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The growth and sugar utilization by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* isolated from market yogurt

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Summary — *Lactobacillus delbrueckii* ssp. *bulgaricus* Yop 12 and *Streptococcus salivarius* ssp. *thermophilus* Yop 9 were isolated from a commercial yogurt of Argentina. Significant differences in growth response to glucose, galactose, fructose, lactose and sucrose were found among these strains in pure and mixed cultures. The development and sugars utilization by these strains are completely different from those found in *Lactobacillus delbrueckii* ssp. *bulgaricus* Ss_1 and *Streptococcus salivarius* ssp. *thermophilus* Ss_2 isolated from another market yogurt of Argentina. In mixed cultures only with glucose as carbon source there is an increase of growth. *Lactobacillus delbrueckii* ssp. *bulgaricus* Yop 12 is unable to ferment galactose and sucrose in pure culture. When combined with *Streptococcus salivarius* ssp. *thermophilus* Yop 9, *Lactobacillus delbrueckii* ssp. *bulgaricus* Yop 12 acquires capacity to grow with galactose. In milk cultures there is a stimulation of growth of both microorganisms. In basal medium with lactose we observed a decrease of growth in *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*. In the presence of fructose or sucrose there is an inhibition of growth of both microorganisms in mixed cultures. Sugars concentrations were measured using high pressure liquid chromatography.

***Lactobacillus delbrueckii* ssp. *bulgaricus* — *Streptococcus salivarius* ssp. *thermophilus* — yogurt — growth and sugar utilization**

Résumé — Croissance et utilisation des sucres par des souches de *Lactobacillus delbrueckii* ssp. *bulgaricus* et *Streptococcus salivarius* ssp. *thermophilus* isolées de yaourt du commerce. Lactobacillus delbrueckii *ssp.* bulgaricus *Yop 12 et* Streptococcus salivarius *ssp.* thermophilus *Yop 9 ont été isolés à partir de yaourt commercial produit en Argentine. Les taux de croissance des souches étaient significativement différents selon que les souches étaient ensemencées séparément ou en mélange dans les cultures contenant les sucres suivants : glucose, galactose, fructose, lactose ou saccharose. Les souches* Lactobacillus delbrueckii *ssp.* bulgaricus *Ss1 et* Streptoccus salivarius *ssp.* thermophilus Ss_2 *isolées d'une autre source de yaourt commercial ont un comportement différent pour l'utilisation des sucres et le taux de croissance. Dans les cultures mixtes avec glucose uniquement il y a une augmentation du taux de croissance.* Lactobacillus delbrueckii *ssp.* bulgaricus *Yop 12 n'est pas capable de fermenter le saccharose quand il est cultivé tout seul. Quand il est mélangé avec* Streptoccus salivarius *ssp.* thermophilius *Yop 9,* Lactobacillus

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delbrueckii *ssp.* bulgaricus *Yop 12 a la capacité de croître dans un milieu contenant du galactose. Dans les cultures sur lait il y a une stimulation pour les deux souches. Sur un milieu de base avec lactose nous avons trouvé une diminution du taux de croissance pour* Lactobacillus delbrueckii *ssp.* bulgaricus *et* Streptococcus salivarius *ssp.* thermophilus*; quand le fructose ou le saccharose sont présents il y a une inhibition du taux de croissance des deux microorganismes dans les cultures mixtes. Les concentrations des sucres ont été mesurées par HLPC.*

Lactobacillus delbrueckii *ssp.* bulgaricus — Streptococcus salivarius *ssp.* thermophilus — *yaourt — croissance — utilisation des sucres*

INTRODUCTION

Yogurt is a fermented milk product made with *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and it has become a highly popular food over the last 15 years. These two organisms have a symbiotic relationship during the manufacture of yogurt (Bautista *et al.*, 1966; Galesloot *et al.*, 1968; Veringa *et al.*, 1968; Radke-Mitchell & Sandine, 1984). In the present text these organisms will be referred to as *S. thermophilus* and *L. bulgaricus*; however, the ratio of the yogurt cultures may range between 1:1—3:1 (Schulz & Hingst, 1954).

Yogurt is an excellent source of protein and the suggested role of the *Lactobacillus* species is to degrade casein, providing the non-proteolytic *S. thermophilus* with a source of peptides and amino-acids (Bautista *et al.*, 1966; Accolas *et al.*, 1971). This is important because the free amino-acids found in milk are in very low concentrations (Thomas & Mills, 1981; Marshall & Law, 1984). Production of formic acid (Galesloot *et al.*, 1968) or carbon dioxide (Driessen *et al.*, 1982) by streptococci has been reported to stimulate *L. bulgaricus*.

The sugar fermentation pattern in milk products containing *S. thermophilus* and *L. bulgaricus* led Turner *et al.* (1983) to suggest another important role of the lactobacilli. They observed that the major role of the thermophilic lactobacilli in Swiss-type cheese is to metabolize the galactose which the *S. thermophilus* culture cannot utilize. Some strains of *S. thermophilus* are unable to ferment galactose (gal^-) and utilize only the glucose portion of lactose, releasing free galactose into the extracellular medium (O'Leary & Woychik, 1976; Tinson *et al.*, 1982; Thomas & Crow, 1984; Hutkins *et al.*, 1985a)

Some yogurt manufacturers believe consumers of plain yogurt prefer a slightly sweetened product and the sweeteners used in the manufacture of the Swiss-style yogurt were sucrose and fructose (Mc Gregor & White, 1986, 1987). The aim of this work was to determine the characteristics of *S. thermophilus* and *L. bulgaricus* (pure and mixed cultures) isolates from commercial yogurt of Argentina to grow and to utilize sugar in order to carry out a comparative study with the results obtained by Amoroso *et al.* (1988) with *S. thermophilus* Ss_2 and *L. bulgaricus* Ss_1 isolated from another commercial source.

MATERIALS AND METHODS

Microorganisms

L. bulgaricus Yop 12 and *S. thermophilus* Yop 9 were isolated from a commercial yogurt in Argentina by Raya *et al.* (1986). These strains were preserved in LAPTg agar medium at 4 °C and lyophilized. For use, the strains were activated by 2 daily transfers in LAPTg medium for 3 days, starting the experiments with a 12-h-old culture.

Culture medium and growth conditions

The basal medium for the growth of these microorganisms was that described by Raibaud *et al.* (1961). The pH was adjusted to 6.5 with 0.2 N NaOH. Glucose, fructose, galactose, sucrose and lactose were added to the medium at the concentrations indicated in each experiment. The basal medium was sterilized by autoclaving for 20 min at 118 °C. The sugars were sterilized by filtration. Lactic acid production was measured by growing the cultures in sterile reconstituted skim-milk powder free from antibiotics (a solution was used of 10% total solids autoclaved at 121 °C for 10 min). In mixed cultures the inoculation rate was 1:1 (*L. bulgaricus* : *S. thermophilus*).

Enumeration of microorganisms

S. thermophilus and *L. bulgaricus* were counted by surface spreading 0.1 ml samples on the LAPTg agar medium with the following composition : 1.0% yeast extract, 1.5% peptone, 1.0% tryptone, 1.0% glucose, 0.1% Tween 80 and 2.0 agar, and by direct microscopic count in the Newbauer chamber.

Developed acidity

Sterile skim milk samples (5 ml) withdrawn from the culture flask at regular intervals were titrated with 0.1 N NaOH using phenolphthalein as colour indicator. The percentage of developed acidity was calculated as lactic acid; pH measurements were carried out using a pH meter pH MD.

Quantification of glucose, fructose and sucrose by high performance liquid chromatography (HPLC)

The cultures were centrifuged at 3 000 rpm for 20 min; 0.5 ml of each supernatant was diluted (4-fold) with acetonitrile-H_2O (85:15). After 10 min at 4 °C it was centrifuged at 3 000 rpm for 10 min. The supernatant was filtered through 0.45 µm filter paper and the concentration measured using high performance liquid chromatography (HPLC) on a Water's Associates (Milford (MA) USA) chromatograph. A model R 401 refractive index detector and a carbohydrate analysis column were used. The oven was heated to room temperature. The mobile phase was acetonitrile-H_2O (85:15). A flow rate of 1.5 ml/min was used; 1% glucose, fructose or sucrose were used as internal standard. Glucose plus galactose were not resolved in our experimental conditions.

Glucose plus galactose were estimated by the method of Nelson & Somogyi (1966) and glucose concentration was determined by the glucose oxidase method (Glucostat, Worthington Diagnostic, Worthington Biochemical Corporation, Free Hold, NJ 07728, USA).

RESULTS AND DISCUSSION

The optimum growth temperature for *L. bulgaricus* Yop 12 was 45 °C and for *S. thermophilus* Yop 9 40—45 °C. The optimum temperature for mixed culture was 45 °C. These results are similar to the data reported by Amoroso *et al.* (1988) for these strains isolated from another commercial yogurt. The growth of our cultures at a different initial pH in LAPTg medium shows that the optimum pH for pure and mixed cultures occurred at 6.8.

Figure 1 (a and b) shows the relationship between growth, developed acidity and pH in *S. thermophilus*, *L. bulgaricus* and mixed culture with 0.5% of each strain in sterile skim milk medium. Both microorganisms grow similarly in pure cultures (Fig. 1 a) and the acid production by *L. bulgaricus* is higher than that of *S. thermophilus* (Fig. 1 b).

The pH changes from 6.6 to 4.4 in 8 h of growth and at the same time the acid production increases up to 0.6% (Fig. 1 b).

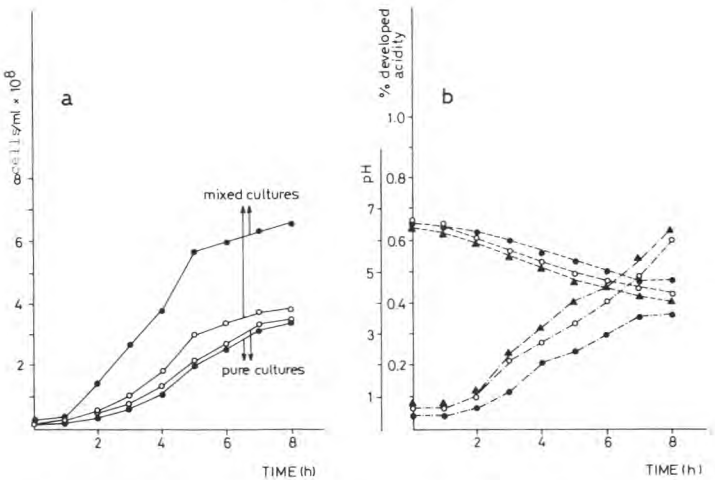

Fig. 1. Growth (a), developed acidity (–•–•–) and pH (– – –) (b) of pure and mixed cultures of *L. bulgaricus* and *S. thermophilus* in milk at 37 °C. *S. thermophilus* (●); *L. bulgaricus* (○); mixed culture (▲). *Croissance (a), acidité développée (–•–•–) et pH (– – –) (b) de cultures pure et mixte de* L. bulgaricus *et* S. thermophilus *dans du lait à 37 °C.*

In mixed culture stimulation of growth in both microorganisms is observed and the increase of *S. thermophilus* is higher than that of *L. bulgarius* (Fig. 1 a). Moon & Reinbold (1976) have shown that stimulation of acid production in mixed culture is due to enhanced growth of streptococci. Accolas *et al.* (1977) reported that the stimulant effect of a strain upon the other can be observed by acid production. These results are different from those reported by Amoroso *et al.* (1988) with strains isolated from another commercial yogurt.

Growth and sugar utilization

In LAPT-glu medium (1% glucose) *L. bulgaricus* and *S. thermophilus* grow similarly (Fig. 2 a). In mixed culture both microorganisms increased their growth : *S. thermophilus* from 2.3 x 10^8 cells/ml to 3.3 x 10^8 cells/ml, and *L. bulgaricus* from 2.28 x 10^8 cells/ml to 3.6 x 10^8 cells/ml in 5 h of incubation at 37 °C. There is a mutualistic effect. With the strains isolated from another commercial yogurt the results reported by Amoroso *et al.* (1988) showed that *L. bulgaricus* growth was more active than *S. thermophilus* in pure culture in this time. In LAPT-gal medium (1% galactose) *L.bulgaricus* does not grow and *S. thermophilus* grows poorly (Fig. 2 b). In mixed cultures, *L. bulgaricus* acquires capacity to grow in LAPT-gal and *S. thermophilus* grows more actively. The unability of many commercial strains of *S. thermophilus* and *L. bulgaricus* to utilize galactose has practical implications in a number of fermented dairy products. Thomas & Crow (1984) and Hutkins *et al.* (1985a) have shown that enzymes of the Leloir pathway are either not inducible or permanently repressed in gal$^-$ strains of *S. thermophilus.* The galactose transport occurred *via* a gal P, which was also present in gal$^-$ strains, but this activity was very low and was de-

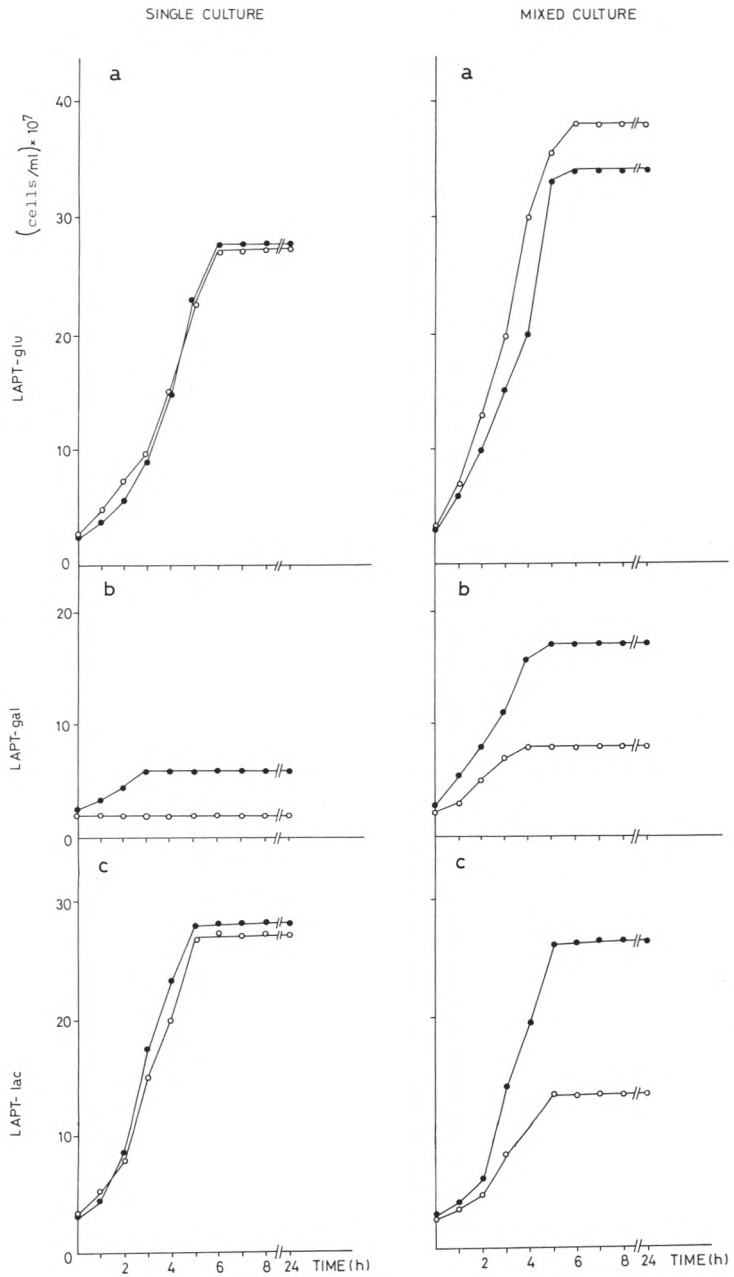

Fig. 2. Growth of *S. thermophilus* (●) and *L. bulgaricus* (○) in pure and mixed cultures in LAPT-glu (a), LAPT-gal (b) and LAPT-lac (c) at 37 °C.
Croissance de S. thermophilus (●) et L. bulgaricus (○) en cultures pure et mixte en milieu LAPT-glu (a), LAPT-gal (b) et LAPT-lac (c) à 37 °C.

pendent on an exogenous energy source. Hutkins *et al.* (1985b) suggest the possibility that some sugars may be bound but not transported by the sugar transport systems in *S. thermophilus*. It is possible that some metabolic products of these *L. bulgaricus* and *S. thermophilus* strains in mixed culture permit to the strains to utilize galactose. In the study with the strains isolated from another commercial source (Amoroso *et al.*, 1988) in mixed culture *L. bulgaricus* grows more actively. In LAPT-lac (1% lactose) single strains of *L. bulgaricus* and *S. thermophilus* have a similar growth pattern and in mixed culture there is an inhibition of growth which is higher in *L. bulgaricus* than in *S. thermophilus* (Fig. 2 c). Lactose is hydrolysed inside the bacterial cell by the β-galactosidase enzyme to glucose and galactose. Both microorganisms utilize glucose but galactose is poorly consumed by these strains in mixed culture. It appears that galactose accumulated in the culture medium may produce an inhibition of β-galactosidase enzyme with a consequent diminution of growth. Bacteria display a remarkable capacity to regulate synthesis of enzyme systems with varying growth conditions. These results are different from those found for the strains isolated from another commercial yogurt (Amoroso *et al.*, 1988) (in mixed culture there is a growth stimulation which is higher in *S. thermophilus* than in *L. bulgaricus*).

The results obtained in milk medium show an increase of growth in both microorganisms in mixed culture (Fig. 1 a). It is known that the lactose utilization for growth of *L. bulgaricus* and *S. thermophilus* in mixed culture depends on the characteristics of the basal medium and we are investigating the effect of different components of LAPT-lac medium on the growth of these microorganisms in milk medium. O'Leary & Woychik (1976) reported one strain of *S. thermophilus* which can simultaneously utilize lactose and glucose in lactose-hydrolyzed milk but only used lactose in broth containing glucose and lactose.

In LAPT-fru medium (1% fructose) *L. bulgaricus* grows more actively than *S. thermophilus*. In mixed culture both microorganisms decreased growth (Fig. 3 a), for example the *L. bulgaricus* viable count decreased from 28 x 10^7 cells/ml to 22 x 10^7 cells/ml and *S. thermophilus* from 20 x 10^7 cells/ml to 13 x 10^7 cells/ml after 5 h of incubation at 37 °C. In LAPT-suc medium (1% sucrose) only *S. thermophilus* grows in pure and mixed cultures and no growth was observed for *L. bulgaricus* (Fig. 3 b).

In LAPT-glu + gal (0.5% glucose and 0.5% galactose) both microorganisms grow similarly but in mixed culture there is an inhibition of growth of the two strains (Fig. 4 a). It is a typical case of inhibition by substrate competition. In the strains isolated by Amoroso *et al.* (1988) the response of growth in LAPT-glu + gal medium is different.

The utilizations of glucose, galactose and lactose by *L. bulgaricus* and *S. thermophilus* in pure and mixed cultures are represented in Table I. Galactose concentration is not modified in the medium with *L. bulgaricus* but in mixed culture this sugar concentration diminished more than in pure culture of *S. thermophilus*. These results are the same as those found for the strains isolated for the other commercial yogurts (Amoroso *et al.*, 1988). Lactose concentration in mixed culture decreased by 2.3 g/l in 8 h of incubation. In pure cultures of *L. bulgaricus* and *S. thermophilus* over the same period the concentration of this sugar decreased by 0.25 g/l and 0.30 g/l, respectively. The response of growth of *L. bulgaricus* and *S. thermophilus* in LAPT-lac shows that in mixed culture there is an example of amensalism in which the growth of one species is inhibited by the presence of the other.

Table II shows the results of glucose + galactose utilization in a culture medium (0.5% glu + 0.5% gal). The consumption of glucose is more rapid in *L. bulgaricus* than in *S. thermophilus* and in mixed culture the residual glucose and galactose is higher than in pure culture.

In LAPT-fru + glu medium (0.5% glucose + 0.5% fructose) both microorganisms grow more actively than in LAPT-fru medium, and in mixed culture we can observe growth inhibition of both microorganisms (Fig. 4b). It can be observed that fructose utilization is not inhibited by glucose and that in mixed culture there is again a growth inhibition by substrate competition.

The utilization of fructose, glucose + fructose and sucrose by *L. bulgaricus* and *S. thermophilus* is represented in Table III. Sucrose concentration is not modified in the medium with *L. bulgaricus* and in mixed culture the sugar concentration is 1.9 g/l after 8 h of incubation at 37 °C.

From these results it can be seen that there are differences in the behavior of strains isolated from two commercial sources of yogurt, as a result of different intracellular control strategies. The mixed cultures of *S. thermophilus* and *L. bulgaricus* isolated by Amoroso *et al.* (1988) showed growth stimulation in respect to pure cultures in medium with glu-

Table I. Utilization of glucose, galactose and lactose* by *L. bulgaricus* and *Str. thermophilus* in pure and mixed cultures.
Utilisation de glucose, galactose et lactose par* L. bulgaricus *et* Str. thermophilus *dans cultures pures et mixtes.*

Sugar Utilization	*Time (h)*	*Cultures* L. bulgaricus	Str. thermophilus	*Mixed Culture*
Glucose	0	10.0	10.0	10.0
	2	5.0	6.0	4.0
	4	2.0	3.4	1.7
	6	0.85	1.4	0.8
	8	0.67	0.9	0.17
Galactose	0		10.0	10.0
	2		8.6	6.6
	4		7.0	4.7
	6		6.7	4.1
	8		6.5	3.3
Lactose	0	10.0	10.0	10.0
	2	2.0	3.0	6.9
	4	1.2	1.8	3.5
	6	0.31	0.5	2.9
	8	0.25	0.3	2.3

* Sugar concentrations determined by HPLC are expressed as g/l.
Values are means of three samples.

Table II. Utilization of glucose + galactose* by *L. bulgaricus* and *Str. thermophilus* in pure and mixed cultures.
Utilisation de glucose plus galactose par* L. bulgaricus *et* Str. thermophilus *dans cultures pures et mixtes.*

Sugar Utilization	*Time (h)*	*Cultures* L. bulgaricus	Str. thermophilus	*Mixed Culture*
Total sugars	0	1.0	1.0	1.0
	2	0.79	0.75	0.81
	4	0.69	0.62	0.76
	6	0.53	0.55	0.64
	8	0.50	0.52	0.60
Residual glucose	0	0.50	0.49	0.48
	2	0.22	0.25	0.34
	4	0.18	0.17	0.24
	6	0.04	0.11	0.11
	8	0.01	0.10	0.03
Residual galactose	0	0.50	0.50	0.50
	2	0.50	0.49	0.50
	4	0.50	0.45	0.49
	6	0.50	0.43	0.46
	8	0.50	0.41	0.45

* Sugar concentrations are expressed as g%. Total sugars determined by Nelson-Somogyi method. Residual glucose determined by the glucose oxidase method.
Values are means of three samples.

Table III. Utilization of fructose, glucose + fructose and sucrose by *L. bulgaricus* and *Str. thermophilus* in pure and mixed cultures.
Utilisation de fructose, glucose plus fructose et saccharose par L. bulgaricus *et* Str. thermophilus *dans cultures pures et mixtes.*

Sugar Utilization	*Time (h)*	*Cultures* L. bulgaricus	Str. thermophilus	*Mixed Culture*
Fructose	0	10.0	10.0	10.0
	2	6.9	8.6	8.7
	4	5.2	7.4	7.9
	6	2.7	5.6	6.1
	8	2.0	4.6	5.2
		glu – fru	glu – fru	glu – fru
Glucose	0	5.0 5.0	5.0 5.0	5.0 5.0
+ fructose	2	2.1 2.4	2.8 2.6	4.6 4.1
	4	1.5 1.7	1.6 1.5	1.9 1.8
	6	1.0 1.2	1.5 1.3	1.6 1.7
	8	0.7 0.9	1.3 1.0	1.4 1.6
			glu – fru – suc	glu – fru – suc
Sucrose	0		0.0 0.0 10.0	0.0 0.0 10.0
	2		1.5 0.9 4.9	2.1 0.2 5.2
	4		0.46 0.08 1.6	0.97 0.17 2.5
	6		0.28 0.07 1.3	0.87 0.12 2.0
	8		0.24 0.05 1.1	0.59 0.10 1.9

* Sugar concentrations determined by HPLC are expressed as g/l.
Values are means of three samples.

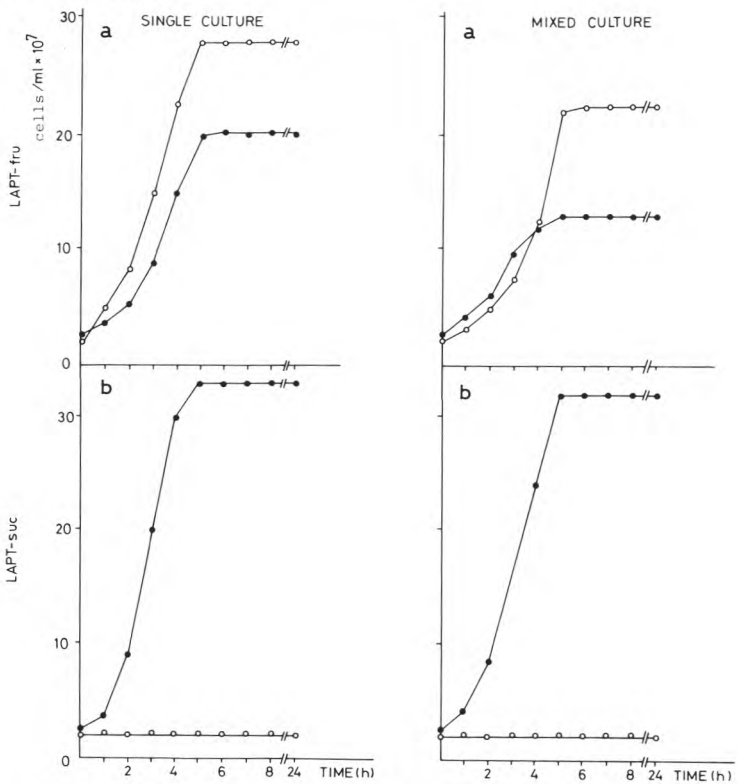

Fig. 3. Growth of *S. thermophilus* (●) and *L. bulgaricus* (○) in pure and mixed cultures in LAPT-fru (a) and LAPT-suc (b) at 37 °C.
Croissance de S. thermophilus *(●) et* L. bulgaricus *(○) en cultures pure et mixte en milieu LAPT-fru (a) et LAPT-suc (b) à 37 °C.*

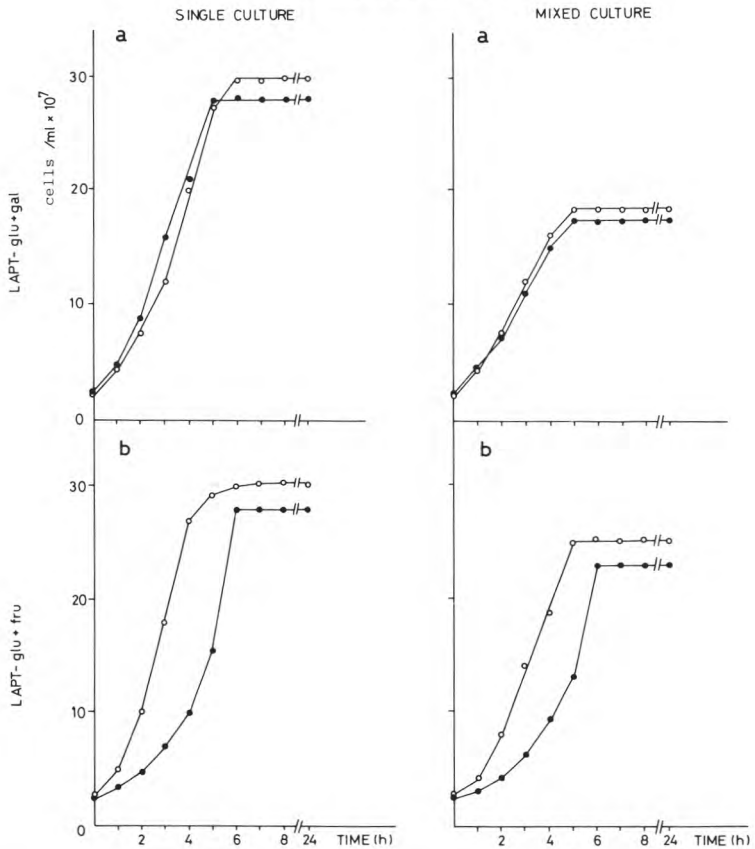

Fig. 4. Growth of *S. thermophilus* (●) and *L. bulgaricus* (○) in pure and mixed cultures in LAPT-glu+gal (a) and LAPT-glu+fru (b) at 37 °C.
Croissance de S. thermophilus *(●) et* L. bulgaricus *(○) en cultures pure et mixte en milieu LAPT-glu+gal (a) et LAPT-glu+fru (b) à 37 °C.*

cose, galactose, lactose, fructose and sucrose. With the strains isolated from another source we observed that the mixed cultures of *S. thermophilus* and *L. bulgaricus* showed a different growth response than pure cultures, according to the sugar present in the medium. Further studies are required with combined mixed cultures of the strains isolated from the two different yogurt sources.

It is important to be aware of the sugar utilization behavior of mixed cultures when choosing starter bacteria for dairy fermentation. The identified interactions could form the basis of later studies on metabolic and genetic control of carbohydrate utilization in these strains isolated from commercial yogurt in Argentina.

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