PAPER

SELECTION OF FOLATE-PRODUCING LACTIC ACID BACTERIA FOR IMPROVING FERMENTED GOAT MILK

SELEZIONE DI BATTERI LATTICI PRODUTTORI DI FOLATI PER IL MIGLIORAMENTO DEI LATTI FERMENTATI CAPRINI

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ABSTRACT

Goat milk is a complete food but its low level of folic acid diminishes its nutritional efficacy. In this study, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains were selected for folate production in goat milk to improve its quality. A suitable HPLC method was developed to directly determine both total folate and its biologically active derivatives such as 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), tetrahy-

RIASSUNTO

Il latte di capra è un alimento completo, tuttavia il suo valore nutrizionale è sminuito dalla mancanza di acido folico. Al fine di migliorare dal punto di vista alimentare la qualità delle produzioni caprine sono stati selezionati ceppi di *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* e *Lb. helveticus* capaci di produrre folati in latte di capra. È stata sviluppata una metodica per la determinazione dei folati in latte di capra mediante HPLC. L'anali-

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drofolate (H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu). Results showed that *S. thermophilus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains produced higher amounts of total folate than *Lb. delbrueckii* subsp. *bulgaricus*, especially 5-CH_3-H_4-PteGlu and H_4-PteGlu. Moreover the use of selected *S. thermophilus* strains in association with *Lb. delbrueckii* subsp. *bulgaricus* resulted in yoghurt with a significant quantity of folate and good organoleptic features.

si ha riguardato sia i folati totali che le forme biologicamente attive quali il 5-metil-tetraidrofolato (5-CH_3-H_4-PteGlu), il tetraidrofolato (H_4-PteGlu) e il 5-formil-tetraidrofolato (5-CHO-H_4-PteGlu). I ceppi delle specie *S. thermophilus*, *Lb. delbrueckii* subsp. *lactis* e *Lb. helveticus* hanno prodotto più folati totali rispetto al *Lb. delbrueckii* subsp. *bulgaricus* e in particolare 5-CH_3-H_4-PteGlu e H_4-PteGlu. L'impiego di ceppi selezionati di *S. thermophilus* in associazione con *Lb. delbrueckii* subsp. *bulgaricus* ha inoltre consentito di ottenere uno yogurt da latte di capra con un elevato tenore di folati e buone caratteristiche sensoriali.

INTRODUCTION

Folic acid and its vitamers such as 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), tetrahydrofolate (H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu), commonly referred to as folate, represent a group of essential vitamins in human metabolism. These metabolites take part in DNA and RNA biosynthesis and repair (FOWLER, 2001). Moreover, during early pregnancy, a diet with insufficient folate is linked with a minor risk of neural tube defects in the foetus (LOCKSMITH and DUFF, 1998; OAKLEY, 1999; BERRY *et al.*, 1999). Folate deficiency is also the most frequent cause of megaloblastic anemia and hyperhomocysteinemia (HERCBERG and GALAN, 1992). Hence, the importance of ingesting a sufficient daily quantity of folate, as well as knowing its content and bioavailability in food has been reviewed by SARMA *et al.* (1995) and WIGERTZ *et al.* (1997).

Milk and its fermented derivatives can be an important source of folate. That is why several methods for extraction and HPLC analysis of folate from milk and dairy products have been proposed in a number of studies also aimed at defining a standard method of determination that is currently lacking (VAHTERISTO *et al.*, 1996; 1997; RUGGERI *et al.*, 1999; KONINGS, 1999; WIGERTZ *et al.*, 1997).

Folate quantity and composition in milk and dairy products is affected by several factors such as the processing technology of milk and the time and incubation temperature (WIGERTZ *et al.*, 1997; FORSSEN *et al.*, 2000; LIN and YOUNG, 2000b; CRITTENDEN *et al.*, 2003). However, differences in folate content are greatly affected by different milk sources. It is generally known that goat milk is excellent for yoghurt and fresh cheese production because of the balanced content of both essential amino acids and fatty acids, hypo-allergenicity and digestibility (PARK, 1994; HAENLEIN, 2004). Despite this, goat milk is dramatically lacking in folate (UBERTALLE, 1986). Lactic Acid Bacteria (LAB) can be another key factor in folate biosynthesis and/or consumption in dairy products (RAO *et al.*, 1984; WIGERTZ *et al.*, 1997). Several researchers have investigated the ability of different LAB spe-

cies (such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) to affect the folate content of skim milk (Oxoid) and full-cream milk (LIN and YOUNG, 2000a; CRITTENDEN *et al.*, 2003). However, there is a substantial lack of information on the folate content of different fermented products made from goat milk, e.g. yoghurt, and at the same time not much is known about the influence of different LAB species (and strains) on the folic acid content and its vitamers (SYBESMA *et al.*, 2003). The aim of this work was to assess folate production by *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains in goat milk. Moreover certain LAB strains were also characterised for suitable technological features essential for yoghurt production. Finally selected cultures of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were used to obtain goat yoghurt with a substantial folate content and with good sensory features.

MATERIALS AND METHODS

Bacterial strains

Different LAB strains autochthonous to goat milk (apart from the reference strains), and belonging to the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agro-Alimentari, University of Sassari (Italy), were used. Twelve *S. thermophilus* strains (SL1, SL2, SL3, SL4, SL7, SF5, SY2, SY4, SS1, SS2, SS4 and NCDO489 as reference strain), three *Lb. delbrueckii* subsp. *bulgaricus* (LY1, LY2 and CNRZ369 as reference strain), one *Lb. delbrueckii* subsp. *lactis* strain (LF14), and one *Lb. helveticus* (LF22) were tested for folate production and characterised for some relevant technological features such as growth speed and acidifying activity. All the tests were carried out in triplicate in sterilised whole goat milk.

Folate analysis by HPLC

The folate production of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains was quantified after inoculation and growth in sterile goat milk. Folate production of mixed cultures made of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains was assessed in goat yoghurt. Single strains and mixed cultures (1:1 ratio) were inoculated at the final rate of 1% in sterile goat milk and folate determination was assessed after 8 hours of incubation at 42°C. The method of VAHTERISTO *et al.* (1996; 1997) was basically employed for folate analysis. However substantial modifications were introduced to adapt the methodologies to goat milk.

Folate extraction: 5 mL samples of fermented milk or yoghurt were homogenised with 25 mL of extraction buffer (0.1 M phosphate buffer containing 0.5% sodium ascorbate and 0.1% (v/v) 2-mercaptoethanol pH 6.0) using an Ultra Turrax Homogenizer (13,500 rpm for 20-30 seconds). The homogenized sample, carefully protected against light, was incubated under nitrogen atmosphere in a boiling water bath for 15 minutes. The extract was rapidly ice-cooled and centrifuged at 4°C for 30 minutes at 12,000 x *g*. The supernatant was separated and the pellet dissolved again in 10 mL of extraction buffer and centrifuged at 12,000 x *g* for another 10 minutes. Finally the supernatant was separated, added to the first supernatant and the mixture brought to volume up to 50 mL with the extraction buffer.

Deconjugation: a large quantity of folate is present in food as polyglutamates (FINGLAS *et al.*, 1999). In order to be detected and identified, they need to be converted into the respective monoglutamates using deconjugation

enzymes. For this purpose, the Human Plasma (HP) enzyme from Sigma-Aldrich Chemical was used. The enzyme activity was tested by adding 50 µL of HP to 30 µg of pteroil-γ-L-polyglutamate ($PteGlu_3$) in 1.3 mL of 0.1 M acetate buffer at pH 4.9. This mixture was incubated at 37°C for 45 minutes, filtered through a 0.45 µm filter and injected into the HPLC. The analysis showed that 90% of the pteroil-γ-L-polyglutamic acid was converted into folic acid. This 90% efficiency rate was used to adjust the total folate readings. For the deconjugation reaction, 5 mL of extract, brought to pH 4.5 with 0.1 M HCl was added to 3 mL of HP and incubated in a gently shaking water bath at 37°C for 3 hours. The extract was put into boiling water for 5 minutes in order to stop the enzyme activity, then ice-cooled and centrifuged at 10,000 x *g* for 10 minutes.

Purification: 3 mL of deconjugated extract were diluted with 6 mL of distilled water and 15 µL of 2-mercaptoehtanol to lower the salt concentration before injecting it through the strong anion exchange columns, Bakerbond SPE, Quaternary Amine (N^+).

Cartridges, previously conditioned with hexane, methanol and distilled water and treated with 0.01 M phosphate buffer containing 1% of 2-mercaptoethanol, were loaded with 3 mL of sample and washed twice with 1.5 mL of the same phosphate buffer as used for column conditioning. Finally, folate were eluted with 2.5 mL of eluant buffer (0.1 M sodium acetate) containing NaCl at 10% w/v and ascorbic acid at 1% w/v.

The eluate was filtered through 0.45 µm filters and 20 µL of this solution were injected into the HPLC. Instruments were: a pump system with three solvents and gradient control (Varian 9010), a variable wavelength uv/vis detector (Varian 9050) and a Linear Fluo LC 305 fluorometer. A Shandon Hypersil ODS (3 µm, 150x4.6 mm) column was used together with a Novapak C18 pre-column, thermostated at 30°C with a thermostat (Timberline Instruments, Inc., Boulder CO, USA).

Operating conditions were as follows: Mobile phase: A = phosphate buffer (pH 2.2); B = acetonitrile; Flow rate: 1 mL/min; Gradient: 0 min. 90% A; 9 min. 90% A; 12 min. 74% A; 20 min. 90% A; Wavelengths for fluorometric determinations: Excitement = 290 nm, emission = 356 nm for determining tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu); Wavelength for determining folic acid and pteroil-γ-L-polyglutamate ($PteGlu_3$) = 290 nm.

Quantitative folate analysis was performed using a calibration curve made up of 5 different concentrations of standard compounds. Standards were tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu), and folic acid (Sigma-Aldrich) and pteroil-γ-L-polyglutamate ($PteGlu_3$) (Schircks Laboratories, Jona, CH). Standard solutions were prepared by dissolving each compound in 0.1 M phosphate buffer at pH 7; aliquots of these solutions were analysed spectrophotometrically to determine the purity level according to KONINGS (1999). Standard mixtures for the calibration curve were prepared using the rates of these solutions, brought to volume in 0.01 M acetate buffer containing 1% w/v sodium ascorbate at pH 4.9. Recovery percentages of 5-CH_3-H_4-PteGlu and folic acid were also determined in order to evaluate the substance losses during the extraction steps: they were 100% and 99% for 5-CH_3-H_4-PteGlu and folic acid, respectively.

Growth and acidification curves in goat milk

The technological suitability of different strains for yoghurt or fresh cheese production was assessed in sterilized

goat milk by inoculating each LAB strain to the final rate of about $1x10^6$ cells mL^{-1}. Growth abilities were assessed by determining the viable LAB count after 2, 4, 6, 8 and 24 h of incubation in goat milk at 42°C. Post-acidification trials were carried out by determining the viable count on plate and the acidifying activity after 8 h and 5, 15 and 30 days of storage at 4°C in goat milk. Colony Forming Units (CFUs) were determined in both the trials on solidified M17 and MRS media. Acidity determinations were carried out in 10 mL of milk titrated with 0.1 N NaOH. Phenolphthalein was used as indicator and acidity is expressed as percent of lactic acid or Dornic degrees (°D = 0.01% lactic acid). These trials were also carried out on mixed cultures (SL1 + LY1 and SL1 + LY2) inoculated in sterile goat milk at a 1:1 ratio.

Yoghurt production

Yoghurt was produced at a pilot scale using goat milk treated at 92°C for 10 min. The mixed strains SL1 + LY1 and SL1 + LY2 were used. A commercial product was also used for comparison. Mixed cultures were inoculated in goat milk at the 1:1 ratio and incubated at 42°C for 8 h. Sensory features of yoghurts were determined according to BATTISTOTTI (1983) and by a panel of untrained tasters consisting of 20 habitual yoghurt consumers.

Statistical analysis

The means and standard deviations were determined for the different folate forms detected in fermented goat milk and yoghurts. Mean values were compared by the Analysis of Variance (ANOVA) followed by the Fisher Least Significant Difference (LSD) method (significant level $p<0.05$). Data analysis were performed using the NCSS statistical analysis software.

RESULTS AND DISCUSSION

Folate production by different LAB species

Although a very low amount of folate is commonly present in goat milk (UBERTALLE, 1986), folate vitamers were not detected in the uninoculated goat milk used in this study, most likely because the goat milk was heat-sterilized before LAB inoculation. This was expected since significant folate losses during UHT treatment have been previously reported (WIGERTZ *et al.*, 1996). The folate level was then measured 8 h after LAB inoculation in goat milk. In preliminary trials this time was found sufficient for all the strains (single and mixed) to reach the maximum growth in goat milk. Moreover LIN and YOUNG (2000b) have already shown that folate production by LAB is basically growth-associated, reaching the highest (folate) content before the stationary phase and then declining during fermentation and storage. Therefore in the present study, the folate level was not assessed during the late fermentation stage and storage.

All the *S. thermophilus* strains evaluated produced different folate vitamers, especially H_4-PteGlu and 5-CH_3-H_4-PteGlu, which were the most abundant forms (Table 1). The high production of 5-CH_3-H_4-PteGlu by several strains (e.g. SL1, SL4 and SS4) is important since this vitamer plays a significant role in human metabolism converting homocysteine into methionine and thus contributing to reducing the risk of cardiovascular disease (RIDDEL *et al.*, 2000; SELHUB *et al.*, 2000; GRUNDY *et al.*, 1999). Moreover as recently reviewed by FORSSEN *et al.* (2000), there are substantial differences in the stability between various reduced folate forms (H_4-PteGlu). Generally the order of stability for the different vitamers considered in this study is as follow: 5-CHO-H_4-PteGlu > 5-CH_3-H_4-PteGlu > H_4-PteGlu (FORSSEN *et al.*,

Table 1 - Production (ng/g) of tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu) in goat milk inoculated with *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus* strains (average values of three determinations ± standard deviations). Values in the same column with different letters are significantly different ($p<0.05$).

	H_4PteGlu	5-CH_3-H_4PteGlu	5-CHO-H_4PteGlu	Total folate
S. thermophilus				
SL1	20.99±1.73^{b}	21.11±2.98^{b}	17.24±2.86^{a}	59.33±7.57^{b}
SL2	12.67±1.77^{d}	18.08±0.18^{c}	18.44±1.43^{a}	49.19±3.02^{b}
SL3	11.85±1.01^{d}	17.76±0.68^{c}	14.83±0.04^{b}	44.43±1.73^{d}
SL4	18.52±2.00^{b}	22.14±4.82^{b}	-	40.66±2.82^{d}
SL7	18.94^{b}	30.72^{a}	-	49.66^{c}
SS1	22.40±1.32^{a}	7.27±0.65^{d}	17.96±0.51^{a}	47.63±2.47^{c}
SS2	20.40±1.70^{b}	4.30±0.42^{e}	-	24.70±2.12^{f}
SS4	14.53±1.68^{c}	23.52±0.17^{b}	21.50±0.08^{a}	59.54±1.77^{b}
SY2	22.72±1.10^{a}	8.01±0.22^{e}	12.48±0.16^{c}	43.20±1.15^{d}
SY4	25.27±4.05^{a}	4.77±1.36^{e}	18.62±0.69^{a}	48.65±6.10^{c}
SF5	18.32±0.11^{b}	16.06±0.85^{c}	12.15±0.81^{c}	46.53±0.15^{d}
NCDO 489	6.50±0.02^{g}	6.95±0.43^{d}	3.31±0.11^{f}	16.76±0.56^{g}
Lb. bulgaricus				
LY1	21.68±0.28^{a}	7.69±0.3^{d}	3.51±0.54^{f}	32.87±0.52^{e}
LY2	16.52±0.64^{c}	5.64±0.37^{e}	5.35±0.01^{e}	27.51±0.28^{f}
CNRZ 369	-	5.45±0.15^{e}	-	5.45^{h}
Lb. lactis				
LF14	28.24±0.17^{a}	24.39±0.25^{a}	10.52±0.49^{d}	63.15±0.91^{a}
Lb. helveticus				
LF22	21.2±0.4^{a}	24.95±0.10^{b}	6.22±0.23^{e}	52.37±0.37^{c}

2000). Table 1 shows that SL1 and SS4 strains produced substantial quantities of total folate and were the most efficient *S. thermophilus* strains. The high production of H_4-PteGlu by SY4 (25.27 ng/g) has to be pointed out since H_4-PteGlu has been reported to be the prevalent form of folate in milk/dairy products (WIGERTZ *et al.*, 1997). The other strains showed less ability to produce the different vitamers especially 5-CHO-H_4-PteGlu. These findings are in contrast with SYBESMA *et al.* (2003) who reported that 5,10-methenyl and 5-formyl tetrahydrofolate were the major folate derivates of *S. thermophilus* grown on M17 medium. It must be pointed out that these authors evaluated the folate production ability of three *S. thermophilus* strains, while in this study, 12 strains were evaluated which gives a more complex picture in which 5-CHO-H_4-PteGlu and 5-CH_3-H_4-PteGlu are the major folate derivates for only some strains (e.g. SL2, SL3 and SS4). The *S. thermophilus* reference strain NCDO489 (not isolated from goat dairy products) produced a lower amount of total folate and showed some substantial difficulties to grow and survive (Table 2).

The *Lb. delbrueckii* subsp. *bulgaricus* strains generally produced a lower amount of total folate than *S. thermophilus* (Table 1). Although some authors have shown the ability of *Lb. delbrueckii* subsp. *bulgaricus* to produce folate on reconstituted Skim Milk (LIN and YOUNG, 2000b), some strains have been reported to be folate-depleting (RAO *et al.*, 1984). The ability of different *Lacto-*

Table 2 - (a): viable count (cfu mL^{-1}) and acidity curves (% of Lactic acid) of *S. thermophilus* (SL1, NCDO489), *Lb. delbrueckii* subsp. *bulgaricus* (LY1, CNRZ369), *Lb. delbrueckii* subsp. *lactis* (LF14), and *Lb. helveticus* (LF22) grown in goat milk at 42°C (average of three determinations). (b): viable count (cfu mL^{-1}) and post-acidification (% Lactic acid) of LAB strains in goat milk stored at 4°C (average of three determinations).

(a) Strain	SL1		NCDO489		LY1		CNRZ 369		LF14		LF22	
	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity
Hours												
0	1.03E+06	0.20	4.70E+05	0.19	2.91E+06	0.16	8.00E+05	0.15	8.00E+05	0.15	6.70E+05	0.16
2	3.00E+07	0.21	1.70E+07	0.28	1.12E+07	0.32	3.00E+06	0.15	3.40E+06	0.17	3.50E+06	0.19
4	4.00E+08	0.26	3.40E+08	0.34	2.00E+07	0.65	4.00E+06	0.19	2.60E+07	0.33	2.20E+07	0.22
6	5.40E+08	0.50	5.60E+08	0.40	2.60E+08	1.10	8.10E+06	0.31	3.20E+07	0.42	2.80E+07	0.30
8	9.40E+08	0.69	8.20E+08	0.55	5.60E+08	1.13	9.00E+07	0.60	4.40E+08	0.57	3.80E+07	0.33
24	4.60E+08	0.84	8.00E+07	0.61	8.40E+06	1.71	8.00E+06	1.30	8.40E+06	0.90	4.60E+06	0.45
(b) Time												
8h	9.40E+08	0.69	8.20E+08	0.55	5.60E+08	1.13	9.00E+07	0.60	4.40E+08	0.57	3.80E+07	0.33
5 d	9.30E+08	0.61	8.10E+08	0.49	3.12E+08	1.18	7.10E+07	0.82	3.40E+08	0.70	1.70E+07	0.52
15 d	9.20E+08	0.71	2.20E+08	0.40	5.90E+07	1.19	4.20E+07	0.81	1.60E+06	0.74	3.80E+07	0.56
30 d	8.04E+08	0.71	1.30E+08	0.44	2.65E+07	1.19	1.40E+06	0.80	3.30E+06	0.81	3.50E+07	0.61

bacillus strains and species to influence the folate level in dairy products is currently under discussion. In a recent study CRITTENDEN *et al.* (2003) showed that starter cultures made of *Lactobacillus* spp. (e.g. *L. helveticus* and *L. acidophilus*) generally did not significantly influence folate levels when grown on reconstituted Skim Milk, even if *Lb. delbrueckii* subsp. *bulgaricus* CSCC2505 and CSCC5168 have been described as folate producer and depleting strains. It seems that at least within the *Lb. delbrueckii* subsp. *bulgaricus* species a certain variability for folate production exists and is strongly strain-specific.

With respect to different vitamers, the H_4-PteGlu production of *Lb. delbrueckii* subsp. *bulgaricus* was 21.68 ng/g, for the LY1 strain, and 16.52 ng/g for LY2. The reference strain CNRZ 369 (not isolated from dairy goat products) was only able to produce 5-CH_3-H_4-PteGlu. The reference cultures used in this study showed evident growth difficulties in goat milk (Table 2), thereby confirming the necessity of using autochthonous starter cultures for goat milk processing and/or improvement of probioticity or nutritional value.

Lb. helveticus produced 52.37 ng/g of total folate and the highest amount of 5-CH_3-H_4-PteGlu (24.95 ng/g). The *Lb. delbrueckii* subsp. *lactis* used in this work was by far the most productive among the tested species, for both total folate (63.15 ng/g) and H_4-PteGlu (28.24 ng/g) production. This is of particular importance since this is the first report (to our knowledge) on folate production by *Lb. delbrueckii* sub-

sp. *lactis*. However further studies are necessary to assess whether the folate-producing ability of *Lb. delbrueckii* subsp. *lactis* is strain-specific, as it seems to be for *Lb. delbrueckii subsp. bulgaricus*, or if it is common to all the members of the species, as in the case of *S. thermophilus*.

Folate production of mixed strains in yoghurt

Folate production of mixed cultures of LAB grown in goat milk is reported in Table 3. The highest production of total folate was 46.93 ng/g (SL1+LY2), significantly lower than those obtained for several *S. thermophilus* strains (Table 1). A comparison between folate contents in Tables 1 and 3 shows that folate produced by SL1 (single culture) was higher, especially for 5-CH_3-H_4-PteGlu and 5-CHO-H_4-PteGlu, than those obtained with the combined strains SL1+LY1/LY2. Total folate production was higher for the mixed cultures SL1+LY2 in which, growth and acidification of the *Lactobacillus* was reduced (Figs. 1 and 2). It is interesting that LY2 alone was found to be a lower folate producer compared to LY1 (Table 1). A lower production or accumulation of different vitamers and/or total folate in the medium could be due to the (partial) utilization of folate by *Lb. delbrueckii* subsp. *bulgaricus* as previously reported (RAO *et al.*, 1984; RAO and SHAHANI, 1987). RAO *et al.* (1984) showed that *Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842) depleted the available folate in reconstituted Skim Milk when inoculated as single and mixed culture together with *S. thermophilus* (RAO *et al.*, 1984). On the contrary in our study it was found that *Lb. delbrueckii* subsp. *bulgaricus*, inoculated as single strains, increased the total folate level of fermented goat milk while no additive effect was observed when they were inoculated together with *S. thermophilus*. CRITTENDEN *et al.* (2003), studying the synthesis and utilization of folate by yoghurt starter cultures, reported similar findings. It seems reasonable to assume that, in mixed culture, strains of *Lb. bulgaricus* influence the folate production of *S. thermophilus* through a possible inhibition mechanism or alternatively by folate consumption.

In the present study, a method, essentially adapted from VAHTERISTO *et al.* (1996, 1997), was set up for folate extraction and determination in dairy products from goat milk. Different extraction procedures have been employed or proposed by several authors to extract folates from different types of food samples (ENGELHARDT and GREGORY, 1990; VAHTERISTO *et al.* 1996; KONINGS, 1999; KONINGS *et al.*, 2001). Basically all of these authors used different γ-glutamyl hydrolases for the deconjugation such as hog kidney (HK), chicken pancreas (CP) and rat plasma (RP) conjugases. Moreover, some of these enzymes (i.e. HK) need an extraction or preparation step before use (ENGELHARDT and GREGORY, 1990). In this

Table 3 - Production (ng/g) of tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu) in goat milk inoculated with mixed cultures of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains (average values of three determinations ± standard deviations). Values in the same column with different letters are significantly different ($p<0.05$).

S. thermophilus + *Lb. bulgaricus*	H_4PteGlu	5-CH_3-H_4PteGlu	5-CHO-H_4PteGlu	Total Folate
SL1 + LY1	20.66±0.31[a]	6.89±0.19[b]	9.21±0.01[c]	36.75±0.49[a]
SL1 + LY2	18.34±2.93[a]	10.97±1[a]	17.62±1.68[b]	46.93±5.61[b]

study, the HP (Human Plasma) enzyme was used for the deconjugation step because of its greater availability compared to the other enzymes. It is currently widely used for folate determination (HPLC methods) in milk and dairy products by several research groups (LIN and YOUNG, 2000b; SYBESMA *et al.*, 2003). KONINGS (1999) and KONINGS *et al.* (2001) determined folate concentrations in different food samples using rat plasma after treatment with protease and amylase, the latter being aimed at detecting the matrix-bound folate. In this study protease and amylase were not used since this treatment is commonly used for folate extraction from food stuffs which contain a considerable amount of protein, starch and carbohydrate matrixes. However the application of this method to different milk and dairy products did not clearly show the presence of substantial amounts of matrix-bound folate in yoghurt (KONINGS *et al.*, 2001).

Growth and acidification of *S. thermophilus* and lactobacilli in goat milk

Results concerning the growth and acidifying activities of selected *S. thermophilus* and lactobacilli strains are reported in Table 2. The growth of *S. thermophilus* in goat milk was rapid during the first hours of incubation, but later a certain slowing down occurred and the microbial population did not reach values higher than 10^9 cells mL^{-1}. The growth rates of *Lb. delbrueckii* subsp. *bulgaricus* were rather slow and reached their maxima after 8 hours of incubation, whereas acidification followed a typical trend for this species. Post-acidification trials highlighted a good tolerance of LAB strains to storage at 4°C while showing the superior ability of autochthonous lactic acid bacteria to survive in goat milk with high numbers for at least 30 days (Table 2).

Growth and acidification of mixed strains in goat milk

Both the speed of growth and the acidification ability of *S. thermophilus* SL1 mixed with *Lb. delbrueckii* subsp. *bulgaricus* LY1 (Figs. 1 and 2) showed a typical trend. Instead *S. thermophilus* SL1 associated with *Lb. delbrueckii* subsp. *bulgaricus* LY2 did not show the characteristic synergism for growth and acidification. In this case LY2 was the slowest strain for both growth and acidification. The presence in goat yoghurt of a high number of living LAB after 4 weeks (Fig. 3), or a substantial concentration of naturally-produced functional metabolites such as folate, can certainly be considered as a positive feature.

Yoghurt production and sensory analyses

Yoghurts made on a pilot scale using LAB strains selected for folate production and technological suitability showed some encouraging results when compared with a commercial product. The sensory features examined were appearance, texture, smell and taste. The experimental yoghurts had a proper thick appearance, a creamy and smooth texture; the whey was not separated and the smell was excellent and sweet. The taste of both the experimental yoghurts was generally judged as good, palatable, not too sour, and similar to the commercial goat yoghurt.

CONCLUSIONS

The ability of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* to produce folic acid or its vitamers in goat milk and their technological suitability for improving goat milk products, particularly goat yoghurt, were examined. They all produced different folate vitam-

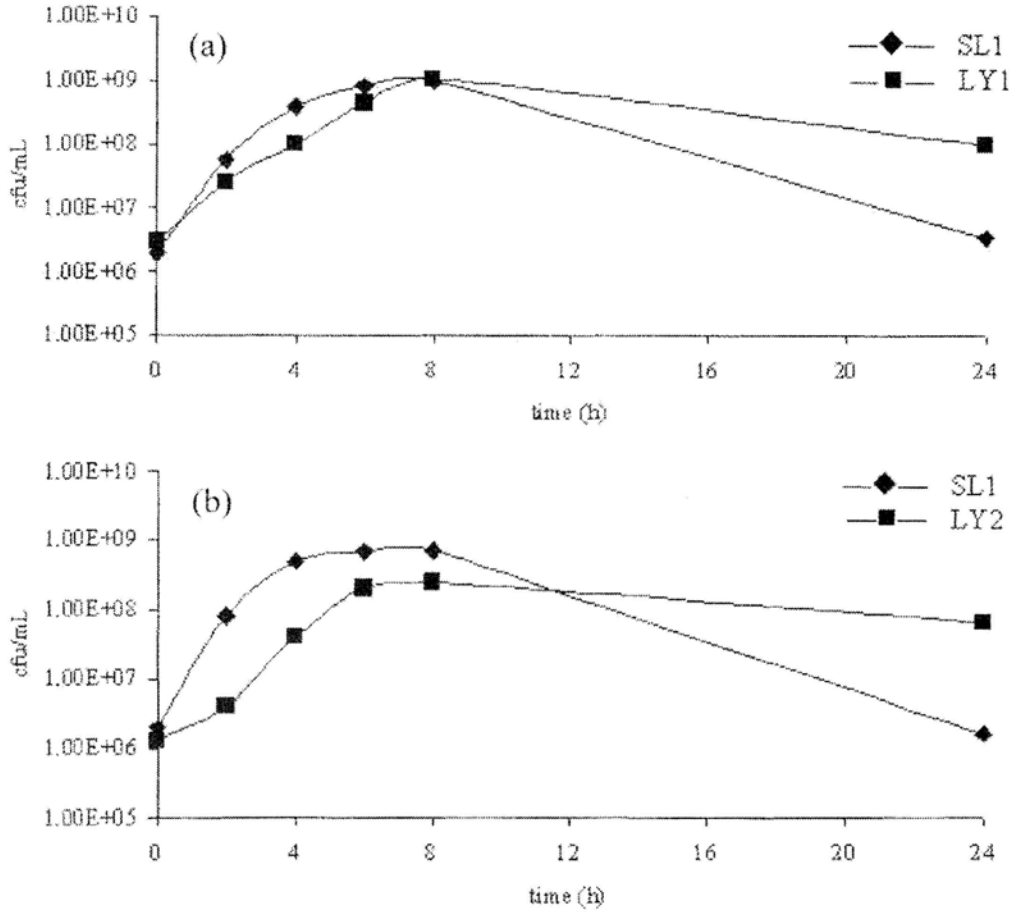

Fig. 1 - Growth curves (cfu mL^{-1}) of mixed *S. thermophilus* SL1 and *Lb. delbrueckii* subsp. *bulgaricus* LY1 (a) and LY2 (b) strains grown in goat milk at 42°C (average of three determinations).

ers (5-CH_3-H_4-PteGlu, 5-CHO-H_4-PteGlu, H_4-PteGlu). In general, 5-CH_3-H_4-PteGlu and H_3-PteGlu, the most important vitamers from a functional point of view, were produced at a higher level. Folate production did not seem to be affected by the physiological features of the LAB examined, which had a different behavior, particularly in speed of growth and acidifying activities.

Results related to folate production by yoghurt starter cultures suggest that consumption of folate by *Lb. delbrueckii* subsp. *bulgaricus* strains may occur. On the other hand, the low amount of folate produced by mixed cultures (compared to single cultures) in fermented goat milk could be related to some inhibitory effects of the lactobacillus towards the *S. thermophilus*.

These results can be considered a useful contribution to the knowledge of folate produced by lactic acid bacteria. A suitable analytical method for determining folate in goat milk and dairy products was also set up. The results from this study are encouraging, especially from an application point of view, since

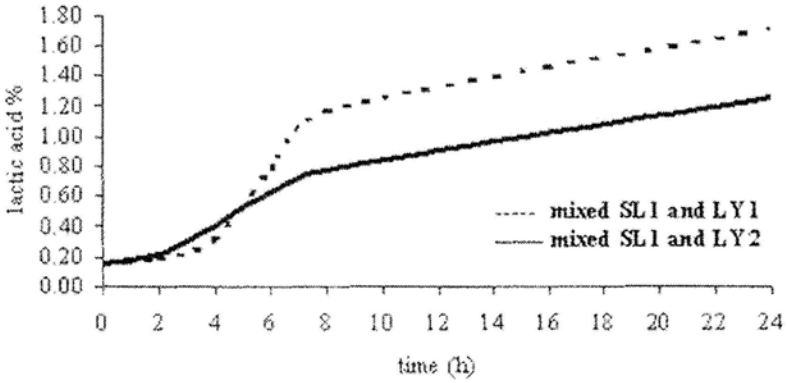

Fig. 2 - Acidity curve (% Lactic acid) of *S. thermophilus* SL1 when mixed with *Lb. delbrueckii* subsp. *bulgaricus* LY1 and LY2 strains grown in goat milk at 42°C (average of three determinations).

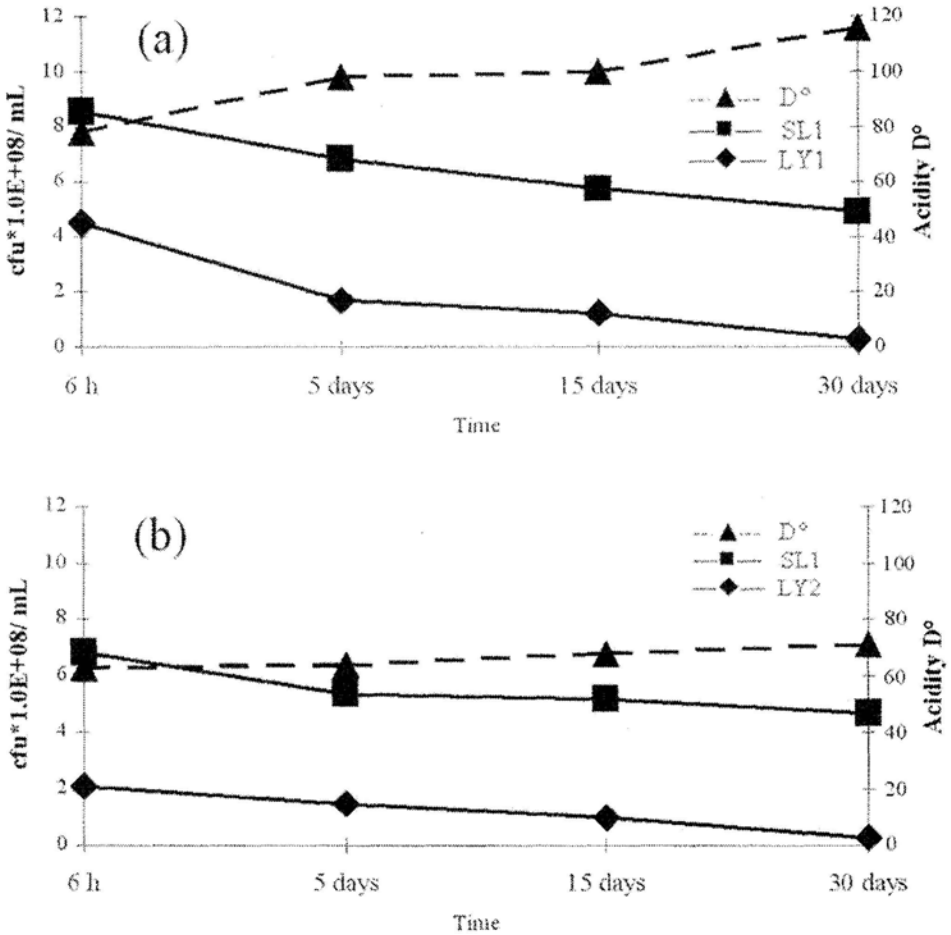

Fig. 3 - Viable count (cfu mL^{-1}) and post-acidification curve expressed as Dornic degree (D°) of mixed cultures of *S. thermophilus* SL1 and *Lb. delbrueckii* subsp. *bulgaricus* LY1 (a) and LY2 (b), during storage at 4°C in goat milk (average of three determinations).

the use of folate-producing LAB isolated from different goat dairy products could contribute to the nutritional improvement of goat milk products.

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