Yogurt: microbiology, organoleptic properties and probiotic potential

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Fermented Food—Part II: Technological Interventions
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1. Introduction: An Ancestral Fermented Food with an Expanding Contemporary Market

Fermentation has been used for thousands of years to preserve food. Thanks to the acidifying activity of bacteria, the shelf life of milk is increased because the growth of undesirable microorganisms is prevented.

Traces of fermented milk products apppear rather quickly after the emergence of agriculture, as early as 8,000 B.C. in Turkey and Eastern Europe. Based on the presence of milk lipids recently discovered on pottery shards, the inhibitants of what is now modern-day Libya were consuming fermented dairy products around 7,000 B.C. (Dunne et al. 2012). Traces of kefir have also been detected on a Bronze Age mummy in China (Yang et al. 2014). Yogurt seems to make an appearance around 5,000 B.C. and was discovered by nomadic peoples living in the Middle East. It has been consumed for thousands of years by different civilizations. "Yogurt" comes from the Turkish word "yogurmak," which means to thicken, coagulate, or curdle.

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In France, yogurt appears around 1542. King Francis I, who suffered from chronic diarrhea, was cured by eating yogurt. In 1905, Stamen Grigorov, a Bulgarian medical student studying in Geneva, Switzerland, was the first to describe the spherical and rod-shaped lactic acid bacterium that is found in Bulgarian yogurt; the species was named *Bacillus bulgaricus*. Then, in the 20th century, Russian Nobel laureate Elie Metchnikoff, a scientist at the Pasteur Institute in Paris, hypothesized that Bulgarians lived unusually long lives because they regularly consumed yogurt; his research helped make yogurt popular in Europe and served as the foundation for the field of probiotics, which is still growing a century later.

Another major event in yogurt's history was the food's transformation into a commercial product by Isaac Carasso in 1919, in Barcelona, Spain. Yogurt's commercialization was taken further by Danone, a private company, and the food was industrialized and spread throughout Europe starting in the 1960s.

A traditional food that is consumed on a daily basis in the Middle East and Europe, yogurt is currently expanding its market across the globe. Demand has grown dramatically in North and South America, as well as in Asia (>100% between 2000 and 2010 for yogurt and fermented dairy products; Mikkelsen 2013). At present, more than 30% of the world's population eats yogurt, and worldwide yogurt consumption has hit around 15 million tons per year. The global yogurt market was projected to surpass $65 billion in 2015 (www.strategyr.com). Traditional yogurts—produced at small scales—currently coexist with industrially produced yogurts, and we are seeing renewed interest in homemade foods. Yogurt's nutritional value and healthful properties are universally recognized. The food has a positive market image, attributable to its specific organoleptic properties (fresh taste, sourness, unique aroma), which has improved following the discovery of its probiotic properties and society's movement toward greater health consciousness.

Producing yogurt requires milk to acidify, whereupon curds are formed. This acidification process, which has to be rapid in industrial settings, largely depends on the growth and activity of bacteria that produce lactic acid by fermenting lactose. The association between the two yogurt lactic acid bacteria (LAB) *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lb. bulgaricus*) is regarded as a protocooperation because it is beneficial for both species, but each bacterium can grow alone in milk (Tamime and Robinson 1999). This protocooperation has industrial importance because it can improve yogurt's properties, such as the texture (*via* exopolysaccharide production; Bouzar et al. 1997), the acidification rate (Pette 1950c, Moon and Reinbold 1976, El-Soda et al. 1986, Amoroso et al. 1988, Beal and Corrieu 1991, Bautista et al. 1996), and the flavor (*via* the production of aromic compounds; Hamdan et al. 1971, Bottazzi et al.

1973, El-Abbassy and Sitohy 1993, Courtin and Rul 2004). This association at least partly relies on metabolite exchanges and involves elements of competition (for the nutrients in the milk) and mutualism (the fellow bacterium synthesizes and hydrolyzes metabolites).

In 1984, the FAO/WHO defined yogurt as "the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lb. bulgaricus*) and *Streptococcus thermophilus* from milk and milk products. The microorganisms in the final product must be viable and abundant." If other bacteria are added, such as probiotics (e.g., *Bidobacteria*, *Lactobacilli* spp.), the product must be called "fermented milk" and cannot carry the yogurt label. The Codex Alimentarius entry for fermented milk (Codex STAN 243-2003) specifies that yogurt should contain a minimum of 2.7% (m/m) milk proteins, a maximum of 15% milk fate, a minimum of 0.6% titratable acidity (expressed as % of lactic acid), and a minimum of 10^7 CFU/g of microorganisms (total microorganisms in the starter culture). Yogurt has highly attractive nutritional properties—it is low in calories (around 90 kcal per serving) but contains enough macro- and micronutrients (proteins, fatty acids, calcium, phosphorus, and vitamins) to cover a person's daily needs.

The purpose of this chapter is to provide an overview of the nature of such bacterial associations in yogurts, describe the interactions among the bacteria involved, and detail how bacterial metabolic activities impact the properties of the end product. We will focus on traditional yogurts; yogurts or fermented milk products that contain probiotic species (such as *Bifidobacterium* or *Lactobacillus* spp.), stabilizers, added aromas, or other additives will not be discussed.

2. A Fermented Food Originating in a Mutually Beneficial Association between Two Thermophilic LAB Species

2.1 How Yogurt Bacteria Grow Together in Milk

Typically, yogurts are produced at temperatures around 42°C, which promotes the optimal growth of both *S. thermophilus* and *Lb. bulgaricus*. When milk is inoculated with these two bacteria, they usually grow in succession and *S. thermophilus* presents diauxic growth (Tamime and Robinson 1999, Courtin et al. 2002, Letort et al. 2002, Courtin and Rul 2004, Sieuwerts et al. 2010; Fig. 1). *S. thermophilus* first grows exponentially (for the first 90 to 120 min) and then experiences a short latency period. *Lb. bulgaricus* stays at inoculation levels. *S. thermophilus* subsequently resumes growth, albeit at a reduced rate, and *Lb. bulgaricus* starts to grow exponentially. As the milk becomes acidified, reaching a pH of around 5.2, *S. thermophilus* stops growing. In contrast, the growth of *Lb. bulgaricus* continues until

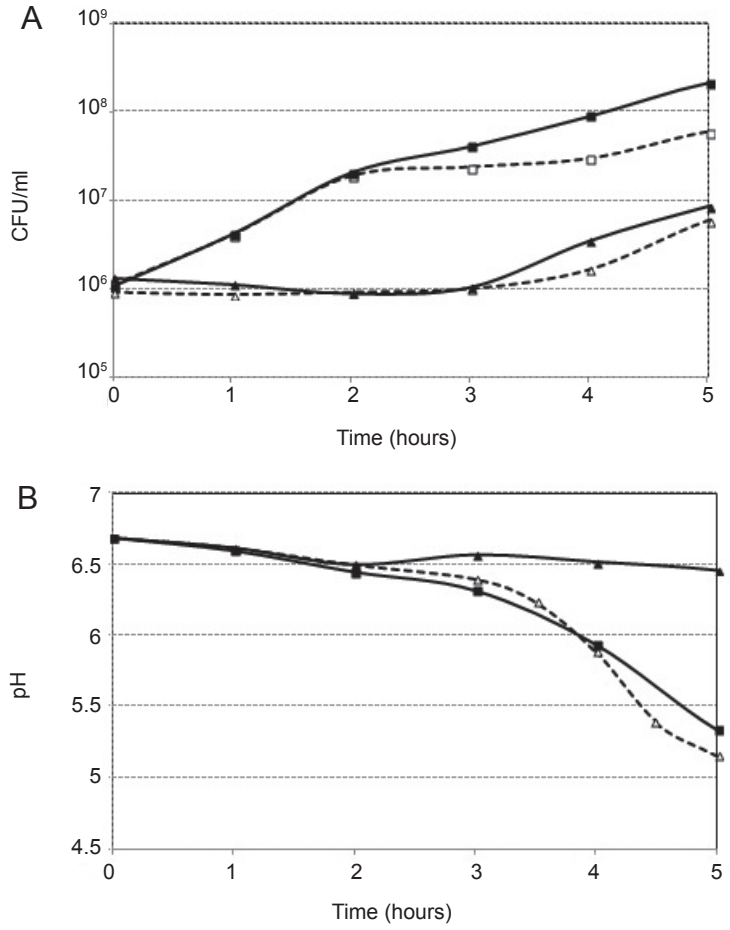

Figure 1. (A) Growth curves of *S. thermophilus* CNRZ385 in pure cultures (□) or co-cultures (■) and of *Lb. bulgaricus* CNRZ398 in pure cultures (▲) or co-cultures (△); cultures were grown in microfiltered milk (Marguerite®).

(B) Acidification curves of milk inoculated with *S. thermophilus* CNRZ385 (■), *Lb. bulgaricus* CNRZ398 (▲), or both bacteria (△).

pH levels drop to around 4.4 (Beal and Corrieu 1991). The acidification curves usually correlate well with these growth patterns (Fig. 1). The first round of acidification corresponds to *S. thermophilus*' period of exponential growth; during the latency period, there is a slowdown in acidification. Then, acidification accelerates with the tandem growth of both bacteria.

Lb. bulgaricus is better than *S. thermophilus* at handling the acidic environment, possibly partly because it can transform ornithine into putrescine, which raises the intracellular pH (Azcarate-Peril et al. 2004).

Despite *S. thermophilus*' greater sensitivity to acidity, this bacterium generally has a numerical advantage over *Lb. bulgaricus* by the end of fermentation (Pette and Lolkema 1950a, Beal et al. 1994, Courtin and Rul 2004, Herve-Jimenez et al. 2008, Ben-Yahia et al. 2012), even when *Lb. bulgaricus* starts off at a higher inoculum level (Béal and Corrieu 1991). This advantage is strain dependent and can be partly explained by the fact that *Lb. bulgaricus* has stricter nutritional requirements. Also, *S. thermophilus* is probably a better competitor than *Lb. bulgaricus* in milk.

2.2 What Genome Analyses Tell us About the (Co)evolution of the Two Yogurt Bacteria?

Growth in yogurt involves several metabolic activities that bacteria have conserved and/or re-enforced over the course of evolution and that are directly related to milk composition. The physiology and metabolic activity of these two LABs have been studied for decades. More recently, the advent of sequencing and post-genomic tools has resulted in a better, more complete picture of how these bacteria evolved and how they have adapted to milk. Analysis of the genomes of *S. thermophilus* and *Lb. bulgaricus* suggests that the two bacteria have coevolved, which has resulted in optimized joint growth. Horizontal gene transfers (HGTs) may be taking place between the two: exopolysaccharide (EPS) genes may be moving from *S. thermophilus* to *Lb. bulgaricus*, and conversely, the *cbs-cblB-cysE* gene cluster—which is involved in sulfur amino acid metabolism—may be moving from *Lb. bulgaricus* or *Lb. helveticus* to *S. thermophilus* (Liu et al. 2009). In the case of *S. thermophilus*, these HGTs could result from the bacterium's natural competence (Gardan et al. 2009) and has allowed the transfer among *S. thermophilus* strains, of the cell-wall protease PrtS, which is essential for growth in milk (see below; Dandoy et al. 2011). In addition, genome analysis suggests that yogurt bacteria have undergone reductive evolution (Bolotin et al. 2004, Makarova et al. 2006): their "domestication" in milk has led to metabolic simplification and specialization. More specifically, among the Lactobacillaceae, the two yogurt LABs have the highest number of pseudogenes, frameshift mutations, nonsense mutations, and deletions (around 10%; Bolotin et al. 2004, Makarova et al. 2006, Goh et al. 2011), leading to a loss of functional genes.

2.3 Yogurt Bacteria are Metabolically Well Adapted to the Composition of Milk

The growth of *S. thermophilus* and *Lb. bulgaricus* in milk largely depends on their ability to efficiently use the medium's major carbon and nitrogen sources (lactose and caseins, respectively), as well as to synthesize any

growth-limiting nucleotide bases that are lacking. These metabolic traits are key for the bacteria's associated growth and thus have a major impact on the properties of the resulting yogurt; they will be discussed further below. Furthermore, *S. thermophilus* produces CO_2 (Driessen et al. 1982, Tinson et al. 1982, Spinnler et al. 1987, Ascon-Reyes et al. 1995), which stimulates the growth of *Lb. bulgaricus*. The CO_2 comes from the decarboxylation of urea—present in milk—by urease (Tinson et al. 1982), which most *S. thermophilus* strains possess (Juillard et al. 1988). Other factors also influence the specifics of this association, such as the production of deleterious compounds like lactate, H_2O_2, or bacteriocins; however, they will not be discussed here.

2.3.1 Lactose metabolism

The main source of carbohydrates in milk is lactose. Because it is still present at high concentrations (around 40 g/L) at the end of fermentation, it is not growth limiting for yogurt bacteria and thus does not directly fuel competition. Yogurt bacteria prefer lactose over other simple sugars, such as glucose or sucrose, as a carbon source (Chervaux et al. 2000, Goh et al. 2011, Thomas et al. 2011), probably because both organisms possess an efficient lactose/galactose antiporter LacS. Lactose is imported into the bacteria and hydrolyzed by β-galactosidase (LacZ) into two compounds: galactose, which is largely exported by LacS permease, and glucose, which feeds the glycolysis pathway. The galactose moiety of lactose is not used by most *S. thermophilus* strains, mainly because they have low galactokinase activity (Vaughan et al. 2001, Vaillancourt et al. 2004) or low levels of induction of the galactose promotor (Van den Bogaard et al. 2004). However, when growth conditions become difficult (e.g., lactose is limited and galactose is present at high concentrations), galactose can be used (Terence and Vaughan 1984, Hutkins et al. 1985, Levander et al. 2002).

Lactose utilization is chromosomally encoded in both yogurt bacteria, which ensures that this trait is maintained. In contrast, it is plasmid encoded in other LABs, such as *Lactococcus lactis*, and is thus less stable. Pyruvate, the end product of glycolysis, is then converted into (L- and D-) lactate, which is excreted, leading to milk acidification. This process is mediated by L-lactate dehydrogenase (L-Ldh) in *S. thermophilus* and by D-lactate dehydrogenase (D-Ldh) in *Lb. bulgaricus*. Even if L-Ldh genes are present in the *Lb. bulgaricus* genome, 90% of pyruvate is nonetheless converted into D-lactate.

2.3.2 Nitrogen metabolism

Optimal bacterial growth depends on efficient protein synthesis and, as a result, on the availability of amino acids (AAs). LABs are auxotrophic

for amino acids: one to several in *S. thermophilus* strains (Hols et al. 2005, Pastink et al. 2009) and 15–20 in *Lb. bulgaricus* strains, which are only able to synthesize 3–4 AAs (Asp, Asn, Thr, +/– Lys; Van de Guchte et al. 2006, Hao et al. 2011).

Milk is poor in nitrogen compounds that can be directly assimilated by LABs (free amino acids and short peptides), but yogurt bacteria possess a complex and efficient proteolytic system that provides them with exogenous nitrogen sources stemming from milk proteins. This multiprotein system has been extensively studied (for reviews, see Christensen et al. 1999, Savijoki et al. 2006, Liu et al. 2010). It is able to hydrolyze caseins—the major proteins in milk. It transports the resulting oligopeptides into the cells and then degrades them into smaller oligopeptides and AAs. The system is composed of a cell-wall protease (Prt), various AA and peptide transporters, and several peptidases, mostly intracellular, the coordinated action of which leads to the recovery of free AAs for protein synthesis (Fig. 2).

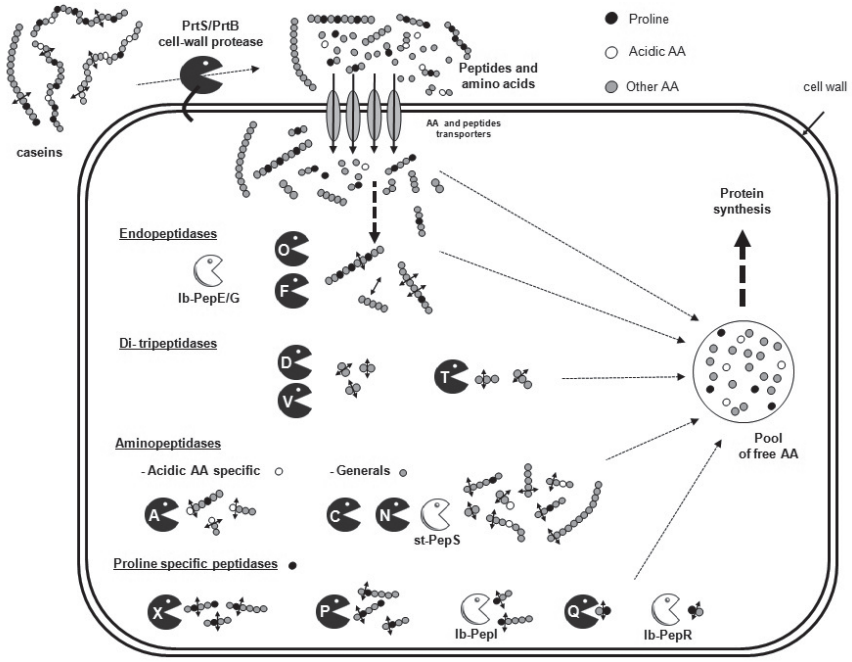

Figure 2. The proteolytic system of *S. thermophilus* and *Lb. bulgaricus.*

: protease/peptidase common to the two bacteria
: bacterium-specific peptidase; "st" stands for *S. thermophilus* and "lb" for *Lb. bulgaricus*

Lb. bulgaricus has higher overall levels of proteolytic activity than *S. thermophilus* (Shankar and Davis 1978, Rajagopal and Sandine 1990, Courtin and Rul 2004). It liberates most of the AAs (Courtin and Rul 2004) that stimulate *S. thermophilus* growth, including valine, histidine, glycine, leucine, isoleucine, methionine, and various dipeptides (Pette and Lolkema 1950b, Bautista et al. 1966, Accolas et al. 1971, Bracquart et al. 1978, 1979, Shankar and Davies 1978, Radke-Mitchell and Sandine 1984, El-Soda et al. 1986, Rajagopal and Sandine 1990, Courtin and Rul 2004). Levels of overall proteolytic activity in *Lb. bulgaricus* vary among strains and differentially promote the growth of *S. thermophilus* when the two bacteria are associated in milk (Courtin and Rul 2004).

Cell-wall protease—PrtB in *Lb. bulgaricus* and PrtS in *S. thermophilus*—plays a major role in stimulating growth in milk because it initiates the breakdown of caseins into various oligopeptides. PrtS is often absent from older strains of *S. thermophilus* (Shahbal et al. 1991) but is frequently found in more recent industrial strains. Prt is particularly important in allowing *Lb. bulgaricus*, which is poorly equipped for AA synthesis (see above), to grow in milk; PrtS- or PrtB-negative mutants develop more slowly than do wild-type strains (Gilbert et al. 1997, Courtin et al. 2002). The PrtS-encoding gene was probably acquired *via* HGT from a species related to *S. suis* (Delorme et al. 2010), and it can be transferred by natural competence to other *S. thermophilus* strains (Dandoy et al. 2011). The expression of PrtS is induced during the latency period (Letort et al. 2002), most probably because available peptides are lacking. In co-cultures, the following are true: (i) PrtB gene expression is probably induced by the presence of *S. thermophilus*, which reduces the peptides available to *Lb. bulgaricus* (as compared to pure cultures; Sieuwerts et al. 2010), and (ii) PrtS is no longer essential to *S. thermophilus* growth if *Lb. bulgaricus* PrtB is present. Indeed, PrtB may be more efficient than PrtS in making nitrogen available because when PrtB-positive *Lb. bulgaricus* strains co-occur with Prt S-negative *S. thermophilus* strains, *S. thermophilus* populations are larger than when PrtS-positive *S. thermophilus* strains co-occur with PrtB-negative *Lb. bulgaricus* strains (Courtin et al. 2002). We also cannot rule out the possibility that the casein-hydrolyzation specificities of PrtB and PrtS are different. For example, the substrate-binding region, which influences Prt specificity, differs between PrtS (Fernandez-Espla et al. 2000) and PrtB (Gilbert et al. 1996).

Yogurt bacteria also possess different transport systems—for AAs, dipeptides, tripeptides, and oligopeptides (the latter have been extensively studied in LABs; for a review, see Savijoki et al. 2006)—that can efficiently target nitrogenous compounds in the medium. *Lb. bulgaricus* lacks the general DtpT di/tri-peptide transporter found in *S. thermophilus*, but its absence may be compensated for by a Dpp transporter that preferentially takes up hydrophobic di/tripeptides. Some *S. thermophilus* transport systems are upregulated in co-cultures, including the oligopeptide carrier

Ami/Opp (Sieuwerts et al. 2010) and potential polar AA transporters (Hervé-Jimenez et al. 2009).

S. thermophilus and *Lb. bulgaricus* have a similar number of protease/peptidase genes (44 [Hols et al. 2005] vs. 45–49 [Hao et al. 2011, Zheng et al. 2012], respectively). Around a dozen have been studied to determine their roles in nitrogen metabolism (Fig. 2). They were found to have various peptide hydrolysis specificity and, *a priori*, are sufficient to meet bacterial needs for AAs. There are even peptidases dedicated to hydrolyzing proline-containing peptide bonds, which are difficult to break down but essential for casein degradation as caseins are rich in proline.

One group of AAs is particularly important in allowing yogurt bacteria to grow in milk: branched-chain AAs (BCAAs), arginine, and cystein (Bracquart and Lorient 1977, Garault et al. 2000). These AAs are predicted to be among the most common in proteins of *S. thermophilus* (Hervé-Jimenez et al. 2009) and *Lb. bulgaricus* (Sieuwerts et al. 2010); in contrast, they are largely lacking from caseins and the two bacteria probably compete for these AAs. This fact may explain why *S. thermophilus* and *Lb. bulgaircus* increases BCAA and arginine biosynthesis, and BCAA permease activity, respectively, in co-cultures as compared to in pure cultures. In addition, when the two yogurt bacteria co-occur in milk, there is an increase in activity along the serine-to-methionine and cysteine-conversion pathways, as compared to in pure cultures (Sieuwerts et al. 2010). Finally, *S. thermophilus* and *Lb. bulgaricus* possess the necessary peptidolytic and transport pathways for casein exploitation; they hydrolyze caseins into free amino acids, thus fulfilling their protein synthesis needs. When the two bacteria co-occur in milk, they both compete and complement each other in terms of their nitrogen metabolisms.

2.3.3 Formate, folate, and purine metabolism

Some of the first metabolic exchanges described in *S. thermophilus* and *Lb. bulgaricus* associations in milk were interactions involving folic acid (Rao et al. 1984, Sybesma et al. 2003), pyruvic acid (Higashio et al. 1978), formic acid (Veringa et al. 1968), and CO_2 (Driessen et al. 1982). These compounds all fed, directly or indirectly, into the purine biosynthesis pathway (Fig. 3). Formate is necessary for the synthesis of purine bases (i.e., xanthine, adenine, and guanine) that are nucleic acid precursors (Suzuki et al. 1986); both yogurt bacteria require it to grow (Galesloot et al. 1968, Suzuki et al. 1986, Derzelle et al. 2005, Horiuchi and Sasaki 2012, Nishimura et al. 2013).

Depending on the strain, *Lb. bulgaricus* gains a boost in growth at formate concentrations ranging from 0.5 to 27 mM (Galesloot et al. 1968, El-Abbassy et al. 1993, Horiuchi and Sasaki 2012). In addition, Courtin and Rul (2004) showed that formate concentrations decreased more strongly in

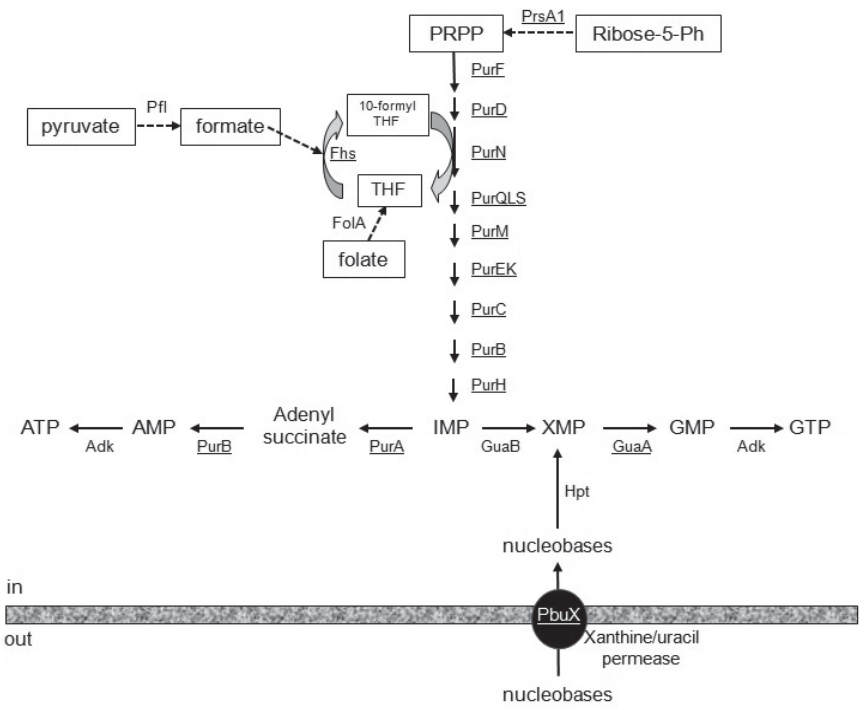

Figure 3. Purine pathway in *S. thermophilus*.

Underlined proteins are proteins or their corresponding genes that were less abundant or repressed during the growth of *S. thermophilus* in co-culture in milk with *Lb. bulgaricus* (data from Hervé-Jimenez et al. 2009).

milk co-cultures than in pure *S. thermophilus* cultures; they differed by a factor of 4 to 8, depending on the *Lb. bulgaricus* strain present and probably because of the latter's consumption of formate.

Pyruvate formate lyase (Pfl), which converts pyruvate into formate, is abundantly expressed in *S. thermophilus* and is induced when the species is grown in milk (as compared to when it is grown in M17-rich medium; Derzelle et al. 2005). If formate (5 mM) or purines (adenine and guanine, 50 µM) are added to the milk, Pfl is no longer overexpressed, and *S. thermophilus* experiences a boost in growth (Derzelle et al. 2005). In contrast, Pfl is absent from the *Lb. bulgaricus* genome (Van de Guchte et al. 2006). In several bacterial species, Pfl activity is oxygen sensitive (Knappe et al. 1974, Yamada et al. 1985, Sawers and Watson 1998). It has recently been suggested that, in *S. thermophilus*, oxygen conversion by NADH oxidase (NOX) could improve growing conditions, by promoting Pfl activity and, as a consequence, formate synthesis (Horiuchi and Sasaki 2012). Interestingly, when added to milk, formate stimulates EPS production in *Lb. bulgaricus*

by a factor of 4, which may be a mechanism contributing to improved growth as well as to enhanced cell-wall synthesis and bacterial division (Nishuimura et al. 2013).

Thanks to post-genomic approaches, it has been discovered that the purine biosynthesis pathway in *S. thermophilus* slows down in co-cultures; almost all the enzymes involved become less abundant or are expressed at lower levels (Hervé-Jimenez et al. 2009) (Fig. 3). This result was unexpected because the pathway is ramped up when the bacterium is grown in pure cultures (Hervé-Jimenez et al. 2008), confirming that purines are essential for *S. thermophilus*' growth in milk. One might hypothesize that, in co-cultures, purines or purine precursors are supplied by *Lb. bulgaricus*; indeed, a potential xanthine/uracil permease (a transporter of purine precursors) was expressed at higher levels when *S. thermophilus* was associated with *Lb. bulgaricus* (Hervé-Jimenez et al. 2009). However, a more recent post-genomic study of yogurt bacteria associations (Sieuwerts et al. 2010) showed that, when fermentation times were similar (around 5 h), purine synthesis and folate cycling pathways were upregulated in *S. thermophilus*, while folic acid and purine synthesis were downregulated in *Lb. bulgaricus*. These contradictory results underscore the importance of milk type and strain identity, as they differed in the two studies (skim milk vs. µ-filtered milk and strains CNRZ1066-ATCC BAA-65 vs. LMG18311-ATCC11842, respectively). However, yogurt bacteria need to utilize purines to grow in milk, and the process can be modulated. It is assumed that *S. thermophilus* supplies *Lb. bulgaricus* with the compounds needed for purine biosynthesis, such as formate, a precursor, and folic acid, which is a co-factor and produced in co-cultures (Crittenden et al. 2002). In turn, *Lb. bulgaricus* may provide *S. thermophilus* with other purine precursors.

The protocooperative association of the two yogurt bacteria has been studied for years (for a review see Sieuwerts et al. 2008), but new genomic and post-genomic approaches have made it possible to gather more detailed knowledge about the general and specific metabolic mechanisms involved (Fig. 4). They have revealed new, entirely unexpected interactions and exchanges. For instance, iron metabolism in *S. thermophilus* (Hervé-Jimenez et al. 2009) and fatty acid metabolism in *Lb. bulgaricus* (Sieuwerts et al. 2010) are modulated when the bacteria are grown in milk co-cultures, but not when they are grown in pure cultures. It has become clear that the association is the sum of a variety of bacterial interactions, both positive ones, such as mutualism and commensalism, as well as negative ones, such as competition and amensalism.

These new findings are crucial when it comes to designing and selecting novel compatible and complementary strains and strain cocktails with specific properties, with a view to creating tailored dairy products or developing entirely new foods.

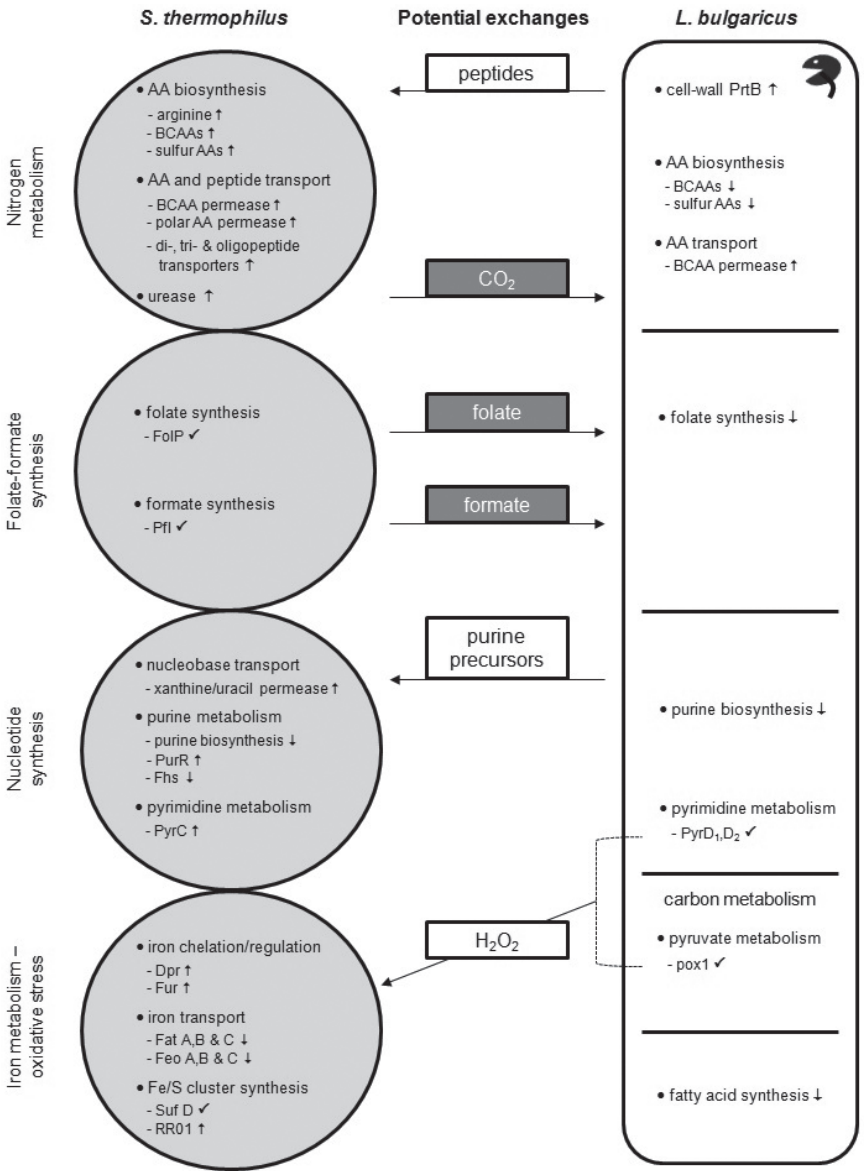

Figure 4. Metabolite exchanges between the two yoghurt bacteria, in particular regarding the metabolism pathways that were modulated during their co-culture in milk.

↑,↓: Proteins or genes that, respectively, were more or less abundant or expressed during the co-culture.

✓: Proteins or genes that were present or expressed without quantitative variation during the co-culture.

3. A Fermented Food with Typical Organoleptic Characteristics

3.1 The Flavor of Yogurt Arises from a Complex Mix of Aroma Compounds Produced by LABs

More than 100 different aroma compounds have been identified in yogurt (Ott et al. 1997, Cheng 2010), as a result of GC (gas chromatography) and GC-MS (gas chromatography-mass spectrometry) analyses, which are sometimes coupled with human olfactory assays (GC-sniffing or GC-olfactory detection) (Ott et al. 1997, Friedrich and Acree 1998). However, most are present at very low concentrations; only a few occur at significant levels. The flavor we typically associate with yogurt comes from its acidity (i.e., the presence of lactic acid) (Ott et al. 2000b). It is also influenced by different carbonyl compounds that were identified quite some time ago and that result from the proteolysis and degradation of amino acids into alcohol, aldehydes, and esters. These compounds are mainly acetaldehyde, acetoin, diacetyl, and 2,3-pentanedione, which are, for the most part, produced by bacterial metabolic activity (Fig. 5). Their production and/or accumulation in milk in co-cultures is strain dependent because, for

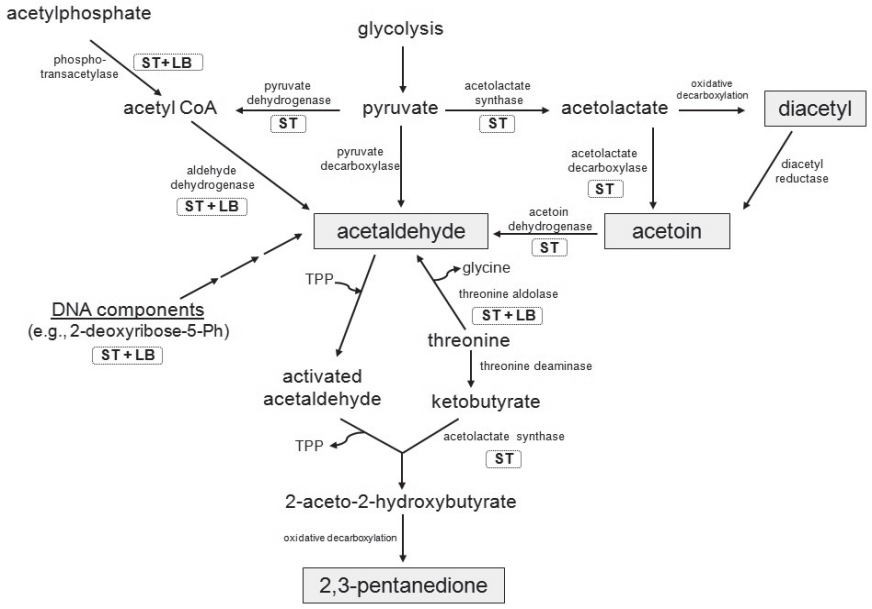

Figure 5. Production pathways of the main aroma compounds found in yogurt bacteria. ST, LB, and ST + LB indicate that the gene or enzyme is present in *S. thermophilus*, *Lb. bulgaricus*, and *S. thermophilus* and *Lb. bulgaricus* together, respectively.

example, when a given strain of *S. thermophilus* co-occurs with different strains of *Lb. bulgaricus*, different levels of acetaldehyde, acetoin, or diacetyl are generated (Courtin and Rul 2004). Their levels do not vary significantly when yogurt is stored at 4°C.

Acetaldehyde is the most typical yogurt flavor component (Pette and Lolkema 1950c, Dumont and Adda 1973, Law 1981) and is responsible for the food's fresh and fruity notes (e.g., hints of green apple and nuts). It is usually found at concentrations of 1 to 25 mg/L (Hamdan et al. 1971, Bottazzi et al. 1973, Rysstad and Abrahamsen 1987, Kneifel et al. 1992, Beshkova et al. 1998). The accumulation of acetaldehyde as yogurt fermentation progresses could be related to the bacteria's limited ability to use it (as hypothesized by Manca de Nadra et al. [1988]) and convert it into ethanol, as other LABs do (Lees and Jago 1976, Chaves et al. 2002).

Acetaldehyde can be produced in several ways: via pyruvate decarboxylation (from DNA) and via alcohol dehydrogenation (Fig. 5). However, the acetaldehyde found in yogurt bacteria probably mainly stems from the transformation of the amino acid threonine (Thr) into glycine (Gly) and acetaldehyde by threonine aldolase (Lees and Jago 1976, Raya et al. 1986, Ott et al. 2000a, Chaves et al. 2002). A threonine aldolase has been purified and characterized in *Lb. bulgaricus* (Manca de Nadra et al. 1987), and there is evidence for its involvement in flavor development (Marshall and Cole 1983). It is inhibited by the presence of glycine, via pH dependent way (Manca de Nadra et al. 1987), similarly to the threonine aldolase found in *S. thermophilus* (Marranzini et al. 1989). When threonine is added to milk, acetaldehyde production in *S. thermophilus* increases (Chaves et al. 2002, Ozer and Atasoy 2002); additionally, the higher Thr to Gly ratio generally enhances acetaldehyde production by both bacteria (Marranzini et al. 1989). Depending on the strain, decarboxylase or aldehyde dehydrogenase activity, which results in acetaldehyde production (Fig. 5), may or may not be present (Lees and Jago 1976 and Raya et al. 1986, respectively).

Apart from acetaldehyde, the other essential aroma compounds are diacetyl, acetoine, and 2–3 pentanedione, which give yogurt its buttery note. Diacetyl is also responsible for yogurt's full, delicate flavor (Rasic and Kurmann 1978); it occurs at concentrations of 0.2 to 3 mg/L (Cheng 2010) and is produced by both yogurt bacteria (Dutta et al. 1973, Rasic and Kurmann 1978). Production of 2–3 pentanedione is ramped up 3 to 5 fold in co-cultures, as compared to its combined production by each bacterium considered separately (1 mg/kg; Imhof et al. 1995).

While diacetyl and acetadehyde are the main compounds that define yogurt's flavor (Ott et al. 1997, Friedrich and Acree 1998), the ratio between the different aromatic compounds matters more than their individual concentrations (Pette and Lolkelma 1950c, Bottazzi and Vescoso 1969). For example, Bottazzi and Vescoso (1969) observed that a stronger flavor was

obtained when the acetaldehyde-to-acetone ratio was 2.8:1 but not when it was 0.4:1. Diacetyl takes on greater importance when the acetaldehyde concentration is low (Groux 1973, Rysstad and Abrahamsen 1987). Although a 1:1 ratio of acetaldehyde to diacetyl yields the preferred, typical yogurt's flavor associated with yogurt (Bottazzi and Dellaglio 1967, Zourari and Desmazeaud 1991), ratios of 7–10:1 can nonetheless create a "good" flavor (Beshkova et al. 1988). In addition, some low-acetaldehyde yogurts present a typical aroma (Hamdan et al. 1971, Groux 1973), possibly thanks to the presence of diacetyl (Kneifel et al. 1992).

Genome analysis has revealed that *Lb. bulgaricus* is probably poorly equipped to produce the aroma compounds mentioned above (Hao et al. 2011). *S. thermophilus* possesses several aminotransferases that convert Asp, aromatic AAs, or BCAAs into flavor compounds and produces alpha ketoglutarate from glutamate dehydrogenase, thus helping generate different volatiles (Pastink et al. 2009).

Finally, yogurt flavor results from a subtle balance between the main flavor compounds, which stem from bacterial proteolytic processes, and from fatty-acid derivatives (Turcic et al. 1969, Dumont and Adda 1973, Beshkova et al. 1998).

3.2 The Texture of Yogurt Results from the Acidification Capacity and Exopolysaccharide Production of LABs

In addition to its unique flavor, yogurt also has a very specific texture, which plays an important role in gustative quality. This texture is largely a function of bacterial activities: the acidification of milk leads to the formation of a coagulated gel and the production of EPSs creates a matrix which participates in shaping yogurt texture.

During milk fermentation, pH drops, and when it reaches the isoelectric point of the caseins (pH of 4.6), the latter precipitate and then aggregate, generating a gel network in which water and fat are embedded. The firmness and viscosity of the curd ultimately depend on the final pH, as well as on bacterial proteolytic activity, which can result in syneresis (Marshall 1987) and change the structure or microstructure of the yogurt.

S. thermophilus and *Lb. bulgaricus* both produce EPSs (Cerning et al. 1986, 1988, Cerning 1990, Laws et al. 2001). These polysaccharides are key players in the development of yogurt's rheological properties and help determine yogurt firmness, unctuosity, stickiness, and mouthfeel (Broadbent et al. 2003, Vaninelgem et al. 2004, Purwandari et al. 2007, Qin et al. 2011). Polysaccharides can bind to casein micelles and thus both increase water retention in the curd and reduce whey exudation at the yogurt's surface (Amatayakul et al. 2006a,b, Purohit et al. 2009). *S. thermophilus* EPSs also act to protect *Lb. bulgaricus* (Ramchandran and Shah 2009).

Fermentation conditions (e.g., temperature, time of incubation), the composition of the medium (e.g., carbon and nitrogen sources, the carbon-to-nitrogen ratio), the level of acidity, and the identity of the strain affect EPS quantity and sugar composition (De Vuyst et al. 1998, Degeest and De Vuyst 1999, Tamime and Robinson 1999, Zisu and Shah 2003, Vaningelgem et al. 2004, Zhang et al. 2014). Depending on the strain and culture conditions, *S. thermophilus* and *Lb. bulgaricus* produce EPSs either during the exponential growth phase (De Vuyst et al. 1998) or late fermentation (Petry et al. 2000, Broadbent et al. 2003, Sieuwerts et al. 2010). Production levels are higher in co-cultures than in pure cultures (Cerning 1990, Frengova et al. 2000, Sieuwerts et al. 2010), possibly because the drop in pH favors EPS production by *Lb. bulgaricus*. EPS production by *Lb. bulgaricus* can be stimulated by formate and other compounds such as vitamins or nucleobases.

EPSs are synthesized in the cytoplasm by polymerization of repeating sugar units that are attached to a lipid carrier. They are translocated to the membrane before being secreted, which is what distinguishes them from capsular polysaccharides (which are permanently attached to the cellular surface). LAB EPSs comprise multiple copies of an oligosaccharide that contains several residues linked in different patterns. All the EPSs characterized in yogurt bacteria up until now are mainly composed of galactose. Galactose's omnipresence is probably a consequence of the ubiquity of lactose in milk—it is the main carbon source and, when hydrolyzed, forms (i) glucose, which is preferentially used for glycolysis-based energy production and (ii) galactose, which can be used to synthesize nucleotide sugars for EPS production.

In *S. thermophilus*, EPSs are most commonly composed of galactose and glucose (De Vuyst et al. 1988, Petit et al. 1991, Laws et al. 2001, Marshall et al. 2001, Nordmark et al. 2005, Sawen et al. 2010, Qin et al. 2011). In some strains, additional mannose (Cerning et al. 1986) or rhamnose (Cerning et al. 1986, Escalante et al. 1998) are also included; in others, it is small amounts of xylose, arabinose, and mannose (Cerning et al. 1988). Alternatively, EPSs can also be solely composed of rhamnose and galactose (Ariga et al. 1992, Faber et al. 1998, 2001) or contain sugar derivatives such as acetylgalactosamine and/or fucose (Doco et al. 1990, Stingele et al. 1996, Laws et al. 2001), D-galactopyranose and L-rhamnopyranose residues (Bubb et al. 1997), or N-acetylglucosamine and glucuronic acid, which are components of hyaluronic acid (Izawa et al. 2009). In *Lb. bulgaricus*, most of EPSs described to date contain galactose, glucose, and rhamnose (Cernning et al. 1986, Zourari et al. 1992, Gruter et al. 1993, Grobben et al. 1995, Petry et al. 2000, Marshall et al. 2001, Lamothe et al. 2002) and occasionally traces of mannose (Petry et al. 2000). However, some *Lb. bulgaricus* EPSs are composed of galactose and glucose (Petry et al. 2000, Faber et al. 2001); galactose,

glucose, and traces of mannose (Bouzar et al. 1996); or xylose and arabinose (Cerning et al. 1988). Even if both bacteria produce EPSs containing similar sugars, the proportions of these sugars differ, generating EPS diversity and shaping yogurt viscosity in different ways (Faber et al. 1998). In addition, EPS composition can vary during fermentation (Bouzar et al. 1997).

Several EPS gene clusters have been described in different yogurt bacterial strains, which often possess two of said clusters. The *eps* clusters usually exhibit a modular organization and a chimeric structure; they are highly diverse in terms of sequence and genetic context across clusters, both within and among strains (Stingele et al. 1996, Bourgoin et al. 1999, Jolly and Stingele 2001, Broadbent et al. 2003, Rasmussen et al. 2008, Goh et al. 2011, Hao et al. 2011). Sequence divergence ranges from 10 to 50% for *S. thermophilus* EPSs (Bourgoin et al. 1999). The diversity of these genes suggests that HGTs are being acquired from other bacteria in the environment. For instance, *S. thermophilus* strain LMD-9 contains some *eps* genes that are very similar to those found in *Lactococcus lactis* (Bourgoin et al. 1999, Goh et al. 2011). In addition, Wu et al. (2014) has proposed that *S. thermophilus* may produce EPSs of different molecular sizes because the researchers detected the presence of two gene pairs that are involved in chain-length determination.

4. A Fermented Food with Probiotic Potential

Yogurt can also be considered to be a probiotic food (Guarner et al. 2005). Indeed, yogurt starters clearly meet the definition of probiotics—"Live microorganisms [that] when administered in adequate amounts confer a health benefit on the host"—proposed by the Joint Food and Agriculture Organization/World Health Organization Working Group (2002) and adopted by the International Scientific Association for Probiotics and Prebiotics (Reid et al. 2003). In addition, yogurt possesses well-documented healthful properties, including the ability to help alleviate lactose intolerance. This latter characteristic is the basis of a health claim recently accepted by EFSA (Section 4.1, 2010).

There are numerous examples in the literature of the probiotic effects of LAB, when delivered in capsules, sprays, or via yogurt. However, the goal here is to focus exclusively on the probiotic effects of traditional yogurt, which includes both and only *S. thermophilus* and *Lb. bulgaricus*.

4.1 Bacterial β-galactosidase Participates in Lactose Digestion in the Gastrointestinal Tract

Lactose maldigestion/intolerance is the main cause of milk intolerance in adults. Yogurt's ability to alleviate the symptoms of lactose maldigestion

is well documented and recognized at the regulatory level (FAO/WHO 2001, 2002, EFSA 2010). Lactose maldigestion results from reduced or absent lactase activity (the hydrolysis of lactose into glucose and galactose) in the brush border membrane of the small intestine. Undigested lactose travels to the colon, where it is subject to fermentation by resident microbiota, leading to excessive gas production (i.e., methane, carbonic gas, hydrogen) and, consequently, symptoms such as abdominal pain, bloating, cramps, or diarrhea. Lactose intolerance/maldigestion can be diagnosed by the breath-hydrogen concentration test. It measures the quantity of exhaled hydrogen, which is proportional to the quantity of ingested lactose reaching the colon and is thus inversely proportional to the level of lactose digestion in the intestine (Savaiano 2014). Hypolactasia (lactase deficiency) is a physiological condition and is affected by age, sex, and ethnic origin. For instance, lactase activity rapidly decreases after weaning in the majority of children. Hypolactasia prevalence is over 50% in adult Africans, American Hispanics, and American Indians, and close to 100% in some Asian populations (Wilt et al. 2010, Lember 2012), because of a genetically programmed loss of lactase after weaning.

Though the lactose concentration in yogurt is similar to that in milk (around 40 g/L), yogurt consumption is recommended for people suffering from lactose intolerance because it alleviates the symptoms of the disorder. For example, lactose intolerance/maldigestion is more severe in young children (e.g., causing acidic diarrhea), but yogurt consumption appears to help (i.e., is associated with a decrease in acidic feces occurrence and volume) (Dewit et al. 1987, Shermak et al. 1995). These benefits can only be obtained if the product contains live yogurt bacteria; they are lost if the yogurt is thermized and the bacteria are killed (Goodenough and Kelyn 1975, Gilliland and Kim 1984, Savaiano et al. 1984, Lerebours et al. 1989, Pochart et al. 1989, de Vrees et al. 2001).

Older work by Alm et al. (1982) suggested that the lactase produced by yogurt bacteria (β-galactosidase, see above)—which all strains of yogurt bacteria have—could promote lactose hydrolysis in the digestive tract. This hypothesis was later experimentally supported in mice by Douault et al. (2002). In lactase-deficient subjects, more than 90% of the lactose found in the small intestine was hydrolyzed by the β-gactactosidase of yogurt starters (Marteau et al. 1990). The results were dependent on the size of the bacterial population present in the yogurt; total hydrolysis occurred when 10^8 UFC/g yogurt were present but was limited when only 10^6 UFC/g were present (Pelletier et al. 2001).

It has been suggested that lactose could enter yogurt bacteria as a result of the permeabilization of their envelopes by bile (Noh and Gilliland 1994), or that bacteria lyze and release their β-galactosidase into the lumen (Marteau et al. 1997). Yogurt's texture—which is more viscous and

thicker than that of milk—could also slow down gastric emptying and gastrointestinal transit times (Marteau et al. 1990), thus favoring the action of residual intestinal lactase on enterocytes by favoring contact with lactose in the lumen. In rats, intestinal lactase activity was higher in animals fed yogurt than in animals fed pasteurized yogurt or milk (Goodenough and Kleyn 1975, Besnier et al. 1983, Garvie et al. 1984).

4.2 Yogurt Consumption Can Have Beneficial Effects on Infections, Inflammatory Diseases, and Cancers

Infections, antibiotic treatments, and tube feeding are major causes of diarrhea. In developing countries, bacterial enteropathogens, such as enterotoxigenic *E. coli*, are frequently responsible for diarrhea in children and travellers (Narayan et al. 2010). The WHO (1995) has recommended using yogurt to treat acute diarrhea because yogurt is associated with the production of antimicrobial compounds (H_2O_2, bacteriocins, or organic acids such as lactic acid), acts via immunomodulation, and inhibits pathogen adhesion to the intestinal epithelium. In particular, yogurt helps limit chronic diarrhea in children (Boudraa et al. 1990, Touhami et al. 1992), by reducing both its frequency and duration (Boudraa et al. 2001).

Maintaining microbial equilibrium is an important part of intestinal physiological health and homeostasis; it also helps eliminate pathogenic enteric bacteria. For patients with chronic liver disease, consuming yogurt can decrease microbiota imbalances (Liu et al. 2010), and in patients with inflammatory bowel disease (IBS), yogurt consumption has been linked to a decline in the pathogen *Bilophila wadworthia* (Veiga et al. 2014). The feces of subjects that have consumed yogurt have higher microbial densities and contain larger amounts of LABs as compared to *Bacteroides* species (Garcia-Albiach et al. 2008). Yogurt consumption resulted in lower *E. coli* counts in patients suffering from chronic liver disease (Liu et al. 2010). It also led to lower levels of *Clostridium* in the elderly, which is beneficial because Clostridia generate putrefactive products that are potentially toxic for the colic mucosa (Canzi et al. 2002). Interestingly, a recent meta-analysis looking at the influence of diet on tooth erosion in children and adolescents has indicated that yogurt has protective effects (Salas et al. 2015). *In vitro* experiments suggest they may stem from bactericidal effects on cariogenic *S. mutans* species (Petti et al. 2008).

In the 1990s, research began to suggest that the administration of live LABs could modify immune responses. The effects of each of the two yogurt bacteria on inflammatory and immune responses have been documented, but a few studies have also shown that yogurt containing both bacteria can have beneficial impacts. Yogurt consumption stimulates the immune system and decreases allergies in young adults (20–40 years old) and older

adults (55–70 years old) (Trapp et al. 1993, Van de Water et al. 1999) and is recommended for the immunocompromised (Meydani and Ha 2000). Based on the results of *in vitro* and *in vivo* studies, one could hypothesize that the immunomodulatory and anti-inflammatory effects of yogurt could stem from the induction of cytokines (γ-interferon, TNFα, IL-12), the production of immunoglobulins (IgA in particular), and an improved barrier effect due to increased mucosa thickness.

Furthermore, in animal models with induced cancers, yogurt consumption reduces tumor number (Narushima et al. 2010); it also inhibits tumor progression and spread of colon cancer by increasing apoptosis (De Moreno de Leblanc and Perdigon 2004), by decreasing the inflammatory immune response (mediated by increases in IgA), and increasing IL-10 expression (Perdigon et al. 2002). In humans, yogurt consumption is associated with a reduction in colorectal cancer (Pala et al. 2011) and may also reduce the risk of breast cancer (Le et al. 1986, Van't Veer et al. 1989). Again, yogurt's cancer-fighting properties are lost if the food is thermized (Pool-Zabel et al. 1993), underscoring that the benefits are due to the presence of live bacteria.

4.3 The Proteolytic Activity of Yogurt Bacteria Contributes to Yogurt's Antihypertensive Potential

The proteolytic activity of yogurt starters is essential for bacterial growth in milk (see above), as well as for the production of peptides that possess varying degrees of functional activity. Several casein-derived peptides produced by yogurt LABs have been identified, and their biological activity has been demonstrated *in vitro*. *In vivo*, following their ingestion, these yogurt peptides must confront the proteolytic enzymes of the gastrointestinal tract (i.e., pepsin, trypsin, chymotrypsin, as well as carbo-, amino- and membrane endopeptidases), which can hydrolyze them and thus prevent them from reaching their target within the host. Nevertheless, proline-rich peptides can withstand the gauntlet of gastrointestinal hydrolysis (Korhonen and Pihlanto 2003) because peptide bonds containing proline can only be hydrolyzed with specific enzymes. Consequently, the tripeptides IPP (Ile-Pro-Pro) and VPP (Val-Pro-Pro), which are produced in yogurt (Donkor et al. 2007) and have antihypertensive properties (Hirota et al. 2007), resist *in vivo* degradation; a product containing these two peptides has been commercialized. The antihypertensive effects of such peptides results from their inhibition of angiotensin-converting enzyme (ACE), which plays a crucial role in regulating blood pressure by modulating the levels of the vasoconstricting peptide angiotensin II and those of the vasodilatory peptide bradykinin. More recently, β-casein(94–123)-derived peptides, which are present in yogurt, have been shown to enhance the expression

of mucin-encoding genes and the number of goblet and Paneth cells in the small intestine (Plaisancié et al. 2013); they may thus play a role in protecting the intestinal epithelium and maintaining its homeostasis. More generally, bioactive peptides with diverse functional properties have been isolated from dairy products (Madureira et al. 2010, Muro Urista et al. 2011).

Apart from the extensive data provided above that demonstrate yogurt's probiotic effects, other findings indicate that yogurt could have promise in treating prevalent pathologies—such as obesity, diabetes II (Margolis et al. 2011), and cardiovascular diseases (Sonestedt et al. 2011)—and maintaining better general health in aging populations (El-Abbadi et al. 2014). Furthermore, the lactate present in yogurt not only acts as an antibacterial agent, but also helps define the food's organoleptic properties (see above) and has bioactive properties (Garrote et al. 2015). It functions as a signaling molecule between bacteria and the host and, in particular, modulates the physiology of the colon epithelium (Rul et al. 2011, Thomas et al. 2011). Recently, Tsilingiri and Rescigno (2013) have proposed the concept of "postbiotic" factors, which are "soluble factors produced by probiotics [that] are sufficient to elicit the desired response" and that "could be a safe alternative for clinical applications, especially in chronic inflammatory conditions like inflammatory bowel disease." However, clinical trials in humans are still lacking, and most of the potential mechanisms involved remain to be specifically elucidated.

5. Conclusions

Yogurt, a healthful, traditional food that has been consumed for millenia, has modern relevance because its combination of nutritional and probiotic proprieties result in unique benefits. It is an interesting food ecosystem which has recently been rediscovered using post-genomic tools. We now have a better understanding of yogurt microbiology, and especially of the metabolic processes that are essential for yogurt production. Nevertheless, data are lacking on some key technical aspects (e.g., the nature of optimal bacterial combinations, the regulation of EPS production) and the factors responsible for yogurt's healthful effects. Research has yet to fully clarify yogurt's probiotic potential and the underlying mechanisms. Indeed, the list of yogurt's possible probiotic properties in the face of various pathologies continues to grow as various animal models are explored. However, findings in humans, particularly in healthy populations, are still needed.

The emergence of new food consumption patterns and of health consciousness on the part of consumers, as well as the global aging of the population, are reasons for continuing to promote fermented foods, such as yogurt, and to develop new ones. For example, vitamin- and/or iron-fortified yogurts, thermized yogurts, and dairy snacks are now available,

and it is easy to imagine that these products will continue to be developed and adapted to different populations (e.g., varying in age, sex, ethnic origin, and geographical location) and different nutritional habits. Traditional yogurt probably has benefits that remain to be identified, and exploring its characteristics in greater detail may yield new probiotic applications. Yogurt bacteria EPSs provide a good example: older studies focused on describing these texturing agents of yogurts, and more recent studies have suggested that texturing agents could be involved in inflammatory diseases (Sengul et al. 2006) and have immunostimulating (Makino et al. 2006), antiviral (Nagai et al. 2011), and antibacterial (Aslim et al. 2007) effects.

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