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Metabolism and biochemical characteristics of yogurt bacteria. A review

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Summary — This review reports recent data regarding the metabolism and biochemistry of *Streptococcus salivarius* subsp *thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* related to yogurt manufacture. The taxonomy of these bacteria is presented. Different proposed pathways for carbohydrate metabolism are then discussed, as well as recent molecular and genetic studies of the enzymes involved. Acetaldehyde is the major aromatic compound in yogurt, and so the different pathways of its formation are briefly described. Recent studies have concerned threonine aldolase which catalyzes acetaldehyde synthesis by yogurt bacteria. Exocellular polysaccharides produced by lactic acid bacteria improve the texture of stirred and liquid yogurts. Some of the polysaccharides of yogurt bacteria are currently known and particular aspects of their production are discussed. Some other properties, *ie* proteolysis, lipolysis, urease, oxygen metabolism, are also briefly presented. Interactions between streptococci and lactobacilli are well established, but more data are required for the complete characterization and control of mixed populations. In particular, little is known about antimicrobial compounds produced by these microorganisms. Bacteriophages of yogurt bacteria are now well characterized, but little is known about lysogeny in thermophilic streptococci. Finally, progress in genetics (on both plasmid and chromosomal DNA) is briefly discussed.

***Streptococcus salivarius* subsp *thermophilus* / *Lactobacillus delbrueckii* subsp *bulgaricus* / yogurt / metabolism / interaction**

Résumé — Métabolisme et caractéristiques biochimiques des bactéries du yaourt. Une revue. Dans le présent article, la position taxonomique de *Streptococcus salivarius* subsp *thermophilus* et de *Lactobacillus delbrueckii* subsp *bulgaricus* est d'abord décrite. Les différentes voies du métabolisme des glucides sont discutées ainsi que les études récentes des enzymes impliquées, sur le plan moléculaire et génétique. L'acétaldéhyde est le composé caractéristique de l'arôme typique du yaourt. Les différentes voies métaboliques de sa synthèse sont brièvement décrites, en particulier celle qui met en jeu la thréonine aldolase. Les bactéries lactiques produisent des polysaccharides exocellulaires qui influencent la texture des yaourts brassés et des yaourts liquides. Des polysaccharides produits par les bactéries du yaourt sont maintenant connus et quelques aspects intéressants de leur production sont actuellement étudiés. D'autres activités (protéolyse, lipolyse, uréase, métabolisme de l'oxygène) sont aussi brièvement présentées, suite à plusieurs études récentes. Les interactions entre streptocoques et lactobacilles sont bien établies, mais davantage de données sont nécessaires pour la caractérisation complète et la maîtrise des populations mixtes. En particulier, les connaissances des substances antimicrobiennes produites par les bactéries du

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yaourt sont encore limitées. Les bactériophages de ces bactéries sont maintenant relativement bien caractérisés mais les données sur la lysogénie du streptocoque demeurent encore incomplètes. Enfin, le progrès des études génétiques (ADN plasmidique et chromosomique) est brièvement discuté.

Streptococcus salivarius subsp thermophilus / Lactobacillus delbrueckii subsp bulgaricus / yaourt / métabolisme / interaction

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INTRODUCTION

Yogurt and similar fermented milk products have been very popular for a long time in Mediterranean countries (the Balkans, North Africa), in central and southwest Asia (Mongolia, Turkey, Iraq, Iran, Syria) and in central Europe. In many of these countries, yogurt is still manufactured using traditional procedures. Since the last world war, yogurt consumption has been steadily increasing not only in European countries, but also in the United States, enhancing its industrial-scale production. At present, new types of fermented milk are available, prepared by adding fruits or flavouring, enriched with vitamins or containing selected intestinal bacteria such as *Lactobacillus acidophilus* and several *Bifidobacterium* species (Kurmann, 1984; Puhan, 1988).

In France, the term "yogurt" can be used legally only to designate the product resulting from milk fermentation brought about exclusively with 2 thermophilic lactic acid bacteria, *Streptococcus salivarius* subsp *thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*, which must be found alive in the final product (≈ 10 million

cfu.g⁻¹ of yogurt). It is noteworthy that these 2 conditions are seldom specified by existing legislation in most other yogurt-producing countries (Anonymous, 1989).

We review here recent data on some of the metabolic and biochemical aspects of these starter bacteria in relation to yogurt manufacture. Tamime and Deeth (1980) have published an excellent review concerning all the technological and biochemical aspects of yogurt, followed by a second article dealing with its nutritional and therapeutic properties (Deeth and Tamime, 1981). These reviews include a detailed presentation of *S salivarius* subsp *thermophilus* and *L delbrueckii* subsp *bulgaricus*. Since 1981, both microorganisms have been extensively studied and reviews dealing with particular aspects of their metabolism and genetics have been published (Hutkins and Morris, 1987; Mercenier and Lemoine, 1989; De Vos, 1990; Mercenier, 1990). The present review is an update in this field and will give the technological and biochemical context of yogurt manufacture. Emphasis is put on bacteria and not on yogurt and only limited information concerning yogurt manufacture will be reported.

TAXONOMY OF *S SALIVARIUS* SUBSP *THERMOPHILUS* AND *L DELBRUECKII* SUBSP *BULGARICUS*

S salivarius subsp *thermophilus*

S salivarius subsp *thermophilus* is the new name proposed to designate *Streptococcus thermophilus* which was originally described by Orla-Jensen (1919) and which stands apart from the other streptococci and especially lactic streptococci, at present designated as lactococci. It is exclusively isolated from the dairy environment, ferments only few carbohydrates, *ie* lactose, sucrose, glucose and sometimes galactose, and is characterized by its thermoresistance and a rather high growth temperature which may reach 50–52 °C. No group-specific antigen has been found (Hardie, 1986).

DNA–DNA homology studies might supply more information on this bacterium and may question its classification. The guanine plus cytosine content (mol G + C%) of DNA ranges from 37.2–40.3% according to Farrow and Collins (1984). These authors obtained 61–78% homology between DNA of several *S thermophilus* strains and a DNA probe of *Streptococcus salivarius* (the homology between DNA of *S salivarius* strains and a DNA probe of *S thermophilus* ranged from 67–91%). They thus confirmed the high DNA homology (70–100%) observed in an earlier study by Kilpper-Bälz *et al* (1982) for 2 strains of each species. On the basis of these results, Farrow and Collins (1984) proposed that *S thermophilus* should be reclassified as a subspecies of *S salivarius* despite the large phenotypic differences between these 2 bacteria. In fact, lactate dehydrogenases of the type-strains of both streptococci have different properties (Garvie,

1978). Furthermore, the thermoresistance of their fructose-diphosphate aldolases is different, as is their level of homology with *Enterococcus faecalis* aldolase assessed by immunodiffusion assays, in spite of very close migration distances (London and Kline, 1973).

In addition, Garvie and Farrow (1981) had previously placed these streptococcal strains in the same group, based on homology between their DNA and ribosomal RNA of the type-strain of *Streptococcus bovis* (this strain now belongs to the *Streptococcus equinus* species; Schleifer and Kilpper-Bälz, 1987), but in different clusters. Besides, DNA–DNA molecular hybridization between several strains of *S thermophilus* and *S salivarius* have only yielded a 60% homology in optimum conditions and 30% under stringent hybridization conditions (Schleifer and Killper-Bälz, 1987). Based on recent hybridization data, phenotypic differences and occurrence in completely different biotopes (mouth/milk), Schleifer and Kilpper-Bälz (1987) suggested maintaining *S thermophilus* and *S salivarius* as 2 separate species. It is obvious that the taxonomy of *S thermophilus* requires further discussion and that more data are required before reaching a definitive decision.

Colmin *et al* (1991) developed a DNA probe which specifically hybridized to 25 strains of subspecies *thermophilus* and to 2 strains of subspecies *salivarius* of *S salivarius*. This cross-hybridization suggests that both species share common DNA sequences, but more data are needed to support a possible close relationship between these 2 streptococci. In addition, the probe easily detects RFLP (restriction fragment length polymorphism) in *S thermophilus* and accordingly can be used for strain characterization.

L. delbrueckii* subsp *bulgaricus

This lactobacillus was first described by Orla-Jensen (1919) and named *Thermobacterium bulgaricum*. It is presently considered as one of the subspecies of *Lactobacillus delbrueckii*. Two other subspecies, subsp *delbrueckii* and subsp *lactis*, also belong to this group. The old terminology *Lactobacillus leichmannii* is no longer in use.

L. delbrueckii subsp *bulgaricus* is homofermentative, ferments few carbohydrates, ie glucose, lactose, fructose, and sometimes galactose or mannose, and has a high growth temperature (up to 48 or 50 °C). Its DNA mol G + C% ranges from 49 to 51% (Kandler and Weiss, 1986).

Simonds *et al* (1971) obtained a DNA homology of 86% between the former *L. bulgaricus* and *L. lactis* species, but only 4.8% with *Lactobacillus helveticus* and no homology with *L. helveticus* var *jugurti*. This was confirmed by Dellaglio *et al* (1973) who obtained < 7% DNA homology between *L. bulgaricus* and *L. helveticus* or *L. helveticus* var *jugurti*. The study of Weiss *et al* (1983) showed 80 to 100% homology (90–100% for type-strains) between the former *L. bulgaricus*, *L. leichmannii*, *L. lactis* and *L. delbrueckii* species. DNA homology led to the reclassification of these 4 lactobacilli as subspecies of *L. delbrueckii* in the most recent classification of *Bergey's Manual* (Kandler and Weiss, 1986).

A specific DNA probe for *L. delbrueckii* has been developed recently, which makes it possible to differentiate this species from other lactobacilli, lactococci and propionibacteria (Delley *et al*, 1990).

In the present article, we shall use the former names of the yogurt organisms, ie *S. thermophilus* and *L. bulgaricus* for purposes of convenience.

IMPORTANT METABOLIC ACTIVITIES FOR YOGURT TECHNOLOGY

The role of streptococci and lactobacilli in yogurt manufacture can be summarized as follows: milk acidification, synthesis of aromatic compounds, development of texture and viscosity. The latter aspect is required mainly for stirred and liquid yogurts. Thus, for industrial yogurt manufacture, starter selection takes into account these 3 properties. Evaluation of acidification properties is difficult because of the high buffering capacity of milk and the lack of a standard procedure. Efforts have been made to develop new methods for an objective evaluation of strains in replacement of former procedures (eg that of Accolas *et al*, 1977) based on the measurement of titratable acidity in milk. For example, the method patented by Corrieu *et al* (1989), and described by Spinnler and Corrieu (1989), uses continuous automatic recording of pH during bacterial growth in milk and the strains are compared on the basis of maximum acidification rates and the corresponding time and pH. In the field of industrial preparation of mixed starter cultures, efforts have been made to control the growth of streptococcus and lactobacillus populations by culture conditions, pH and temperature (Béal *et al*, 1989). Chamba and Prost (1989) evaluated the acidifying properties of strains used for cheesemaking, based on pH measurements during growth following the temperature profile of a standard cheesemaking operation.

The evaluation of aroma formation is generally based on the production of acetaldehyde, a major aromatic compound of yogurt, whereas the thickening character is based on measurements of milk viscosity (Bouillanne and Desmazeaud, 1980, 1981; Zourari *et al*, 1991; Zourari and Desmazeaud, 1991).

In the first part of this review, we present the metabolic activities responsible for the acidification, flavouring and thickening properties of yogurt bacteria. We then describe some metabolic activities of lesser importance for yogurt manufacture, but which determine the growth of bacteria in milk and their interactions, *ie* proteolysis, lipolysis, urease activity, oxygen effect.

Acid production.

Carbohydrate metabolism

The main role of *S thermophilus* and *L bulgaricus* in yogurt manufacture is to acidify milk by producing a large amount of lactic acid from lactose. Lactic acid reduces the pH of the milk and leads to a progressive solubilization of micellar calcium phosphate. This causes the demineralization of casein micelles and their destabilization, which generates the complete precipitation of casein in a pH range of 4.6–4.7 (Fox, 1989). In addition, lactic acid provides yogurt with its sharp, acid taste and contributes to flavour.

Lactic acid production may also occur during yogurt storage at low temperature. It may lead to an excessive acidification which affects the organoleptic properties of the product. This activity depends on the strains used and especially on the lactobacilli (Accolas *et al*, 1977; Bouillanne and Desmazeaud, 1980, 1981).

Carbohydrate metabolism of *S thermophilus*

Lactose and galactose transport

The mechanism of lactose transport in *S thermophilus* differs from that of lactococci, which possess a specific system for lactose transport, the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Lactose-6-phosphate

formed during transport is hydrolyzed by an intracellular phospho- β -galactosidase into glucose and galactose-6-phosphate which are then metabolized into lactic acid (Thompson, 1987). *S thermophilus* does not possess lactose PTS or phospho- β -galactosidase (Tinson *et al*, 1982a; Thomas and Crow, 1984), but has a lactose permease system including a proton-dependent membrane-located permease and an intracellular β -galactosidase (Poolman *et al*, 1989).

The lactose permease gene of *S thermophilus* (*lacS*) was cloned and expressed in *Escherichia coli* (Poolman *et al*, 1989). The comparison of amino acid sequences showed that the streptococcal lactose transport protein (*LacS*) differs from the lactose permease of *E coli* (*LacY*) and appears to be a hybrid protein of 69 kDa. The NH₂-terminal region is homologous with the melibiose carrier (*MelB*) of *E coli* (23% overall similarity) whereas the COOH-terminal end is partially homologous (34–41%) with enzyme III (*E III*) of 3 different PEP-PTS systems.

Galactose seems to be directly involved in lactose transport. In fact, the presence of a large amount of galactose in the growth medium inhibits lactose transport (Somkuti and Steinberg, 1979). Biochemical studies have shown that galactose has a considerable affinity for lactose permease and acts as a competitive inhibitor of the lactose transport system (Hutkins and Morris, 1987), but more recent data supply another explanation. In membrane vesicles of *E coli*, *lacS* permease not only catalyzes a proton motive force-linked transport system, but also exchange of β -galactosides. This means that the lactose transport reaction proceeds as a lactose-galactose antiport independent of the proton motive force (Poolman *et al*, 1989; Hutkins and Ponne, 1991).

Galactose transport requires an exogenous energy source and involves the action of a galactose permease. A PEP-PTS-like system has not been detected in *S thermophilus*. Glucose and galactose transport is slower than that of lactose (Hutkins *et al*, 1985a). The preferential utilization of lactose could be due to limitations in monosaccharide transport (Somkuti and Steinberg, 1979). In light of the involvement of galactose in lactose transport, however, these data should be re-evaluated.

Metabolic pathways of lactose, glucose and galactose utilization

Figure 1 summarizes the proposed pathways for lactose and galactose transport and their utilization by *S thermophilus*. Intracellular β -galactosidase (β -gal) hydrolyzes lactose to glucose and galactose. Glucose represses synthesis of this enzyme (Tinson *et al*, 1982a). Streptococcal β -gal has been purified and characterized (Ramana Rao and Dutta, 1981; Greenberg and Mahoney, 1982; Smart *et al*, 1985). The β -gal-encoding gene has been cloned and expressed in *E coli* (Herman and McKay, 1986) and in *Saccharomyces cerevisiae* (Lee *et al*, 1990). Streptococcal β -gal is a protein of about 105 kDa and the β -gal-encoding gene is located in a 3.85-kb region of the chromosomal DNA (Herman and McKay, 1986). This enzyme is useful as a potential selective marker in food-grade cloning vectors, and also for its possible industrial production because of its higher thermostability compared to currently produced yeast enzymes.

The glucose moiety of lactose is directly used *via* the Embden-Meyerhof-Parnas pathway (fig 1). *S thermophilus* possesses 2 fructose-1,6-diphosphate-independent (FDP-independent) lactate dehydrogenases (LDH) which reduce pyruvate to lactic acid (Garvie, 1978).

The galactose moiety is released into the extracellular medium by most *S thermophilus* strains which are not able to use it, *ie* Gal⁻ phenotype (Hutkins and Morris, 1987). Gal⁺ strains have been isolated (Somkuti and Steinberg, 1979; Thomas and Crow, 1984), however, in which galactose is metabolized *via* the Leloir pathway involving the enzymes galactokinase, galactose-1-phosphate-uridylyl transferase and uridine-5-diphospho-glucose-4-epimerase. The 2 latter enzymes are constitutive and present in Gal⁻ as well as in Gal⁺ strains (Thomas and Crown, 1984). The difference between the 2 phenotypes seems to be due to a difference in galactokinase and/or galactose permease activity. Both enzymes are induced by galactose and repressed by lactose (Hutkins *et al*, 1985a, b). Nucleotide sequences of the genes encoding the 2 constitutive enzymes are homologous with the analogous genes of other bacteria and yeasts. Their expression and regulation have been studied (Poolman *et al*, 1990). Until the present time, the use of galactose *via* the tagatose pathway has not been reported for either *S thermophilus* or *L bulgaricus*.

It is possible to obtain Gal⁺ derivatives of *S thermophilus* strains after growth of Gal⁻ cultures in a chemostat under lactose limitation (Thomas and Crow, 1984). The use of Gal⁺ strains in cheesemaking decreases residual galactose in Cheddar, Mozzarella, and Emmental cheeses and thus avoids some serious technological problems. As mentioned by Hutkins *et al* (1986), high residual galactose in cheese curd: (i) may enable a heterofermentative metabolism of galactose during ripening, with concomitant carbon dioxide production, and may also lead to the development of a harmful flora generating off-flavours and texture defects and (ii) has been implicated in browning reactions which occur during processing or cooking at high tem-

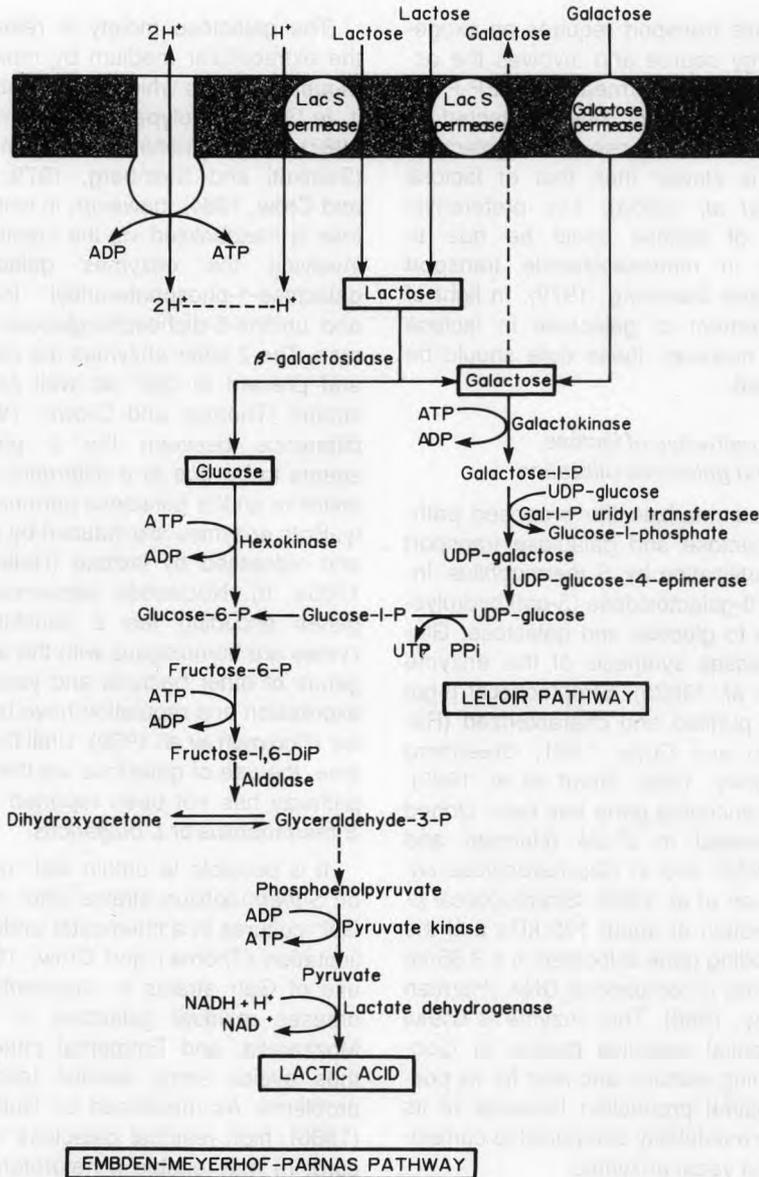


Fig 1. Proposed pathways for lactose (lac) and galactose (gal) utilization in *S. thermophilus*. From Hutkins and Morris (1987), Thompson (1987) and De Vos (1990). P: phosphate, PPI: inorganic pyrophosphate, ATP: adenosine triphosphate, ADP: adenosine diphosphate, UTP: uridine triphosphate, UDP: uridine diphosphate.

Voies métaboliques proposées pour l'utilisation du lactose (lac) et du galactose (gal) par S. thermophilus. D'après Hutkins et Morris (1987), Thompson (1987) et De Vos (1990). P : phosphate, PPI : pyrophosphate inorganique, ATP : adénosine triphosphate, ADP : adénosine diphosphate, UTP : uridine triphosphate, UDP : uridine diphosphate.

peratures of cheeses such as Cheddar or Mozzarella.

Sucrose utilization

Sucrose metabolism has been poorly documented, especially at the molecular and genetic levels. During the growth of *S thermophilus* on sucrose, both glucose and fructose are used simultaneously. Fructose accumulates in the growth medium, however, even when the strain can use it (Thomas and Crow, 1983). The higher rate of sucrose utilization compared to those of glucose and fructose might be due to a different uptake rate (Hutkins and Morris, 1987).

An inhibitory effect of high sucrose content in milk (10–12%), on the growth of yogurt bacteria has often been reported. It is due to both an adverse osmotic effect of the solutes in milk and a low water activity (Tamime and Robinson, 1985).

Carbohydrate metabolism of *L bulgaricus*

L bulgaricus is characterized by the utilization of a small number of carbohydrates, but their metabolism has not been studied enough. This lactobacillus uses only the glucose moiety of lactose and releases galactose into the growth medium. Some strains can use galactose in a growth medium containing limiting concentrations of lactose. Glucose transport occurs via a PTS-like system, while lactose and galactose uptake occurs by means of permeases (Hickey *et al*, 1986). Recent findings suggest that intracellular galactose is exchanged with lactose, enabling the transport of lactose as in *S thermophilus*. In fact, preliminary results indicate that galactose efflux from the cells is stimulated almost 100-fold by the presence of lactose in the external medium (Poolman *et al*, 1990). The lactose genes and gene organi-

zation are well conserved in *S thermophilus* and *L bulgaricus*. Their lactose permeases and β -galactosidases share 60 and 48% common amino acids, respectively (Mercenier, 1990).

Once inside the cell, lactose is hydrolyzed by a β -gal. Glucose represses lactose transport and β -gal synthesis. Purified β -gal of an *L bulgaricus* strain has a maximum activity at 50 °C and pH 7 (Itoh *et al*, 1980). The β -gal-encoding gene has been cloned in *E coli* and expressed using its own promoter. The molecular mass of the enzyme is about 114 kDa, deduced from the nucleotide sequence of the corresponding gene (Schmidt *et al*, 1989). Galactose is metabolized by *L bulgaricus* via the Leloir pathway. A galactokinase activity has been detected in Gal⁺ strains (Hickey *et al*, 1986).

Lactic acid isomers in yogurt

S thermophilus possesses 2 FDP-independent L-LDH (Garvie, 1978; Hemme *et al*, 1981a) and produces L(+) lactic acid, while *L bulgaricus* produces D(-) lactic acid and possesses an NAD-dependent stereospecific LDH (Gasser, 1970). Thus, both lactic acid isomers are simultaneously produced in yogurt.

The D-LDH of *L bulgaricus* purified by Le Bras and Garel (1991) is a dimer composed of 2 identical 37-kDa chains. The amino-terminal sequence determined on 50 residues is the first D-LDH sequence available and does not show significant homology with any known LDH. This suggests that the evolutionary process of D-LDH from *L bulgaricus* is not identical to that of the NAD-dependent L-LDH family.

D(-) lactic acid is metabolized only very slowly in man compared to the L(+) isomer and may cause metabolic disorders if ingested in excess. The World Health Organization therefore recommends a limited

daily consumption of D(-) lactic acid to 100 mg.kg⁻¹ bodyweight and some countries, *eg* Germany, have decided to minimize the D(-) lactic acid content of fermented dairy products (Kondratenko *et al*, 1989). In yogurt, this can only be obtained in the absence of *L bulgaricus* producing the D(-) isomer, which accounts for 30–50% of the total lactic acid content of yogurt (Kunath and Kandler, 1980; Alm, 1982a). Nevertheless, the D(-) lactic acid content may reach critical concentrations that can lead to disturbances especially when the diet is extremely unbalanced, but this finding has not yet been reported (Gurr, 1986). Rare cases of D(-)-lactic acidosis have been reported in children and adults, in association with altered bowel physiology (short gut syndrome) and proliferation of active D-lactate bacterial producers (Szyllit and Nugon-Baudon, 1985).

Detection of a weak L-LDH activity in the type-strain of *L bulgaricus* grown in a chemostat is therefore very interesting (Ragout *et al*, 1989). The identification of its mechanism of regulation could contribute to increase L(+) lactic acid synthesis by this species and to reduce the D(-) lactic acid content of yogurt.

Formation of flavour compounds

Flavour compounds of yogurt

The typical flavour of yogurt is due to lactic acid and various carbonyl compounds, *ie* acetaldehyde, acetone and diacetyl, produced by *S thermophilus* and *L bulgaricus*. In addition to carbonyl substances, many volatile compounds have also been identified in yogurt, *ie* volatile fatty acids (Turcic *et al*, 1969; Dumont and Adda, 1973) and several compounds derived from the thermal degradation of lipids, lactose and proteins during the heat treatment of milk be-

fore yogurt manufacture *eg* aldehydes, ketones, alcohols, lactones, sulfur compounds (Tamime and Deeth, 1980).

Acetaldehyde is considered as the major flavour component of yogurt (Pette and Lolkema, 1950c; Dumont and Adda, 1973; Law, 1981) while high concentrations of this compound in other dairy products (cheese or cream) lead to flavour defects described as "green" or "yogurt-like" (Lindsay *et al*, 1965). Bottazzi and Vescovo (1969), however, underlined the importance of the acetaldehyde to acetone ratio.

Diacetyl contributes to the delicate, full flavour of yogurt and seems to be important when the acetaldehyde content is low (Groux, 1973a). According to Rasic and Kurmann (1978) *S thermophilus* is the only microorganism responsible for diacetyl production in yogurt, whereas Dutta *et al* (1973) reported that diacetyl is also largely produced by *L bulgaricus* (12–13 ppm for both species in pasteurized milk). Rysstad and Abrahamsen (1987) detected diacetyl in goat's milk yogurt with a low acetaldehyde content and suggested that diacetyl might be very important for the flavour of goat's milk yogurt.

Even though the importance of acetaldehyde, acetone and diacetyl for yogurt flavour is well established, it is very difficult to evaluate the contribution of several identified volatile compounds because their sensory perception thresholds vary considerably (Dumont and Adda, 1973).

In spite of important progress in chromatographic methods such as head-space techniques which increase the detection sensitivity of volatile compounds (Degorce-Dumas *et al*, 1986), only few recent studies are available in this field and none of them has involved a large number of strains examined using the recently developed techniques. Thus, it is difficult to compare results and conflicting findings

may be due to the different sensitivity of the methods previously used.

Metabolic pathways of acetaldehyde synthesis

Formation of acetaldehyde from glucose

Two possible pathways exist:

– The Embden–Meyerhof–Parnas pathway generates pyruvate. An α -carboxylase catalyzes the formation of acetaldehyde from pyruvate. An aldehyde dehydrogenase may also generate acetaldehyde from acetyl-CoA which is formed from pyruvate by the action of a pyruvate dehydrogenase. Neither significant α -carboxylase nor aldehyde dehydrogenase activities have been detected by Raya *et al* (1986) in 2 strains of *S thermophilus* and 2 strains of *L bulgaricus*. Nevertheless, Lees and Jago (1976a) found an aldehyde dehydrogenase activity in 4 strains of each species. Therefore it is difficult to conclude whether this pathway occurs rarely or frequently in yogurt bacteria.

– The hexose monophosphate (HMP) pathway generates acetylphosphate. A phosphotransacetylase catalyzes the formation of acetyl-CoA from acetylphosphate and an acetate kinase leads to the formation of acetate. Although Raya *et al* (1986) detected these 2 activities in both yogurt bacteria, acetaldehyde cannot be formed by this pathway since their strains did not possess aldehyde dehydrogenase which catalyzes the biosynthesis of acetaldehyde from acetyl-CoA or acetate.

Formation of acetaldehyde from threonine

Threonine aldolase catalyzes the cleavage of threonine to acetaldehyde and glycine and appears to be the most important pathway for acetaldehyde production in yogurt. In fact, Raya *et al* (1986) detected

this enzyme in 2 strains of *L bulgaricus*, but not in the 2 strains of *S thermophilus* investigated. In other studies, however, this enzyme was detected in both species (Lees and Jago, 1976b; Wilkins *et al*, 1986a; Marranzini *et al*, 1989). Streptococcal threonine aldolase activity decreases significantly when growth temperature increases from 30 to 37 °C (Lees and Jago, 1976b) or from 30 to 42 °C (Wilkins *et al*, 1986b), but that of *L bulgaricus* remains almost identical. Thus, at 37 and 42 °C threonine aldolase activity of the streptococci is significantly lower than that of the lactobacilli, whereas it is identical at 30 °C. Since yogurt is generally manufactured at high temperatures (43 °C), acetaldehyde is probably produced mainly by *L bulgaricus*.

The addition of threonine (0.6%) or glycine (2%) to the growth medium does not significantly affect the specific activity of threonine aldolase in cell extracts (Lees and Jago, 1976b). High threonine concentrations combined with low glycine concentrations in the growth medium increase the biosynthesis of the enzyme. When this ratio is inverted, a decrease is observed. However, the threonine concentration during growth may have a greater effect on increasing threonine aldolase activity than the glycine concentration may have on inhibiting it. Streptococcal threonine aldolase is more affected by high glycine concentrations than the lactobacillus enzyme (Marranzini *et al*, 1989). The threonine aldolase of a *L bulgaricus* strain has been partially purified and studied by Manca de Nadra *et al* (1987). Maximum enzyme activity is observed at 40 °C and pH 6.5 as well as a strong allosteric pH-dependent inhibition by glycine. Inhibition of threonine aldolase by glycine could explain the low acetaldehyde content of yogurt from goat's milk, which is richer in glycine than cow's milk (2.4 versus 0.84 mg.100 ml⁻¹; Rysstad *et al*, 1990).

Formation of acetaldehyde from DNA components

Two strains of *S thermophilus* and one *L bulgaricus* strain studied by Raya *et al* (1986) possess a deoxyriboaldolase which catalyzes the synthesis of acetaldehyde from 2-deoxyribose-5-phosphate. This enzyme could be involved in the degradation rather than the synthesis of DNA. The 4 lactobacilli strains and 3 out of the 4 streptococcal strains examined by Lees and Jago (1977) did not contain this enzyme.

Alcohol dehydrogenase

Alcohol dehydrogenase reduces acetaldehyde to ethanol. This enzyme has not been detected in *L bulgaricus* but has been found in 2 of 4 strains of *S thermophilus* (Lees and Jago, 1976a; Marshall and Cole, 1983; Raya *et al*, 1986).

Production of polysaccharides

Several Gram-negative and Gram-positive bacteria, including lactic acid bacteria, produce exocellular polysaccharides. The "ropy" character is often required for the manufacture of many fermented milk products. Ropy strains of lactococci have been isolated from Scandinavian fermented dairy products (Makura and Townsley, 1984; Nakajima *et al*, 1990). In stirred yogurt, yogurt beverages and low milk solids yogurts, production of polysaccharides can improve viscosity and texture, increase resistance to mechanical handling and decrease susceptibility to syneresis. The use of ropy strains is particularly important in France and in the Netherlands where the addition of stabilizers in yogurt is prohibited. In fact, some strains of *S thermophilus* and *L bulgaricus* produce neutral exopolysaccharides. Only few studies are available on the characterization and production of these polymers (Cerning, 1990).

Schellhaass (1983) isolated ropy strains of some thermophilic lactic acid bacteria. She observed higher exopolysaccharide production in milk at suboptimal growth temperatures, as measured by an increase of relative viscosity. The polymer material obtained contained galactose and glucose in the ratio 2:1. The slime secreted by a strain of *L bulgaricus* studied by Groux (1973b) contained mainly galactose and some glucose, mannose and arabinose. Cerning *et al* (1988) studied an exocellular polysaccharide produced by *S thermophilus*, which contains galactose (55%), glucose (25%), mannose (15%) and small amounts of rhamnose, xylose and arabinose. The *L bulgaricus* exopolysaccharide studied by Cerning *et al* (1986) contains galactose, glucose and rhamnose in an approximate molar ratio of 4:1:1 with a molecular mass of about 500 kDa and intrinsic viscosity of 4.7 dl.g⁻¹. Doco *et al* (1990) studied an exopolysaccharide produced by *S thermophilus* after 3–4 h of incubation and obtained a polymer composed of galactose, glucose and *N*-acetylgalactosamine in a ratio of 2:1:1. The molecular mass of the polymer is 1 x 10³ kDa and intrinsic viscosity 1.54 dl.g⁻¹. Rhamnose, present mostly in the exopolysaccharides of *L bulgaricus*, has not been identified in the exocellular polymer produced by 3 lactobacillus strains isolated from traditional Greek yogurts (Zourari, 1991). These molecules are composed of galactose (50–70%), glucose (20–40%) and some mannose and arabinose.

The quantities of polymer formed by ropy strains of both species vary considerably even under identical experimental conditions (Giraffa and Bergère, 1987; Cerning *et al*, 1990; Zourari, 1991). It is difficult to establish a good correlation between the quantity of polysaccharide produced and the corresponding viscosity. This difficulty may be due to changes in

the 3-dimensional configuration of polymers and to their interactions with some milk compounds, mainly caseins that are precipitated at low pH (Olsen, 1989). In addition, viscosity measurements are difficult to interpret for non-Newtonian solutions such as milk and fermented milk products. Bottazzi and Bianchi (1986) studied by scanning electron microscopy (SEM) the structure of milk fermented with a ropy *L. bulgaricus* strain. It was shown that a part of the polysaccharide covers the cells with a uniform layer, and the rest of the polymer binds cells together and to milk casein via a dense network of visible filaments. Recently, Teggatz and Morris (1990) used SEM to explain changes occurring in the microstructure of ropy yogurt when it is subjected to a shear force. Their aim was to understand the interactions of exopolysaccharide material with its surroundings, and how they may influence viscosity. It was observed that an increase of shear rate first disrupts the attachment of polymer to the bacterial surface, but the polysaccharide material remains incorporated with the casein where it continues to influence viscosity.

The ropy character of *S. thermophilus* is often unstable (Cerning *et al.*, 1988). This may be partially due to the presence of glycohydrolase enzymes capable of hydrolyzing the polysaccharide material. There is no evidence of a possible loss of plasmids encoding this character as in *Lactococcus lactis* subsp. *cremoris* (Vedamuthu and Neville, 1986) or in *Lactobacillus casei* subsp. *casei* (Vescovo *et al.*, 1989). In fact, ropy strains of yogurt bacteria studied by Cerning *et al.* (1986, 1988, 1990) and Zourari (1991) are plasmid-free.

OTHER METABOLIC ACTIVITIES

Proteolytic activity

Amino acids in milk and yogurt

In yogurt, proteolysis is not determinant for organoleptic properties, but it is an important factor for the selection of strains for cheesemaking. On the other hand, proteolytic activity is greatly involved in both nutrition and interactions of yogurt bacteria, since lactic acid bacteria cannot synthesize essential amino acids. Therefore, they require an exogenous nitrogen source and utilize peptides and proteins in their growth medium by more or less complete enzyme systems.

S. thermophilus primarily requires glutamic acid, histidine and methionine, as well as cystine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine for growth (Shankar and Davies, 1977; Bracquart *et al.*, 1978). The uptake of branched-chain amino acids has been studied. It is an active transport which requires an exogenous energy source, depends on temperature and pH and is inhibited by L-cysteine (Akpemado and Bracquart, 1983).

The free amino acid content of cow's milk generally does not exceed 10 mg·100 ml⁻¹ (Rasic and Kurmann, 1978; Miller *et al.*, 1964; Alm, 1982c). In yogurt, the free amino acid pattern depends on the type of milk (animal species, season), its heat treatment, manufacturing techniques, bacterial strains used and storage conditions (Rasic *et al.*, 1971a, b). The free amino acid content generally increases in yogurt compared to that of milk. *L. bulgaricus* ap-

pears to be the main species responsible for these changes (Miller *et al*, 1964; Alm, 1982c; Feller *et al*, 1990).

Utilization of proteins

Milk proteins (caseins, whey proteins) are the main nitrogen source for lactic acid bacteria, which utilize them with the action of exocellular proteinases, membrane-bound aminopeptidases and intracellular exopeptidases and proteinases.

Proteinase activity has been detected in several strains of lactobacilli and streptococci by Ezzat *et al* (1985) and Kalantzopoulos *et al* (1990). *L bulgaricus* possesses a firmly cell-bound proteinase with optimum activity between 45 and 50 °C and pH values ranging from 5.2 to 5.8 (Argyle *et al*, 1976). The partially purified cell-wall-associated proteinase studied by Ezzat *et al* (1987) has maximum activity at 35 °C and pH 5.5. The proteinase of *L bulgaricus* is more active on β -casein than on whey proteins (Chandan *et al*, 1982). El Soda and Desmazeaud (1982) and Laloï (1989) revealed a preferential but partial hydrolysis of β - and α_{s1} -caseins. Laloï (1989) also observed that after growth in milk, caseinolytic activity is 3 times higher than that measured after growth in a complex medium rich in short peptides.

Desmazeaud (1974) studied an intracellular metalloproteinase of *S thermophilus* which was more active on the carboxymethylated B-chain of insulin or on glucagon than on caseins. A weak caseinolytic activity was detected in both the cell envelopes and cytoplasm of *S thermophilus*. As the 2 corresponding enzymes had the same electrophoretic mobility they were considered to be identical (Meyer *et al*, 1989). A high cell-wall-associated proteinase activity characterizes 2 strains of *S thermophilus* studied by Shahbal *et al*

(1991) who also obtained a proteinase-negative mutant. Strains which possess this proteolytic activity have a significantly higher acidification rate in milk compared with 13 other randomly chosen strains.

Utilization of peptides

The low molecular weight peptide fraction of milk is an important nitrogen source for yogurt bacteria. The importance of peptides for their growth stimulation and their acidification is now well established, especially for *S thermophilus* (Desmazeaud and Devoyod, 1970; Desmazeaud and Hermier, 1973; Bracquart and Lorient, 1979; Hemme *et al*, 1981b).

S thermophilus generally possesses a leucine-aminopeptidase activity (Bouillanne and Desmazeaud, 1980). Some strains possess an arginine-amino-peptidase activity which is usually inactive against dipeptides. Two intracellular metalloenzymes of this species have been purified and characterized, *ie* an amino-peptidase with broad specificity and a dipeptidase which hydrolyzes dipeptides with a hydrophobic and bulky residue at the NH₂ terminal end (Rabier and Desmazeaud, 1973). A non specific prolyl-dipeptidase hydrolyzing several dipeptides has also been detected (Desmazeaud and Jugé, 1976). An X-prolyl-dipeptidylamino-peptidase (X-pro-DPAP) of a *S thermophilus* strain has been purified, characterized and compared with that of a *L bulgaricus* strain. The streptococcal enzyme is composed of 2 subunits, has a molecular mass of 165 kDa, an isoelectric point of 4.5 and optimum pH values ranging from 6.5 to 8.2 (Meyer and Jordi, 1987).

Two intracellular exopeptidases of *L bulgaricus* have been characterized by El Soda and Desmazeaud (1982), an amino-peptidase with specificity limited to argi-

nine- β -naphthylamide, and a dipeptidase with broad specificity. Laloi (1989) revealed the presence of 4 aminopeptidases (API, II, III, IV) and an X-pro-DPAP in the same species. API and APIII (cytoplasmic enzymes) are induced after growth in a complex medium, while APII and APIV are constitutive. Mutants partially or totally free of APII or X-pro-DPAP have been isolated after chemical mutagenesis. Deficiency of these 2 enzymes significantly increases the caseinolytic activity of mutants compared to the parental strain (Atlan *et al*, 1989, 1990).

Lipolytic activity

Lipolysis is generally low in yogurt and is therefore not significant in terms of flavour. The free fatty acid content of yogurt differs only slightly from that of milk (Rasic and Vucurovic, 1973; Alm, 1982b). Therefore, only few studies with these enzyme systems have been reported in yogurt bacteria.

Tamime and Deeth (1980) have summarized the main lipolytic activities as follows: the hydrolytic activity of streptococci on tributyrin and triolein is high and, inversely, is low on milk fat and on Tween 40 or 60. The hydrolytic activity of lactobacilli on tributyrin is weaker, while esterases hydrolyzing several soluble substrates, eg α -naphthylacetate, are active in both bacteria.

L. bulgaricus possesses intracellular esterases active on *ortho*- and *para*-nitrophenyl derivatives of short-chain fatty acids and activity levels vary only slightly between different strains (El Soda *et al*, 1986). Many strains of *S. thermophilus* (30 out of 32 tested) possess a lipase more active towards tributyrin than towards natural lipids (De Moraes and Chandan, 1982) and maximum activity was observed at 45 °C and pH 9. Kalantzopoulos *et al*

(1990) detected esterase-1 and esterase-2 activities in both yogurt bacteria, using 2- and 4-nitrophenylbutyrate, respectively.

Urease activity

In milk, *S. thermophilus* produces a large amount of carbon dioxide (CO₂) which is not formed from lactose metabolism since this microorganism is a strictly homofermentative species (Driessen *et al*, 1982). It also produces a large quantity of ammonia (NH₃) which was initially explained as resulting from the deamination of some amino acids (Groux, 1973a). CO₂ production is due to the activity of urease, which breaks down milk urea (about 250 mg.l⁻¹) into CO₂ and NH₃ (Miller and Kandler, 1967b; Tinson *et al*, 1982b; Juillard *et al*, 1988). This leads to the alkalization of the growth medium and directly affects acidification rate measurements in milk (Spinnler and Corrieu, 1989; Famelart and Maubois, 1988; Zourari *et al*, 1991).

Urease activity is of technological interest for 3 reasons: (i) it enables streptococcal numbers in mixed cultures with *L. bulgaricus* (this lactobacillus has no urease activity) to be followed by measuring the amount of CO₂ produced (Spinnler *et al*, 1987; Miller and Kandler, 1967a); (ii) NH₃ production affects the evaluation of streptococcal acidifying properties by pH measurements; (iii) this activity may also be involved in the stimulation of lactobacilli by CO₂ produced by the streptococci (see discussion below).

Besides its technological interest, the presence of urease activity in *S. thermophilus* also has taxonomic relevance. Although Farrow and Collins (1984) reported that *S. thermophilus* does not degrade urea, the presence of an urease activity seems to characterize most strains examined until now. It also characterizes oral

S salivarius strains (Sissons *et al*, 1989) but lactococci do not possess this activity (Miller and Kandler, 1967b). The absence of urease activity in the 6 strains of *S thermophilus* studied by Bridge and Sneath (1983) could be due to the prolonged incubation time (5 days at 37 °C) before urease assays were carried out, since Juillard *et al* (1988) demonstrated that urease activity dropped during advanced stationary phase.

Oxygen and metabolism

Lactic acid bacteria are facultative anaerobes with a preference for anaerobic conditions (Whittenbury, 1978). They cannot synthesize porphyrins and consequently they do not synthesize cytochromes or catalase. Oxygen is sometimes used for the formation of hydrogen peroxide (H_2O_2) which is toxic for lactic acid bacteria which do not contain catalase to break it down. Some species possess protection mechanisms, *eg* a high content of manganese which is oxidized by O_2^- radicals in *Lactobacillus plantarum* (Archibald and Fridovich, 1981).

L bulgaricus is among the least oxygen-tolerant lactic acid bacteria (Archibald and Fridovich, 1981). It can produce a very large amount of H_2O_2 which activates the LPS system of milk (lactoperoxidase-hydrogen peroxide-thiocyanate system) and inhibits its own growth as well as the growth of some other bacteria such as *Lactobacillus acidophilus*, associated in the manufacture of some fermented milk products (Gilliland and Speck, 1977). *S thermophilus* does not produce enough H_2O_2 to activate the LPS system and therefore inhibition occurs only when exogenous H_2O_2 is added to milk (Premi and Bottazzi, 1972; Guirguis and Hickey, 1987). On the other hand, some strepto-

coccal strains can degrade H_2O_2 , *eg* strain TS2 studied by Smart and Thomas (1987). Nevertheless, no further experiments have been performed and the cleaving activity of this strain was attributed by the authors to a mechanism independent of active metabolism.

The differences generally observed between strains regarding their tolerance to and utilization of oxygen are due to variabilities in their enzymatic systems. Ritchey and Seeley (1976) detected no NADH-oxidase activity in 3 *S thermophilus* strains, while many lactococcal strains possess this enzyme. Nevertheless, another well studied strain possesses this enzyme as well as NADH-peroxidase, superoxide dismutase, pyruvate and lactate dehydrogenases (Smart and Thomas, 1987).

Teraguchi *et al* (1987) compared the *S thermophilus* STH 450 strain which has a high oxygen consumption, with the type-strain, ATCC 19258. Strain STH 450 has NADH-oxidase, but no pyruvate oxidase, and very low NADH-peroxidase activity. These enzyme activities are much lower in the type-strain. Strain STH 450 accumulates α -acetolactate, acetoin and diacetyl as end-products of aerobic glucose metabolism.

PARTICULAR ASPECTS OF YOGURT FERMENTATION

Interactions between yogurt bacteria

A positive interaction is generally observed between *S thermophilus* and *L bulgaricus* in mixed culture, leading to the stimulation of growth and acid production of both bacteria compared to their single-strain cultures (Pette and Lolkema, 1950a; Bautista *et al*, 1966; Accolas *et al*, 1977). In addi-

tion, total proteolysis in mixed culture (expressed in μg of released tyrosine per ml of culture) exceeds the sum of the values obtained by each strain alone (Rajagopal and Sandine, 1990). Mixed yogurt cultures may also stimulate the production of some metabolites such as acetaldehyde (Hamdan *et al*, 1971; Bottazzi *et al*, 1973) and influence carbohydrate utilization. For instance, one *L bulgaricus* strain studied which cannot use galactose in pure culture metabolizes this sugar when it is associated with one strain of *S thermophilus* (Amoroso *et al*, 1988, 1989). Contrary behavior was observed with one streptococcal strain (Oner and Erickson, 1986) indicating that interactions between yogurt bacteria are very complex and are greatly dependent on the strains involved.

S thermophilus does not possess substantial extracellular proteolytic activity and the amino acid and free peptide content of milk is not high enough to promote its full growth. Lactobacillus proteases break down caseins and supply the streptococcus with amino acids and peptides (Pette and Lolkema, 1950b, Bautista *et al*, 1966; Shankar and Davies, 1978; Radke-Mitchell and Sandine, 1984).

The growth of *L bulgaricus* is stimulated by a compound produced by *S thermophilus*, which seems to be formic acid (Galesloot *et al*, 1968; Veringa *et al*, 1968; Accolas *et al*, 1971; Shankar and Davies, 1978). Higashio *et al* (1978) reported a combined stimulating effect of formic and pyruvic acids. Suzuki *et al* (1986) observed that addition of formic acid to boiled milk prevents abnormal cell elongation in *L bulgaricus* (filamentous forms). Formic acid synthesis from pyruvate is a limiting step in purine synthesis and this explains the combined action of the 2 acids. When formate is lacking, ribonucleic acid (RNA) synthesis is depressed. Elongated cells contain less RNA than normal cells while DNA contents

of both cells are almost equal. The combined growth of *L bulgaricus* and *S thermophilus* has the same effect as the addition of formic acid. A better knowledge of pathways and kinetics of formic acid production by *S thermophilus* is required to definitively establish the stimulating effect of this acid, questioned by Juillard *et al* (1987).

Driessen *et al* (1982) observed that stimulation of lactobacilli growth could also result from an increased CO_2 content in the growth medium, during continuous culture at constant pH. *S thermophilus* produces a large quantity of CO_2 from milk urea (Tinson *et al*, 1982b). In this way, it can stimulate lactobacilli, since the quantity of CO_2 dissolved in milk decreases after heat treatment and so remaining CO_2 is too low to meet the requirements of the lactobacillus (Driessen *et al*, 1982). Carbon dioxide might be involved in the synthesis of aspartic acid (Reiter and Oram, 1962).

In conclusion, interaction between yogurt bacteria is a good example of integrated metabolism in a mixed culture of lactic acid bacteria, but our knowledge on the stimulation of *L bulgaricus* by *S thermophilus* is still incomplete.

Production of antimicrobial compounds by yogurt bacteria

As mentioned above, there is generally a symbiotic relationship between yogurt bacteria, but growth inhibition is sometimes observed (Moon and Reinbold, 1974; Suzuki *et al*, 1982; Pereira Martins and Luchese, 1988). This should be taken into account when selecting starters. Inhibition may be due to competition for one or more nutrients of the growth medium (Moon and Reinbold, 1976) or to inhibitory compounds produced by the strains, such as bacteriocins and inhibitory peptides (Pereira Martins and Luchese, 1988).

Pulusani *et al* (1979) extracted at least 3 fractions of ≈ 700 Da from milk cultured with *S thermophilus*, which inhibited the growth of *Pseudomonas*, *Bacillus*, *E coli*, *Flavobacterium*, *Shigella*, *Salmonella* and *Lactococcus* strains. These fractions are most likely aromatic amines released in the growth medium since cells are free of any antimicrobial activity. Glucose and lactose, but not sucrose, are essential for production of these compounds which are also obtained in milk-free media, *eg* "soy-milk" (Rao and Pulusani, 1981).

Smaczny and Krämer (1984a) studied 2 bacteriocins of 10–20 kDa produced by 2 *S thermophilus* strains which inhibited the growth of strains of the same species and to a lesser extent of enterobacteria. These molecules are sensitive to several proteases and to a lipase, suggesting that a lipid component participates in their active sites.

An antimicrobial substance named "bulgarican" produced by an *L bulgaricus* strain has been partially purified (Reddy and Shahani, 1971; Reddy *et al*, 1983). At neutral or acid pH, this heat-stable substance was active against several strains of *Bacillus*, *Streptococcus*, *Staphylococcus*, *Sarcina*, *Pseudomonas*, *Escherichia* and *Serratia* species. Protonated carboxyl groups may be important for the activity of this molecule. Spillmann *et al* (1978) could not confirm, however, bulgarican production.

A newly isolated antibacterial substance from *L bulgaricus*, active against a *Pseudomonas fragi* and a *Staphylococcus aureus* strains has optimum pH values close to 4. It is a di- or a tripeptide (molecular mass < 700 Da) which probably contains an aromatic group (Abdel-Bar *et al*, 1987).

Knowledge of antibacterial substances produced by yogurt bacteria is still fragmentary and the results are often contradictory. Available studies are few and give

only little information on the nature of the compounds involved, their spectrum and mode of action. These antibacterial substances often seem to be peptides. More extensive study is required for a better understanding of interactions of yogurt bacteria with other lactic acid bacteria and with non-lactic cheese flora.

Bacteriophages of *L bulgaricus* and *S thermophilus*

Knowledge of the specific bacteriophages (phages) of thermophilic lactic acid bacteria has been well documented, primarily on and after 1980. Specific phages may attack *S thermophilus* and *L bulgaricus* strains during yogurt or cheese manufacture and seriously affect product quality. Moreover, even if phage attacks do not delay acidification during yogurt manufacture, they can lead to an important decrease in the streptococci and to a lower flavour score of the resulting yogurt (Stadhouders *et al*, 1988). Phage outbreaks are less frequent in modern yogurt production units than in cheese factories because: (a) the high heat treatment of milk destroys phages present in raw milk; (b) aseptic processing and packaging may prevent contaminations by airborne bacteriophages, bacteria, yeasts or moulds; and (c) according to Lawrence and Heap (1986), an increasing viscosity of the yogurt milk below pH 5.2 and the fact that no whey is drained off during yogurt manufacture could efficiently reduce the spreading of phages in yogurt plants. Thus, phage attacks are due either to accidental contaminations (Stadhouders *et al*, 1984) or to the presence of lysogenic starter bacteria which might be the major source of phages by two possible routes: (i) and temperate phage particles derived from a lysogenic strain may infect a sensitive (indicator) strain of the same starter;

(ii) temperate phages may give rise to virulent mutants able to superinfect and lyse the parental lysogenic and other related strains. A typical example of the latter incidence has been well documented for a lysogenic strain of *Lactobacillus casei* subsp *casei* used in Japan for the industrial manufacture of a fermented milk beverage, Yakult (Shimizu-Kadota and Sakurai, 1982; Shimizu-Kadota *et al*, 1985). Substitution of a prophage-free strain for the lysogenic strain immediately stopped all phage infections in the different Yakult production units.

Bacteriophages of *L bulgaricus* are very closely related to those of *L delbrueckii* subsp *lactis* (*L lactis*) but they are completely different from those of *L helveticus*, which is usually used in cheesemaking (Séchaud, 1990). Two surveys of *L bulgaricus* and *L lactis* phages (Cluzel, 1986; Mata *et al*, 1986) led to the description of 4 groups named a, b, c and d. All these phages belong to the *Siphoviridae* family of Matthews (1982) which corresponds to Bradley's group B (Bradley, 1967). The head of these phages is isometric or prolate, collars are frequent, and the tails often have diverse terminal structures (pronged or plain plates, fibers) and occasionally harbour regularly spaced cross-bars. Figures 2 and 3 illustrate some of these typical morphologies. Group a is the largest phage group (20 of the 26 phages studied by Cluzel, 1986) and includes temperate phages of both subspecies (8 of the 20 phages of Cluzel's group a) along with lytic phages isolated in cheese plants or yogurt production units (12 of the 20 phages of Cluzel's group a). All phages of group a share common molecular characteristics, serological properties, lytic spectra and are specific towards both subspecies (typical morphology in figure 2). The close relationship between temperate and lytic phages of group a suggests that lysogeny may be

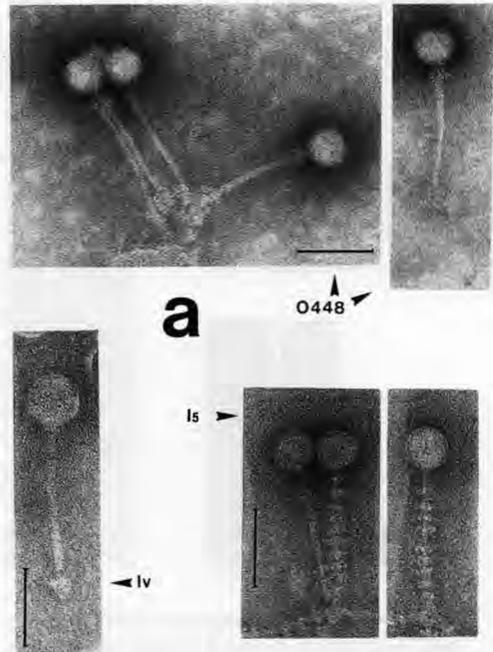


Fig 2. Morphology of bacteriophages which belong to group a. Their host-ranges include *L bulgaricus* and *L lactis* strains. Phage 15 possesses fragile cross-bars on its tail which may be easily removed during phage preparation. Collars are visible at the head-tail junctions of phage 0448 (triple collar) and phage 1v (single collar). Fibers are present at the tail tip of phages 1v and 0448. Bars = 100 nm; the magnification factor is the same for all the pictures (micrographs from INRA, Jouy-en-Josas, France).

Morphologie de bactériophages du groupe a. Ces phages attaquent indifféremment un certain nombre de souches de L bulgaricus et L lactis. Le phage 15 possède des structures transversales fragiles tout le long de la queue, qui peuvent se détacher facilement au cours de la préparation du phage. Des colliers sont visibles à la jonction de la tête et de la queue des phages 0448 (triple collier) et 1v (collier simple). Des fibres sont présentes à l'extrémité distale de la queue des phages 1v et 0448. Barres = 100 nm; l'agrandissement est le même pour toutes les images (micrographies de l'INRA, Jouy-en-Josas, France).

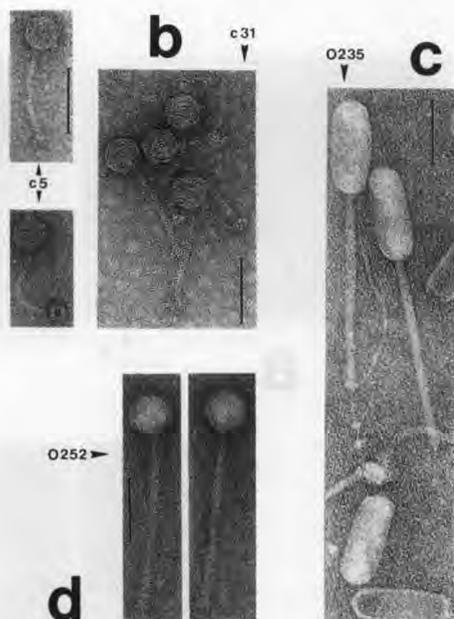


Fig 3. Morphology of bacteriophages which belong to groups **b**, **c** and **d**. Phages of group **b**, such as **c31** and **c5**, have a host-range restricted to *L. bulgaricus* strains whereas phages **0235** (group **c**) and **0252** (group **d**) have host-ranges similar to those of group **a** phages. Phages **c31** and **c5** (groupe **b**) possess a non-contractile tail fitted with a pronged-base plate and at least one fiber. Tail tip of prolate-headed temperate phage **0235** (group **c**) exhibits a small base plate and a fiber with 2 swellings. Isometric-headed temperate phage **0252** (group **d**) possesses a fairly long tail with several fibers. Bars = 100 nm; the magnification factor is the same for all the pictures (phages **c31**, **0235** and **0252**: micrographs from INRA; phage **c5**: micrographs from INRA/ETH-Zürich).

Morphologie des phages des groupes b, c et d. Les phages du groupe b, tels que c31 et c5, ont un spectre d'hôtes limité à quelques souches de L. bulgaricus, tandis que les phages 0235 (groupe c) et 0252 (groupe d) ont des spectres d'hôtes similaires à ceux des phages du groupe a. Les phages c31 et c5 (groupe b) possèdent une queue non contractile pourvue d'une plaque basale dentée et d'au moins une fibre. L'extrémité distale de la queue du phage tempéré à tête allongée 0235 (groupe c) porte une petite plaque basale et une fibre avec deux renflements.

a determining factor in the occurrence and spread of these phages. Each of the groups **c** and **d** contains a single representative temperate phage of *L. lactis*. These 2 phages have lytic spectra similar to those of group **a** phages and consequently can infect some *L. lactis* as well as *L. bulgaricus* strains. However, no morphological (fig 3), molecular or serological similarities between them or with group **a** phages have been observed. Their low incidence may limit their importance in technology.

Group **b** is composed of 4 closely related virulent phages. They seem less widespread than group **a** phages and attack only *L. bulgaricus* strains. They have different lytic spectra and do not share molecular and serological relationships with phages of the 3 other groups. Some *L. bulgaricus* strains sensitive to group **b** phages are lysogenic and harbour prophages that belong to group **a**. Prophage-free (cured) bacterial clones become sensitive to the corresponding temperate phage and to all other phages of groups **a**, **c** and **d** but are unexpectedly resistant to group **b** phages. Inversion of phage sensitivity occurs along with changes in morphology of bacterial colonies (rough form for cured clones/smooth form the parental lysogenic strains; Cluzel *et al*, 1987). This suggests that these strains could possess specific receptors for phages of all four groups. In the presence of prophage, only receptors for group **b** phages are functional while those for groups **a**, **c** and **d** phages are masked or non-functional. It could be a lysogenic conversion.

Le phage à tête isométrique 0252 (groupe d) possède une queue assez longue, munie de plusieurs fibres terminales. Barres = 100 nm; l'agrandissement est le même pour toutes les images (phages c31, 0235 et 0252 : micrographies de l'INRA; phage c5 : micrographies de l'INRA / ETH-Zürich).

The large number of group a phages underlines the risk resulting from the presence of lysogenic bacteria in lactic starters. Therefore, the use of cured strains appears to be a satisfactory solution, but requires strictly aseptic conditions during starter preparation and fermentation in order to prevent attacks by the widespread group a phages.

Among phages of *L. bulgaricus* and *L. lactis*, only phage LL-H, isolated in Finland and active against *L. lactis* strain LL 23, has been extensively studied (see Alatossava, 1987). Its morphology is representative of group a, and is closely related to that of phage lv illustrated in figure 2. The average burst size reaches 10^2 phages per infected cell under optimal conditions. Phage LL-H contains 2 major structural proteins (34 kDa for the head and 19 kDa for the tail) and no less than 5 minor proteins. The DNA molecules are linear (34 kbp) with constant ends. A restriction map of LL-H DNA has been established and a library of restriction fragments has been prepared by molecular cloning into *E. coli*. This had led to the localization of 5 genes encoding structural proteins and phage lysin. Phage LL-H DNA contains 2 polycistronic clusters of genes *ie* "early" genes which are involved in phage replication and "late" genes which encode structural proteins and phage lysin. Bivalent cations (Ca^{2+} or Mg^{2+}) stabilize the coiled DNA into the capsid, improve adsorption rate and control penetration efficiency of phage DNA into the bacterial cells. A molecular study of the LysA-encoding gene of *L. bulgaricus* temperate phage mv1 classified in group a (Boizet *et al.*, 1990), revealed a close relationship between all lysins of group a phages (including LL-H and mv1 enzymes). The *lysA* gene has a nucleotide sequence of 585 bp corresponding to a 21,120 Da protein. Significant homology with the N-terminal domain of known muramidases has been observed.

Recent studies in Germany and France have demonstrated the existence of an apparent homogeneity of the specific phages of *S. thermophilus* at both the morphological and molecular levels (Krush *et al.*, 1987; Neve *et al.*, 1989; Prévots *et al.*, 1989; Benbadis *et al.*, 1990; Larbi *et al.*, 1990). These phages have been isolated as lytic and belong to the *Siphoviridae* family of Matthews (1982) or to Bradley's group B1 (Bradley, 1967), as shown in figure 4. They contain linear double-stranded DNA (34–44 kb) with complementary cohesive ends. Grouping of these phages has been attempted on the basis of protein patterns and DNA restriction profiles. It is likely that most *S. thermophilus* phages studied until now were derived from a common ancestor. As indicated by Mercenier (1990), their genomes have non-homologous sequences flanked by homologous regions. It is consistent to apply the hypothesis of Botstein (1980) who postulated that the observed diversity of phages might result from recombination and exchange of "modules" *ie* genome fragments encoding for particular biological functions.

The existence of lysogenic strains in *S. thermophilus* has been reported several times since 1970 (Ciblis, 1970; Kurmann, 1979, 1983; Smaczny and Krämer, 1984b), but these studies remained incomplete. Neve *et al.* (1990) detected prophages in 2 strains by probing chromosomal DNA of 17 strains with genomic DNA of virulent phages. Subsequently, the same authors demonstrated that these 2 strains were inducible by mitomycin C ($0.2\text{--}1\ \mu\text{g}\cdot\text{ml}^{-1}$) and released mostly defective phage particles. However, these phages were able to relysogenize cured clones and formed turbid plaques on agar medium. Presence of prophage led to a modification of some phenotypic traits of the strains, *ie* homogeneous non-clumping cultures in broth instead of clumping in cured

strains, and autolysis at 45 °C probably due to the thermoinducibility of prophage at elevated temperature.

A survey of *S thermophilus* phages is currently being carried out at the INRA laboratory at Jouy-en-Josas (B Fayard and M Haefliger, unpublished data). Ten of the 120 strains examined were shown to be lysogenic. Induction by mitomycin C gave temperate phages active on one or several indicator strains for 7 of them. Temperate phage DNA exhibited 5 different restriction patterns but shared homology. DNA homology also existed between temperate phages and 52 phages isolated as lytic following acidification failures. Preliminary results suggest that prophages might represent a phage source for *S thermophilus* and the impact of lysogeny in current phage outbreaks should not be underestimated.

Starter suppliers and yogurt producers still lack basic information about the origin of disturbing phages and phage-bacteria relationships. This explains why they often adopt a pragmatic approach to the phage problem. Thus, an infected strain is replaced by an insensitive strain, sometimes by a spontaneously resistant mutant, with similar technological properties. This is the case for Australian yogurt manufacture (Hull, 1983). In day-to-day practice, phage control requires experience and "know-how", but progress in the study of yogurt bacteria phages will certainly help to prevent phage attacks. For instance, improvement of phage resistance by genetic engineering might be the solution to phage problems in the future (Sanders, 1989).

Genetics of *S thermophilus* and *L bulgaricus*

Only few studies have been performed on the genetics of *S thermophilus*, reviewed

by Mercenier and Lemoine (1989) and Mercenier (1990). This is mainly due to important methodological problems, but also to the fact that the major functions are carried by chromosomal genes making studies more difficult. For this reason, many efforts have been made to adapt DNA transfer methods to this species. The organization and expression of genes involved in lactose and galactose metabolism were then studied by Herman and McKay (1986), Poolman *et al* (1989, 1990), and Lee *et al* (1990).

The plasmids of lactic acid bacteria encode important characters for dairy technology, *eg* carbohydrate metabolism, proteolytic activity, citrate utilization, production of antimicrobial compounds, bacteriophage and antibiotic resistance, polysaccharide production (McKay, 1983; Vedamuthu and Neville, 1986). *S thermophilus* and *L bulgaricus* seem to be naturally poor in plasmids (table I).

All plasmids mentioned in table I are considered as being cryptic. Most of the replicons isolated from *S thermophilus* would be too small to carry a phenotypic trait (Mercenier, 1990). Until the present, the largest extrachromosomal element described is the 22.5-kb plasmid harboured by strain IP6631, along with 2 other small replicons (Gavoille, 1989).

Only one endogenous plasmid of *S thermophilus* has been fully sequenced, *ie* plasmid pA33 (6.9 kb) from strain AO33 (Mercenier, 1990). Preliminary results indicate that this plasmid integrates and re-excises from chromosomal DNA. Its presence or absence as an autonomously replicating extrachromosomal element provides the host-cells with phenotypes which differ by their colony morphology, cell chain length, growth and acidification rates in milk, antibiotic resistance and phage sensitivity. The nucleotide sequence of this plasmid showed limited similarity with coding

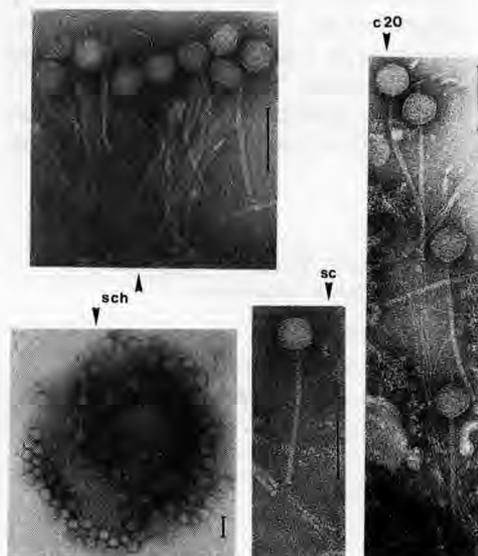


Fig 4. Morphology of *S thermophilus* bacteriophages. All phages possess an isometric head and a fairly long and flexible tail, generally fitted with a small base plate. Tails of phage c20 exhibit a long fiber, whereas those of phages sch and sc are fitted with a shorter one. The cluster of phage sch particles adsorbed on a streptococcal cell debris is reminiscent of lysis from without. Bars = 100 nm; the magnification factor is the same for all the pictures except phage cluster sch (micrographs from INRA/ETH-Zürich).

Morphologie de bactériophages spécifiques de S thermophilus. Tous les phages possèdent une tête isométrique et une queue longue et flexible, généralement pourvue d'une petite plaque basale. La queue du phage c20 se prolonge par une longue fibre, tandis que celles des phages sch et sc sont munies d'une fibre plus courte. Le groupe de particules du phage sch adsorbées sur un débris cellulaire du streptocoque constitue une image évocatrice de la lyse externe. Barres = 100 nm; l'agrandissement est le même pour toutes les images, à l'exception du groupe de particules du phage sch (micrographies de l'INRA/ETH-Zürich).

sequences of some proteins of the outer membrane or of proteins involved in antibiotic resistance. This plasmid seems to be

responsible for cell wall changes, but its role remains to be demonstrated. These observations should be considered in relation to specific chromosomal DNA rearrangements in *S thermophilus*, which lead to morphologically different colonies (Pébay *et al*, 1989).

These general considerations also apply to *L bulgaricus*. The rare plasmids are cryptic and genetic studies of different metabolic functions have just started. Morelli *et al* (1983) tried to correlate antibiotic resistance with the presence of plasmids in three lactobacillus strains. After treatment with curing agents (ethidium bromide, acriflavin) these strains lost several plasmids and their resistance to various antibiotics. However, the 2 observations cannot be correlated since the loss of plasmids and of antibiotic resistance was obtained by powerful mutagens which may also act on the chromosome.

CONCLUSION

Since 1980 many studies on *S thermophilus* and *L bulgaricus* have led to a better understanding of some metabolic and biochemical aspects of these bacteria that control their growth, especially in mixed cultures. For instance, lactose and galactose metabolism have been well studied at both the biochemical and molecular levels. In addition, some enzymes involved in the production of aroma compounds have been investigated. Production of exocellular polysaccharides by yogurt bacteria is now well established and some of these polymers have been studied. Several enzymes such as aminopeptidases, proteases, esterases and lipases have been characterized in both bacteria, especially proteases hydrolyzing caseins as well as urease activity of *S thermophilus* because of their technological and taxonomical in-

terest. The taxonomy of *S thermophilus* is still debated and its definitive classification as an *S salivarius* subspecies requires further consideration. Many bacteriophages of yogurt bacteria have been well characterized and their interactions with host-

strains better established. In particular, lysogenic strains seem to be a major source of phages for *L bulgaricus* and probably for *S thermophilus*.

Many questions still remain unanswered. The molecular aspects of the me-

Table I. Distribution and characterization of plasmids from *S thermophilus* and *L bulgaricus*.
Distribution et caractérisation de plasmides de S thermophilus et de L bulgaricus.

Reference	No of plasmids per strain	Plasmid size (kb)	Plasmid characterization
<i>S thermophilus</i>			
Pechmann <i>et al</i> (1982)	+ (1/8) ¹	-	-
Herman and McKay (1985)	1 (5/23)	2.1 to 3.3	2 plasmids in duplicate (restriction profiles) Homology of all plasmids (DNA-DNA hybridization)
Somkuti and Steinberg (1986a)	1 (10/35) 2 (3/35)	2.20 to 14.75	9 distinct types (restriction profiles, obtained using 19 endonucleases)
Girard <i>et al</i> (1987)	1 (6/50) ² 2 (3/50) ³	2.9 ± 0.1 to 7.6 ± 0.2	8 types (size, restriction profiles) - 3 groups (DNA-DNA hybridization)
<i>L bulgaricus</i>			
Vescovo <i>et al</i> (1981)	+ (1/19)	-	-
Klaenhammer <i>et al</i> (1979)	+ (1/1)	52.5, 40.5, 13.5 and 6	-
Somkuti and Steinberg (1986b)	4 (1/1)	-	-
Bosi <i>et al</i> (1990)	1 (1/3) 2 (2/3)	8.2 to 16.1	-

+ : No of plasmids not given; - no data available. ¹ In parentheses, number of plasmid-containing strains on tested strains. ² Four strains isolated from raw milk and 2 industrial starter strains. ³ Two strains isolated from raw milk and strain CNRZ 312.

* : Le nombre de plasmides n'est pas donné. - Pas de données disponibles. ¹ Entre parenthèses, nombre de souches contenant des plasmides sur le nombre de souches testées. ² Quatre souches isolées du lait cru et deux souches provenant de levains industriels. ³ Deux souches isolées du lait cru et la souche CNRZ 312.

tabolism of sucrose and other carbohydrates should be better studied. This is particularly true for *L. bulgaricus*, since metabolic studies are less advanced than in *S. thermophilus*. Volatile compounds produced by *S. thermophilus* and *L. bulgaricus* remain to be identified because of their importance for the organoleptic properties of yogurt. The study of the different biosynthesis pathways of acetaldehyde and other volatile compounds, especially at the molecular level, is also necessary. Further research is needed in the field of exopolysaccharide production. In particular the genetics of the metabolic pathways (enzymes involved, and their regulation) require investigation in order to control and stabilize polysaccharide production in milk. The nutritional and possible therapeutic roles of these polymers remain to be confirmed.

In mixed cultures, the growth of *L. bulgaricus* and *S. thermophilus* is not yet fully controllable. Even if the interactions of these bacteria are well established, many aspects are still unknown. In particular, the real influence of associated growth on secondary metabolic activities has not been studied sufficiently. In addition, the characterization of inhibitory substances which may be produced would be very useful (a) for a better understanding of the relationship between the yogurt bacteria, and (b) for their action on other micro-organisms associated with yogurt bacteria for the manufacture of several fermented milk products.

Finally, progress in genetic studies should contribute to better knowledge on the yogurt bacteria and their growth and activity in milk.

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