the entire GAL genetic pathway and that this pathway has been progressively lost because of adaptation to new ecological niches (Hittinger *et al*. 2004). Most of the studies of genetic regulation of genes involved in the utilization of lactose and/or galactose have been carried out in *K. lactis* and *S. cerevisiae*, therefore, an extensive part of this section will describe the main characteristics found for this regulatory network in the two yeasts (for a more extensive review, see Johnston 1987; Lohr *et al*. 1995; Schaffrath and Breunig 2000; Bhat and Murthy 2001; Rubio-Texeira 2005, 2006; Traven *et al*. 2006; Rubio-Texeira 2007).

S. cerevisiae internalizes galactose through the major galactose permease encoded by *GAL2* (**Fig. 7**; Tschopp *et al*. 1996). Once in the cytosol, galactose is converted into the glycolytic intermediate glucose-6-phosphate through the concerted action of Gal1p (EC 2.7.1.6), a galactokinase that phosphorylates galactose to galactose-1-phosphate; Gal10p (EC 5.1.3.2), an uridine diphosphoglucose 4-epimerase that interconverts UDP-glucose into UDP-galactose, and *vice versa*; Gal7p (EC 2.7.7.12), a galactose-1-phosphate uridylyltransferase that uses the UDP-glucose to produce glucose-1-phosphate; and, finally, Gal5p, a glycolytic enzyme that converts glucose-1-phosphate into glucose-6-phosphate (Leloir pathway; Leloir 1971). The expression of the genes encoding the first three activities, *GAL1*, *GAL7*, and *GAL10*, is tightly regulated by carbon sources through the interplay of three major regulator proteins: Gal4p, the transcriptional activator; Gal80, Gal4p repressor; and Gal3p, Gal80p repressor and inducer of the *GAL* regulon.

Gal4p is expressed constitutively and binds as a dimer to specific galactose upstream activating sequences, UAS_G (Marmorstein *et al*. 1992; Liang *et al*. 1996). Gal80p binds to Gal4p with 1:1 stoichiometry and blocks its interaction with the transcriptional machinery (Johnston *et al*. 1987; Ma and Ptashne 1987). *GAL* genes that have more than one UAS_G are more tightly regulated since interactions between adjacent Gal80p dimers increase shielding of the activation domains fo Gal4p (Melcher and Xu 2001). According to this, levels of induction of the GAL genes are variable, depending on the number of UAS_G. Genes with more than one site (*GAL2*, *GAL1*, *GAL7*, *GAL10*) are more tightly repressed in the absence of galactose and show the highest difference between basal and induced states (up to 1,000-fold increase). The regulatory genes only have one UAS_G, so their basal levels of expression are higher and their induction is relatively much lower (Lohr *et al*. 1995).

Gal3p interacts with Gal80p in the cytosol in such way that, when galactose is present, it binds galactose and ATP adopting a conformation which binds Gal80p with high affinity (Suzuki-Fujimoto *et al*. 1996; Blank *et al*. 1997; Peng and Hopper 2000; Timson *et al*. 2002; Lakshminarasimhan and Bhat 2005). This interaction shifts the equilibrium of Gal80p molecules towards the cytosol, releasing Gal4p from Gal80p-mediated inhibition (Pilauri *et al*. 2005).

Gal3p is a paralogue of the Gal1p that has lost the galactokinase activity (Bhat and Hopper 1990). Gal1p is, therefore, a bifunctional galactokinase/galactose sensor and inducer, although its sensor/inducer activity is weaker than that of Gal3p (Bhat and Hopper 1991; Platt *et al*. 2000).

Upon induction, Gal4p is able to recruit the transcriptional pre-initiation complex, RNA polymerase II, the mediator coactivator complex, and the chromatin-remodeling complex, SAGA (Traven *et al*. 2006). Using the *GAL* genes as the model, it has recently been found that members of these complexes act as scaffold to confine genes to the internal nuclear envelope, close to the nuclear pores, in order to couple transcription to nuclear export of the transcripts, 'gene gating' hypothesis (Cabal *et al*. 2006; Taddei *et al*. 2006). It has also been shown that, for transcription elongation to proceed, Gal4p needs to be turnovered by the ubiquitin proteasome system (Muratani *et al*. 2005). Nuclear recruitment by active Gal4p of proteasomal subunits seems to also contribute to the expression of *GAL* genes but in a way that is, apparently, independent from proteolytic turnover of Gal4p (González *et al*. 2002; Nalley *et al*. 2006; Sulahian *et al*. 2006).

GAL genes are repressed when *S. cerevisiae* is in the presence of its favorite carbon source, glucose. Glucose repression is, in a major extent, mediated by the action of the repressor protein Mig1, which binds to the *GAL* promoters in upstream repressor sequences, URS_G (Nehlin and Ronne 1990; Cassart *et al*. 1995). Mig1p binding to the URS_G in the *GAL3*, *GAL1*, and *GAL4* promoters reduces by ~5-fold the levels of inducer, galactokinase, and transcriptional activator, which enhances Gal80p-mediated repression, causing, overall, up to 1000-fold repression in some of the *GAL* genes (Lohr *et al*. 1995). Mig1p represses *GAL* expression because of recruiting the general co-repressor complex Cyc8-Tup1 to the promoters (Papamichos-Chronakis *et al*. 2004). In the absence of glucose, the Snf1p kinase complex is able to travel to the nucleus, where it phosphorylates Mig1p, favoring its export back to the cytosol and, this way, releasing *GAL* and other genes from glucose repression (Treitel *et al*. 1998; DeVit and Johnston 1999; Vincent *et al*. 2001; Papamichos-Chronakis *et al*. 2004).

K. lactis shares most of the components acting in the GAL pathway from *S. cerevisiae*, with a few but important exceptions. *K. lactis* is fundamentally adapted to like in milk and dairy products and it has, therefore, developed a system for the transport and intracellular hydrolysis of lactose, based in the lactose permease gene, *LAC12*, and the β-galactosidase gene, *LAC4* (Sreekrishna and Dickson 1985; Leonardo *et al*. 1987; Gödecke *et al*. 1991). A new hexose transporter has recently been found in this yeast, Kht3p, with galactose transporter activity. However, this activity is much weaker than that of *LAC12* (Wiedemuth and Breunig 2005). *S. cerevisiae* naturally lacks the system for lactose transport and hydrolysis and, therefore, relies in the metabolism of Lac^+ bacteria and yeasts present on milk and dairy products. Another major difference between these two yeasts is that, in *K. lactis* the galactokinase and sensor/inducer functions have not been splited in two paralogs but are instead carried out by the bifunctional KlGal1p (Meyer *et al*. 1991; Zenke *et al*. 1996; Vollenbroich *et al*. 1999; Menezes *et al*. 2003). The fundament inherent to this difference seems to be the fact that an active galactokinase acting in early stages of the GAL induction pathway may lead to the toxic accumulation of galactose-1-phosphate before the other enzymes of the Leloir pathway reach enough levels to eliminate the compound. This would not happen in *K. lactis* because induction of the Leloir pathway is subjected to concomitant induction of the *LAC4*, and *LAC12* genes, so that not enough galactose would accumulate in the cells as to produce a build-up of the toxic intermediate before the Leloir pathway becomes fully activated.

Further differences also exist in the interaction of KlGal1 with KlGal80. In *K. lactis*, KlGal1p is apparently able to reach the nucleus, so that it competes for KlGal80p binding both in the nucleus and in the cytosol. This has been proposed as a feedback mechanism of KlGal80p to control the levels of galactokinase activity, a phenomenon that seems more relevant in *K. lactis* and that it may be helping the yeast to use first the glucose that is simultaneously released from lactose hydrolysis (Anders *et al*. 2006).

The *K. lactis GAL4* gene has an UAS_G on its promoter, which causes autogenous regulation and higher basal levels of the transcriptional factor, and consequently of the remaining *GAL* genes (Dong and Dickson 1997). Because of this, induction only cause up to 100-fold increases in the levels of *GAL* gene expression, although the net amounts of the galactose enzymes are comparable to the levels reached by those of *S. cerevisiae* upon induction. This phenomenon, along with the fact that only *KlGAL1* gene has one URS_G, indicates that this yeast has a permanently semi-induced galactose pathway, less sensitive to glucose repression, and ready to rapidly respond to the presence of galactose, in accordance with the particular ecological niche in which *K. lactis* has evolved.

Sensitivity to glucose is, in yeasts, largely dependent on the type and relative substrate-affinity of their sugar transporters. *S. cerevisiae* has a constitutive low-affinity glucose uptake and a glucose-repressible high-affinity glucose uptake, which result from the combined expression a total of 20 genes encoding for glucose/hexose transporters (HXT) in this yeast (Özcan and Johnston 1999). *K. lactis* has instead a gene encoding a high-affinity, constitutively expressed glucose transporter, *HGT1*, and a gene encoding a low-affinity, glucose-inducible transporter, *RAG1* (Chen *et al*. 1992; Wésolowski-Louvel *et al*. 1992). Certain *K. lactis* isolates have been found that have two hexose transporter encoding genes, *KHT1*, and *KHT2*, instead of *RAG1* (Breunig 1989). *KHT1* is almost identical to *RAG1*, and it is believed that *RAG1* may have arisen by recombination between former *KHT1* and *KHT2*. Strains carrying *KHT1* and *KHT2* genes show the highest level of glucose repression (Chen *et al*. 1992; Weirich *et al*. 1997). Natural isolates have also been found that are totally insensitive to glucose repression because of carrying a defective *rag1* allele (Goffrini *et al*. 1989; Suleau *et al*. 2006).

S. cerevisiae and *K. lactis* sense extracellular levels of glucose through the action of a glucose-sensor system, which is encoded by *SNF3* and *RGT2* in *S. cerevisiae*, and by *RAG4* in *K. lactis* (Betina *et al*. 2001; Santangelo 2006). In the presence of low concentrations of glucose, *S. cerevisiae* glucose sensor stimulates phosphorylation of the regulators Mth1p and Std1p by Yck1/2p, targeting them for degradation by the ubiquitin-proteasome system. This derepresses Rgt1p, stimulating expression of the *HXT* genes. *K. lactis* glucose sensing has similar components but seems to work in a different way. In this yeast, KlRgt1p normally represses *RAG1* expression in low levels of glucose, unless it is repressed by Rag4p- and Rag8p (casein I-like protein)-dependent phosphorylation, in response to high levels of glucose. It has been argued that this difference is due to the fact that, in normal conditions, *K. lactis* is rather faced to low levels of glucose, relying mainly in respiration because of the low glycolytic flux determined by the sole expression of the high-affinity transporter Hgt1p. The low-affinity transporter Rag1p is, instead, tightly regulated because it responds to unusual conditions for this yeast, i.e. high environmental levels of glucose (Betina *et al*. 2001).

Additional properties that make yeasts 'good milk growers'

Besides the ability to metabolize milk carbon sources, there are additional characteristics that help certain yeasts to thrive on milk and dairy products. Optimal properties for growth on these media include the ability to grow at relatively high/low temperatures, in acidic pH, to resist conditions of high salinity, and to produce strong lipolytic and proteolytic activities to use fat and protein from milk as additional sources of carbon, nitrogen, and energy.

Regarding the first of these characteristics, most yeasts

mentioned in **Table 3** have an optimum growth temperature ranging from 25-30ºC, but can also sustain growth at temperatures as low as 5-10ºC, and as high as 37-40ºC (or even higher, as in the case of *Pichia jadinii*, that can grow at 44ºC; Fröhlich-Wyder 2003). This ability is of particular interest for the contribution of yeasts to the organoleptic properties of dairy products, since part of the processing of these products occurs at the two extremes of this wide temperature interval.

Most yeasts are also able to grow, and some of them even have optimum growth rates, at slightly acidic pH (Fröhlich-Wyder 2003). They are, in fact, able to use lactic, citric, and succinic acids produced by the previous metabolism of LAB. As mentioned before, this ability is crucial for the further maturation of certain dairy products since it leads to an increase in pH that enables acid-sensitive LAB to continue growth and, therefore, to continue their participation to the properties of the final product. Perhaps the only exception in this regard is *Ya. lipolytica*, which uses lactic and citric acids but has been reported to release formic acid, therefore, not contributing as much to the increase in pH generally caused by yeasts (Freitas *et al*. 1999; Wyder and Puhan 1999).

Manufacturing of products such as cheese involves addition of salts. Certain yeasts and, in particular *Deb. hansenii*, are exceptionally adapted to life in high-salt environments (Prista *et al*. 2005). This yeast can be considered halophilic rather than halotolerant. It does not seem to have evolved specific genes for the ability to grow in salts but it has, instead, developed particular mechanisms that, altogether, account for its unique adaptation to growth in the presence of high concentrations of NaCl. For instance, enzymes leading to pyruvate in the glycolytic pathway are inhibited by high salinity in the medium, which causes the glycolytic flux to be diverted to glycerol synthesis (Neves *et al*. 1997). The synthesis of additional osmotic stress protective compounds like arabinitol, trehalose, glutamic acid, and alanine, are also enhanced under these conditions (Jovall *et al*. 1990). *Deb. hansenii* also accumulates glycerol inside the cells through an active glycerol-Na^+ symporter, and it has been argued that a special composition of the plasma mebrane and cell wall may prevent leakage of glycerol out of the cells, at the same time as protecting them in case of hypoosmotic shock (Lucas *et al*. 1990). *Deb. hansenii* has a stronger extrusion mechanism for sodium than *S. cerevisiae*, and potassium uptake is not inhibited by sodium as it is in *S. cerevisiae* (Norkrans and Kylin 1969). Finally, it seems that sodium protein targets are, in *Deb. hansenii*, more resistant to sodium (Prista *et al*. 2005).

As it has previously been mentioned, several yeasts present in dairy products have an important contribution to the maturation of the products because of their lipolytic and proteolytic activities, which greatly influence the definitive texture, aromas, and flavors (and that this, in an uncontrolled manner, may also lead to spoilage of the product). *Ya. lipolytica* has a particularly active glyoxylate pathway that allows it to aerobically use alkanes, fatty acids, alcohols, and acetate as source of carbon and energy (Barth and Gaillardin 1997). This yeast produces potent extracellular lipases and esterases. In order to be able to internalize lipids it produces liposan, an extracellular emulsifier that forms small fat droplets at the cell surface which can, this way, be internalized (Cirigliano and Carman 1985). Once inside the cells, lipids are hydroxylated by the endoplasmic reticulum-associated cytochrome P-450, and further oxidized to acetyl-CoA (Iida *et al*. 2000). Other yeasts like *Geo. candidum* also produce numerous extracellular lipases, some of them showing specificity for unsaturated fatty acids (Boutrou and Guéguen 2005). Lipases are very important contributors to flavor since the degradation of lipids leads to the production of many potential flavor and fragrance compounds (e.g. esters, carboxylic acids, lactones, esters, ketones, alcohol, ethyl acetate, isoamyl acetate, and macrocyclic musk fragrances; Jollivet *et al*. 1994; Marilley and Casey 2004; Waché *et al*. 2006).

These and other yeasts and moulds (in cheese) have strong proteolytic activities as well. Yeasts, like *Ya. Lipolytica* and *Geo. candidum*, have both extracellular and intracellular proteases and aminopeptidases with convenient neutral or slightly acidic optimum pH (Boutrou and Guéguen 2005). *Deb. hansenii*, *K. lactis*, and *K. marxianus* produce intracellular and extracellular proteinases that hydrolyze the milk caseins (Kumura *et al*. 2004). These proteolytic activities have an important role in the generation of aromas and flavors. *Geo. candidum* has a particular effect in the reduction of bitterness through the activity of its aminopeptidases, which hydrolyze low molecular weight-peptides originated from the degradation of β-caseins by other microorganisms like moulds (Boutrou and Guéguen 2005). These yeasts also produce flavoring compounds through amino acids catabolism. Amino acids can be converted into α-ketoacids, then to aldehydes, by oxidative deamination. Aldehydes are reduced to primary alcohols (alcoholic/floral flavors) or oxidized to acids (Marilley and Casey 2004). For example, methyl ketones with fruity, floral, mouldy, cheesy, wine aromas, and 2-phenylethanol (fade-rose odor) were found in an analysis of the spectrum of volatile compounds from cheese deacidified by *Deb. hansenii* (Leclercq-Perlat *et al*. 2004). Generation of methyl ketones by *Geo. candidum* also intensifies flavors in mould-ripened cheeses, such as Camembert (Molimard and Spinnler 1996). *Deb. hansenii* and other cheese-ripening yeasts also synthesize *S*-methylthioacetate, an additional sulfur volatile responsible for the strong flavor of Cheddar and Camembert (Ferreira and Viljoen 2003). *Deb. hansenii* is also responsible for the production of the flavoring and aroma compound, methylthiopropanal (Arfi *et al*. 2002). Other products of amino acid catabolism, biogenic amines, are also responsible for the less pleasant fishy and putrid odors from certain cheeses like blue cheese (Gardini *et al*. 2006). *Ya. lipolytica* and *Geo. candidum* produce aminotransferases, and these enzymes are responsible for the conversion of L-methionine into methanethiol (cabbage flavor) and other sulfides which also contribute to the aroma of smear-ripened cheeses (Bondar *et al*. 2005).

In summary it can be said that, at the same time as yeasts being able to grow in the milk carbon sources –along with LAB- help Lac^-/Gal^- yeasts to grow by producing assimilating carbon sources, strong lipolytic/proteolytic yeasts/moulds also help the Lac^+/Gal^+ organisms to assimilate the lipids and nitrogen sources that are present on milk, in such way that every nutrient is efficiently utilized by this diverse ecosystem.

METABOLIC ENGINEERING AS AN EMERGING TOOL TO IMPROVE PROPERTIES OF INDUSTRIAL INTEREST IN MILK-GROWING MICROORGANISMS

Biotechnological solutions for the utilization/ elimination of lactose from industrial residues

Because of its high contents in lactose, worldwide dumping of vast amounts of the liquid whey residual fraction from cheese manufacturing industries poses a serious environmental problem. Many interesting reviews are available that discuss the most recent technologies involved in the ideation of new strategies to solve this problem in ways that can be affordable for dairy industries (Moulin and Galzy 1984; Mawson 1994; Yang and Silva 1995; Siso 1996; Rubio-Texeira 2005). Here we will briefly review a few examples of each of the most relevant approaches currently being explored and applied for the elimination of lactose from cheese-whey.

The most successful and less expensive strategies to eliminate lactose from whey usually imply the use of microbial fermentation of this by-product, either by bacteria, fungi, or yeast. Microbial fermentation of whey must lead to products of high-added value whose commercialization ultimately compensates for the cost of the fermentation process used to decontaminate whey from its organic residues. On

the brightest side, cheese-whey is one of the cheapest and more abundant substrates available for industrial microbial fermentations, and its effect in the physiological state of the microbial biomass does not seem to be as hazardous as when other traditional media, such as beer wort and beet molasses, are used (Verstrepen *et al*. 2004). Perhaps its main disadvantage is the low content in organic nitrogen source (since most of the milk proteins were previously separated from the liquid fraction by curdling; see Moulin and Galzy 1984). This inconvenient is usually solved through the addition of nitrogen supplements (e.g. yeast extract) or proteases that hydrolyze the proteins remaining in whey. Concentrative methods to obtain dried or whey permeate are also alternatives that improve the use of whey as microbial growth substrate for fermentation (see reviews above).

Most of the fermentation products have further applications in food and/or health-related industries. This poses a limit in the selection of the microorganism for the fermentative process. Although several microbes are very efficient in the assimilation lactose as a carbon source, only those considered by the United States Food and Drug Administration as GRAS (generally regarded as safe) organisms, such as certain non-pathogenic bacteria, moulds, and yeast, are legally approved for this purpose. Nevertheless, laboratory trials are constantly being carried out using new organisms that may in the future be considered as additional alternatives. For example, a laboratory has analyzed the ability of the ligninolytic enzyme-producer fungus Bjerkandera sp BOS55 to grow on cheese whey as a growth substrate (Feijoo *et al*. 1999). In another case, a strain of *Clostridium acetobutylicum* has recently been tested for growth on whey (Yu *et al*. 2007).

Regarding the use of lactose-positive bacteria, several interesting approaches have, over the years, been mentioned in the literature. For example, the use of methanogenic bacteria has been described, with the ability to digest cheese-whey anaerobically, producing biogas that partially covers for the energy cost of the overall processing (Mawson 1994; Ergüder 2001). The use of LAB, such as *Lactobacillus casei*, or *Lactococcus lactis*, has recently been reported with optimal results (see Flôres and Alegre 2001; or Mondragon-Parada *et al*. 2006, among other examples). Bacterial biomass produced in these fermentation processes has potential applications in the preparation of starter cultures for the production of dairy products such as yogurt, and as probiotic food supplements. Moreover, in several of these approaches, bacteriocins are a natural product of lactic acid-producing bacteria which can be obtained in significant amounts out of cheese-whey fermentation, and that have further applications as food preservatives (e.g. in canned foods) by preventing growth of pathogenic bacteria like Clostridia. In other cases, mixed cultures of bacteria and yeasts, or moulds, are also an option upon analysis. For example, in one case, co-culture of yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) with the lactose-negative yeast *Rhodotorula rubra* has allowed a more efficient assimilation of cheese whey and production of exopolysaccharides by LAB. Exopolysaccharides have industrial interest as thickeners, emulsifiers, gelling and water-binding agents in food products (Simova *et al*. 2004).

The use of lactose-assimilating yeasts and moulds is, perhaps, the most popular in this field. In principle, yeasts with the strongest ability to grow using lactose as carbon source are mostly within the *Kluyveromyces* genus. As explained in a previous section, their Crabtree negative and predominantly oxidative metabolism allows rapid growth of these yeasts in cheese whey. In principle, the generation of biomass from *Kluyveromyces* was aimed at its utilization as source of nutrients in food supplements for domestic livestock and humans (SCP, single cell protein), since this yeast is categorized as a GRAS microorganism (Bonekamp and Oosterom 1994; Belem and Lee 1998). This is largely due to the fact that, twenty years ago, the knowledge of the genetics and physiology of yeasts alternative to *S. cerevisiae* was very limited, whereas *S. cerevisiae* had already multiple applications as industrial biomass in the production of alcoholic beverages and in the generation of bakery food products. The extensive knowledge of *S. cerevisiae* at the molecular level allowed more versatility in terms of its genetic manipulation leading to a microorganism with improved properties. For all of these reasons, between the 80s and 90s several laboratories decided to pursue an interesting new approach: the transfer of genes from lactose-assimilating microorganisms into *S. cerevisiae* by genetic manipulation (for a more extensive review, see Rubio-Texeira 2005, 2006). The approaches followed can be divided into two main classes: the first class consisted in the ideation of strategies for the extracellular hydrolysis of lactose. In the earlier approaches, *S. cerevisiae* was cultured in lactose-pre-hydrolyzed cheese whey either by combining a first phase of microbial fermentation involving an alternative microorganism, or by previous addition of purified β-galactosidase onto the medium (Champagne and Goulet 1988). Later advances in genetic engineering enabled the expression of heterologous β-galactosidases, either secretable or intracellular, in *S. cerevisiae*. In the latter case, simultaneous coupling to a process of controlled cell-permeabilization is required (Porro *et al*. 1991; Compagno *et al*. 1993).

The second class of strategies involved the expression of a genetic system in *S. cerevisiae* that allowed internalization and further intracellular metabolization of lactose. In this latter category, most of the laboratories opted for the genetic transfer of the *LAC12* and *LAC4* genes of the closely related Lac^+ yeast *K. lactis* into *S. cerevisiae* (Sreekrishna and Dickson 1985; Rubio-Texeira *et al*. 1998). Continuous exploration of variants from these two main classes of strategies is still an ongoing (Jurascik *et al*. 2006; Rodriguez *et al*. 2006).

Homologous and heterologous production of high added-value products of industrial interest from the milk sugar lactose

Ideas behind the development of Lac^+ strains of *S. cerevisiae* range from the generation of cell biomass for further industrial applicability (e.g. baker yeast in Adam *et al*. 1999), to the generation of ethanol from lactose (Porro *et al*. 1991; Domingues *et al*. 2001). In some cases, attempts have been made to improve the recollection of cell biomass at the end of the fermentative process by combining in cells the ability to ferment lactose with the property of flocculence (Domingues *et al*. 1999; Rubio-Texeira *et al*. 2000). Elimination of lactose from whey by genetically engineered *S. cerevisiae* strains has, in several cases, been coupled to the simultaneous production of enzymes and/or chemical products of pharmaceutical interest, e.g. human lysozime (Maullu *et al*. 1999), or fructose-1,6-bisphosphate (Compagno *et al*. 1993). One of the most important heterologous products obtained is β-galactosidase itself, either from bacterial (e.g. Porro *et al*. 1992), fungal (Ramakrishnan and Hartley 1993), or alternative yeast sources (Becerra *et al*. 2004).

While part of the effort was initially concentrated in providing the most traditional yeast, *S. cerevisiae*, with interesting properties from less characterized microorganisms, the opposite strategy has not been given up. Several laboratories have continued working on the characterization of alternative microorganisms with the aim to obtain further advantage of all of these important characteristics that are missing in *S. cerevisiae*. Progressive advancement in the genetic and molecular characterization of the physiologies of less conventional yeasts, like *Kluyveromyces lactis*, has led to a gradual shift of preferences, from the use of *S. cerevisiae* back to the use of naturally Lac^+ microorganisms for the assimilation of lactose (Wésolowski-Louvel *et al*. 1996; Gellisen and Hollenberg 1997; Dominguez *et al*. 1998; Schaffrath and Breunig 2000). A more extensive knowledge of these alternative yeasts has allowed the adaptation of techniques for their genetic manipulation, which has been

key for this switch of biotechnological interests. *K. lactis* can now be used to eliminate lactose at the same time as being able to synthesize competitive amounts of heterologous products in fermentative processes (van Ooyen *et al*. 2006). When ethanol is intended as the final product, *S. cerevisiae* is still the best microorganism. However, because of its particular physiology, *K. lactis* is equally hard to beat as generator of biomass and concomitants products, when the growth of both yeasts in cheese whey, or in any other lactose-containing medium, is compared.

As we have seen in a previous section, although *S. cerevisiae* lacks the genetic tools to assimilate lactose, it shares with *K. lactis* many components of the genetic pathway for the assimilation of galactose. Induction of the expression of genes in the *GAL* pathway picked up an immediate interest in the early 80s, soon after molecular studies of *S. cerevisiae GAL/MEL* regulon were initiated (Johnston 1987; Lohr *et al*. 1995). The ability to switch expression of genes such as *GAL1* from very low levels up to 1,000-fold, led to the idea of using *GAL* promoters for heterologous gene expression (Romanos *et al*. 1992; Stagoj *et al*. 2006). The *GAL* inducible system is, so far, one of the most effective methods to control expression in yeast of homologous and heterologous genes because of its tight regulation by the carbon source present in the growth medium. Several laboratories have recently started to use lactose and galactose promoters, like the *LAC4* promoter from *K. lactis*, to couple lactose assimilation by this yeast to heterologous protein production, obtaining very promising results (van Ooyen *et al*. 2006). In terms of homologous production, the native intracellular *K. lactis* lactase has already been on the market for decades under the trade name Maxilact™ and has a history of safe use (DSM Food Specialties, Delft, The Netherlands; AMFEP 1997). Neutralact®, is the DSM brandname of another commercially available lactase preparation, also produced from a *K. lactis* strain containing extra copies of the *LAC4* gene inserted in the rDNA locus (Coenen *et al*. 2000).

Lactases: relevant enzymes for food and pharmaceutical industries

β-galactosidases are very important enzymes for both food and pharmaceutical industries. In particular, yeast β-galactosidases have special physicochemical properties that makes them especially suitable for certain food biotechnology purposes (as discussed in Siso 1996). Lactose *per se* has important uses as an additive that modifies properties of certain foods, and is also used as a coating component of pills by pharmaceutical industry (Yang and Silva 1995). However, as discussed at the beginning of this review, only a small percentage of humans have enough levels of expression of human intestinal lactase in adulthood (Beja-Pereira *et al*. 2003). Most human populations suffer of variable levels of lactose-intolerance because of deficiencies in the expression and/or activity of this enzyme. Fortunately, many dairy foods no longer contain high levels of lactose, either because the sugar has been eluted in the liquid whey fraction or because of microbial assimilation. However, for the consumption of milk, isolation of β-galactosidases from GRAS sources for their further use in the generation of the more digestible glucose and galactose from lactose has become an important way to solve lactose intolerance (for a review about the problematic of lactose intolerance and associated disorders in humans see Adam *et al*. 2004; for a more detailed review of the most traditional mechanisms developed for the purification of this enzyme from different sources see Gekas and Lopez-Leiva 1985).

In a more recent research, interest has also been focused in the ability of β-galactosidases to generate lactose derivatives by transgalactosylation. Galactooligosaccharides (GOS) consist in a variable number of oligosaccharides bound through β-glycosidic linkages. For example, the utilization of mixtures of fructose and lactose leads to the synthesis of lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). Both GOS and lactulose have great importance as dietary supplements, in special in infant foods, because of their prebiotic activities. Prebiotics are substances that are able to surpass the digestive barriers of the stomach and to reach, practically intact, the intestine where they are metabolized by beneficial intestinal flora. GOS are, therefore, important in the maintenance of this benign microflora, which as mentioned before, prevents colonization by pathogens and helps to strengthen our immune system (Szilagyi 2002).

Transfer of new traits to microbes and other organisms to improve the quality of dairy products

According to Wikipedia (http://en.wikipedia.org/wiki/Metabolic_engineering), 'metabolic engineering' can be defined as the practice of optimizing genetic and regulatory processes within cells to increase the cell's production of a certain substance. Metabolic engineering follows two main different aproaches: in the 'constructive' approach, the metabolism in a wild-type representative of a determined organism is first characterized so that this knowledge can be then applied to make genetic modifications with the aim to improve a relevant phenotype. In constrast, in the alternative approach, 'inverse metabolic engineering', a particular host is genetically modified by transferrence onto that host of genes from a different organism with the aim to express in the host the particular phenotype associated with these genes in the original organism (Bailey *et al*. 1996). In the latter case, full characterization of the host of interest is not necessarily required. Examples of pioneer work in this newly emerging scientific field, that in several cases have led to the desired results, are precisely some of the attempts to eliminate lactose from cheese-whey through the design of genetically-engineered yeasts expressing heterologous genes (**Fig. 8**). More ambitious projects now imply the displacement of entire biosynthetic pathways into new host organisms. As an example, an artificial and fully self-sufficient mammalian biosynthetic pathway for the synthesis of hydrocortisone, involving the transfer of 13 engineered genes, has recently been assembled in yeast (Szcebara *et al*. 2003). Properties of the inducible *GAL* system, both in *S. cerevisiae* and in *K. lactis*, have also been modified either by overexpression of the main regulator genes (Sil *et al*. 2000), by knocking-out the repressor genes (Ostergaard *et al*. 2000), or by elimination of the *GAL1* gene, which allows

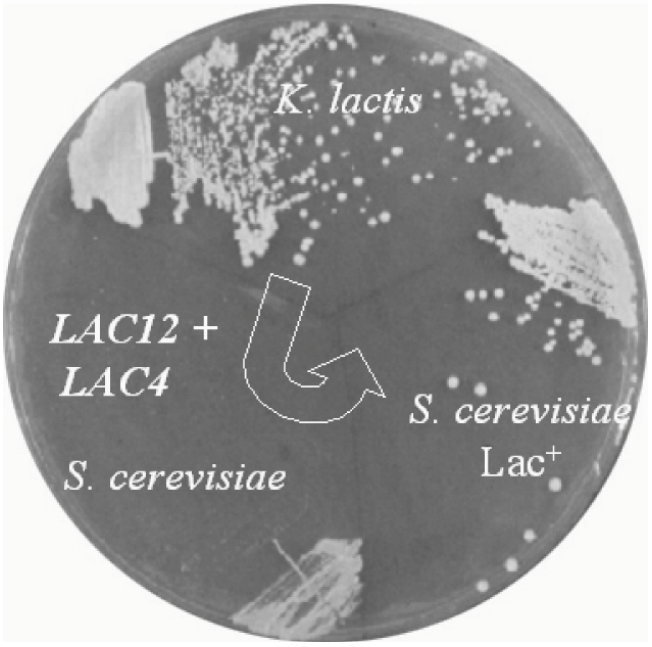

Fig. 8 Example of inverse metabolic engineering to reduce lactose contents of cheese-whey. Development of a *S. cerevisiae* Lac$^+$ strain expressing mitotically stable high levels of *K. lactis LAC4* and *LAC12* genes. (For more information see the text and Rubio-Texeira *et al*. (1998).

induction of the *GAL* genes in the presence of much lower levels of galactose because of the inability of the mutant to metabolize this carbon source (Hsieh and da Silva 2000; Panuwatsuk and da Silva 2003; Kang *et al*. 2005; Stagoj *et al*. 2006). In all of these cases, significant improvements in the levels of production of heterologous proteins from the *GAL* promoter have been observed. New strategies are also being applied to 'humanize' the glycosylation pattern of heterologous yeasts expressed in *S. cerevisiae* or in more efficient secretory yeasts, like *K. lactis*, by genetic modifications in mannosyltransferase genes (reviewed by Gerngross 2004). The recent completion of the genomic sequences of yeasts like *Ya. lipolytica* and *Deb. hansenii* (Dujon *et al*. 2004) also makes now possible to improve the role of these yeasts in the manufacturing of dairy and other fermented products by means of metabolic engineering. For example, both yeasts have the ability to accumulate lipids, serving as source of single-cell oil that can be used as alternative food ingredient (Merdinger and Devine 1965; Papanikolaou *et al*. 2003). This, and other properties, like the synthesis of volatile flavors/aromas, may be modulated through the generation of mutants and/or by modification of different steps of the metabolic pathways involved (Fickers *et al*. 2005; Breuer and Harms 2006).

LAB are also current targets for metabolic engineering with the aim to improve properties of dairy and other products (Teusink and Smid 2005). For example, a group has recently modified two biosynthetic pathways in the dairy starter bacteria *Lactococcus lactis* for the simultaneous overproduction of both folate and riboflavin (Sybesma *et al*. 2004). Novel foods, enriched through fermentation using these multivitamin-producing starters, could compensate the B-vitamin-deficiencies that are common even in highly developed countries, and could specifically be used in dietary foods for the large fraction of the Caucasian people (10-15%) with mutations in the methylene tetrahydrofolate reductase gene. In another case, a *Lactococcus lactis* ssp. *cremoris* has been engineered to make it only able to metabolize galactose but not glucose, with the aim of reducing lactose levels in food derivates at the same time as increasing sweetness of the product, which would prevent for the need to add sweeteners (Pool *et al*. 2006). The glycolytic pathway of LAB is also an interesting target for metabolic engineering since it is possible to divert it at the pyruvate branch from lactate to the production of diacetyl, acetaldehyde, and other important food ingredients (de Vos and Hugenholtz 2004). The ability of LAB to resist digestive enzymes makes them also a suitable vehicle for the oral delivery of heterologously-expressed vaccine antigens (Robinson *et al*. 1997; Mercenier *et al*. 2000).

But metabolic engineering has not remained at the level of microorganisms: transgenic cows have already been created that express human protein in their milk (Whitelaw 1999). Moreover, transgenic mice ectopically expressing rat intestinal lactase-phlorizin hydrolase in the mammary gland have been created that produce milk with a reduction in lactose contents of up to 85% (Jost *et al*. 1999), and chickens expressing β-galactosidase in their intestine have also been generated (Mozdziak *et al*. 2003). These achievements inspire hope for a future in which some of the restrictions applied to genetical engineering of livestock for food purposes may be finally overcome. For instance, lactose-intolerance could be solved through the use of modified cattle expressing lactase in their mammary gland to reduce the contents of lactose in milk. Generation of such clones would avoid the expenses resulting from the microbial fermentations and/or purification in large quantities of β-galactosidase for its use in the elimination of lactose from milk. However, years will go by before legislations worldwide ensure that this ultimate jump to mammalian systems is safe for human health.

Meanwhile, advances in all of the newest functional genomics ('ome') technologies are key for the develoment of succesful metabolic engineering strategies to solve biotechnological problems in the use of microbial systems. Whole-genome sequence analysis, genome-wide genetic screens, transcriptional profiling (transcriptome), analysis of the whole protein contents through microarrays, 2D-gels, and mass spectrometry (proteomic analyses), analysis of the rate at which cellular pathways work and interact (fluxome analysis), analysis of the total cellular metabolite profiling (metabolome), and integration of all of these data through informatic analysis (systems biology), are the fields where most efforts are now being focused and from which we will get in a near future, further advantages in the use of microbes to improve food processing and human health.

CONCLUDING REMARKS

After thousands of years of unintended use of microbes for the manufacture of dairy and other food products, we have arrived to an era in which we can finally start to understand the properties of milk and of the microorganisms that modify it, at a molecular level. Physicochemical characterization of milk has allowed us to find the causes behind conditions such as lactose intolerance, and learn how to put remedy to this problem. The secrets of the healthiness conferred by the consumption of certain dairy products are now unveiled after the isolation, identification, and physiological characterization of the beneficial microflora responsible for their making. Our advances in microbiology have also led us to be able to differentiate between microorganisms on milk that may do us good from those that might get us sick. In response to this knowledge, humans have evolved methods to sterilize, sanitize, and prevent contaminations by these harmful microorganisms, while promoting growth of beneficial flora.

Furthermore, the recent progress in our molecular understanding of the genes and biochemical pathways behind the generation of compounds improving the organoleptic qualities of food now enables us to manipulate the production of such desirable traits by means of the emergent science of metabolic engineering. Completion of 'Omic' analyses combined to systems biology approaches is all we need next in order to be able to generate all of these engineered microbial cell factories that will allow us to improve the industrial processing of fermented food products.

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