Journal of Industrial Microbiology & Biotechnology (2000) 25, 288–297

www.nature.com/jim

ORIGINAL PAPERS

The production of mixed cultures containing strains of *Lactococcus lactis, Leuconostoc cremoris* and *Lactobacillus rhamnosus*, on commercial starter media

J Barrette, CP Champagne, D Roy and N Rodrigue

Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant Blvd. West, Saint-Hyacinthe, Quebec, Canada J2S 8E3

Mixed starters containing *Lactococcus lactis*, *Leuconostoc cremoris* and *Lactobacillus rhamnosus* strains were produced on commercial starter media (MB Complete, Thermolac, Marlac), as well as on milk. With the exception of Marlac, the starters were cultured under pH control. The effect of media and incubation temperature (22 or 32°C) on population ratios, on specific acidifying activities (SAA) of the cultures as well as on their ability to produce aroma compounds in milk was studied. The starters had higher contents in lactobacilli when they were produced at 32°C, whereas a tendency to obtain higher *Leuconostoc* populations was observed at 22°C. With respect to the lactococci, there was a significant interaction between temperature and growth medium for both strains. Thus, *Le. cremoris* T2 reached higher populations at 32°C if grown in MB complete and Thermolac, whereas in Marlac and skim milk, viable counts were higher at 22°C. The lactococci represented 50% of the total population of the culture at the beginning of the incubation, but they composed between 80% and 99% of the total population following fermentation. The best medium for growth of *Leuconostoc* was milk, but populations of only 10^8 cfu/ml were reached. The lactobacilli did not grow well in MB Complete, and their development was best in the low-phosphate Marlac medium. The cultures grown on Marlac had the highest SAA values, whereas those grown on MB complete had the lowest. Overall, more ethanol and diacetyl were detected in the fermented milks when the starters used to inoculate them were produced at 22°C.
Journal of Industrial Microbiology & Biotechnology (2000) **25,** 288–297.

Keywords: dairy starters; pH control; MB Complete; Marlac; Thermolac; acetaldehyde; ethanol

Introduction

Traditionally, mixed starter cultures containing various lactococci and *Leuconostoc* have been prepared from milk and nonfat dry milk, without pH control. Starter bacteria convert lactose to lactic acid and the pH drops until acidic conditions become detrimental to lactic cultures, usually around pH 4.3–4.7 [15,36].

In the mid 1970s, pH-controlled systems were introduced [17]. Commercial media were designed to be used with external control of pH (addition of alkali), or contained high levels of buffering agents and were thus considered to have "internal pH control" [26]. Although cultures grown in commercial media with pH control may have lower specific acidifying activities (SAAs) than those grown in milk without pH control [3,34], pH-controlled starters contain up to 10 times more cells than traditional starters [25,31,36,38], which largely compensates for the slight reduction in SAA. As a consequence, there is a widespread use of this technology in North America. With respect to mesophilic starters, the success of the pH-control technology is partially related to the fact that it is mainly applied to cultures used for cheddar, which are less complex than those used for fresh/Quarg, Camembert/Brie or Edam/Gouda cheeses. Indeed, cheddar cultures generally contain only lactococci whereas the latter often have *Leuconostoc* strains in addition. Although some information is available on production of mixed *Leuconostoc*-containing cultures under pH control [31], most of the information is proprietary [39], and no published data are available on population ratios obtained with the commercial starter media.

Some thermophilic cultures, used for yoghurt or Mozzarella production, contain lactobacilli, but mesophilic starters generally do not. *Lactobacillus casei* and *Lactobacillus rhamnosus* grow at 15°C and could thus potentially be included in mesophilic starters. Interest in such lactobacilli is increasing because of their potential probiotic properties [30]. However, as for *Leuconostoc*, there is no information on the development of population ratios when *La. rhamnosus* is added to lactococci and grown in commercial starter media under conditions used for the production of mesophilic cultures.

The aim of this study was to examine population ratios as well as SAA (the cell population required to inoculate milk to obtain a pH of 5.0 in a given incubation time) or secondary metabolite (diacetyl, ethanol, acetaldehyde, acetoin) production properties of mixed starters containing *Lactococcus lactis, Leuconostoc cremoris* and *La. rhamnosus* strains produced on commercial starter media.

Correspondence: CP Champagne, Agriculture and Agri-Food Canada, Food Research and Development Center, 3600 Casavant Blvd. West, Saint-Hyacinthe, Quebec, Canada J2S 8E3
Received 23 June 2000; accepted

Materials and methods

Microorganisms

The starter was composed of *L. lactis* subsp. *lactis* Type I (*L. lactis* T1), *L. lactis* subsp. *cremoris* Type II (*Le. cremoris* T2), obtained from Rosell Institute (Montréal, QC, Canada), *La. rhamnosus* ATCC 9595M (*La. rhamnosus* 9595M) and *Leuconostoc mesenteroides* subsp. *cremoris* CAF-500 (*Le. cremoris* CAF-500) obtained from Rhôdia (Madison, WI). The lyophilized cultures of *Lactococcus* were stored at $-18°C$. The populations were estimated, respectively at 4.1×10^{10} cfu/ml for *L. lactis* T1 and 1.6×10^{10} cfu/ml for *Le. cremoris* T2. A frozen can (70 ml) of *Le. cremoris* was thawed in cold water, and the bacterial concentrate was immediately fractioned in 5-ml units and frozen at $-70°C$. The total population after the thaw–freeze operation was 1.0×10^{10} cfu/ml. The *La. rhamnosus* 9595M strain was grown and freeze-dried in our laboratory. It was subcultured twice on MRS both (Oxoid; Nepean, ON, Canada) for 16 h at 37°C, and a 2-l batch was produced in a bioreactor (New Brunswick Scientific, NY) on MRS broth. Fermentation conditions in the bioreactor were: inoculation level 1% (v/v), agitation 100 rpm, 37°C, 16 h without pH control. When the fermentation was completed, cells were harvested by centrifugation at $5600\times g$ for 20 min at 4°C. The cell pellet was resuspended in a sterile freeze-drying medium composed at 20% nonfat milk, 5% sucrose and 1% casitone, previously heated at 110°C, 10 min, as well as 0.35% of ascorbic acid, added after heating from a filtered (0.2 μm) stock solution at 17.5%. Resuspended cells were frozen at $-70°C$ and dried on a Lyo-Tech freeze dryer (LYO-SAN, Lachute, QC, Canada), for 48 h at 25°C in a 10-μm vacuum. The lyophilized culture was stored at $-18°C$, and its viable population was 1×10^{11} cfu/ml.

Biocompatibility

Because the cultures were obtained from various suppliers, compatibility between strains were examined.

(1) Biomass. Cultures for seeding the agars as well as for production of cell-free extracts were obtained with the following procedures. The lactococci were produced on M17 broth (Oxoid), with an incubation of 12 h at 21°C; *La. rhamnosus* 9595M on MRS broth (Oxoid), 16 h at 37°C; *Le. cremoris* on tomato juice (TJ) broth (Difco), 16 h 30°C. The same media were used to support growth of the test organisms in compatibility tests.

(2) Cell-free extracts. Ten milliliters of each culture was centrifuged at $3000\times g$ for 20 min at 4°C. The supernatants were filtered (0.45-μm pore-size filters, nylon HVLP, Millipore) and the filtrates were kept at 4°C until used.

(3) Acid controls. Since the acidity of the cell-free extracts was not neutralized, acid solutions were added as controls, to determine if any inhibition in the compatibility tests was due to acid or to other metabolites (H_2O_2 or bacteriocins). The media were adjusted to the same pH as the cultures with 85% lactic acid or with 40% acetic acid. All media and broth were sterilized at 121°C, 15 min.

(4) Test. The indicator organism (1 ml) was mixed with 25 ml of the appropriate molten agar in a Petri plate. When the media had solidified, three sterile bacto-disks (Difco) were placed on each plate and 30 μl of each cell-free extract or of the acidified broths was added on the disk. The plates were incubated for 48 h at optimal temperatures for the indicator bacteria, after which the diameters of the inhibition zones were measured. Two independent assays were carried out.

Culture media

The four starter media were: (1) MB Complete, a commercial medium destined for mesophilic cultures grown under external pH control (Rhôdia) rehydrated, (2) Thermolac, a commercial medium destined for thermophilic cultures grown under external pH control (Rhôdia), (3) Marlac, a commercial medium destined for mesophilic cultures grown under internal pH control (Rhôdia Canada), and (4) rehydrated skim milk (Agropur; Granby, QC, Canada) serving as a control. Rehydration conditions as well as some composition features of these media are found in Table 1. All media were pasteurized at 90°C for 45 min.

Inoculum

Each of the four bacteria was inoculated at 1×10^7 cfu/ml. The lyophilized cultures (1 g) were rehydrated during 10 min at 25°C in 4 ml of a sterile medium (121°C, 15 min) composed of 1.5% peptone (Difco), 1.0% tryptone (Difco) and 0.5% meat extract (Oxoid) before inoculation in the media.

Fermentation

Fermentations were carried out in two 2.5-l reactors: BioFlo III and 3000 (New Brunswick Scientific), controlled by a Biocommand (New Brunswick Scientific) software system. The working volume was 2 l. Each medium was inoculated with the mixed starter culture described above. The temperature was set at 22 or 32°C depending on the experimental design. The agitation was set to 65 rpm. A zone-pH procedure common in industrial starter production, was applied with MB Complete, Thermolac and skim milk media. The fermentation was allowed to proceed until pH of 5.8 was reached.

Table 1 Some characteristics of the growth media following rehydration

Medium	Used under external pH control	Solids (g/l)	Lactose (g/l)	Phosphates (g/l)	Carbonates (g/l)	Citrates (g/l)	Mg (ppm)	Mn (ppm)	Contain	
									Yeast extracts	Whey
MB Complete	Yes	80	26.3	9.8	ND[1]	0.13	71	<0.1	Yes	Yes
Thermolac	Yes	74	33	4.6	ND	0.16	70	<0.1	Yes	Yes
Marlac	No	70	13.6	9	8	0.1	78	0.2	Yes	Yes
Milk	Yes	110	47.6	3.2	ND	0.28	102	<0.1	No	No

[a]Not determined

At this set point, agitation was increased to 150 rpm and the alkali pump (5 N NH_4OH) was activated until a pH of 6.0 was reached. At the second set point, alkali addition was stopped and agitation returned to 65 rpm. The pH was then kept at 5.8–6.0, until acidification stopped. With Marlac medium, the supplier's recommendation was followed, and no external pH control was applied, because the medium relied on the buffering action of the carbonates. This fermentation was allowed to proceed until acidification stopped. Samples were taken at the end of the fermentation for SAA, high pressure liquid chromatography (HPLC) analyses, population counts and for the production of aroma compounds.

Differential bacterial enumerations

All four bacteria were estimated by plating appropriate peptone (0.1%) water dilutions on selective and differential media: *Le. cremoris* on MSE medium [24] following a 30°C/72-h incubation under anaerobic conditions (85% N_2, 5% CO_2 and 10% H_2); *La. rhamnosus* 9595M on MRS medium (Difco) following a 42°C/48 h incubation; *L. lactis* (T1) and *Le. cremoris* (T2) on Turner medium [37] after a 24°C/48-h incubation. On the Turner medium, colonies of *L. lactis* (T1) appeared bright red, whereas those of *Le. cremoris* (T2) were light pink.

Specific acidifying activities

SAAs were carried out at 25 and 32°C with the procedure described by Champagne *et al* [3,4]. In this study, the test was based on the determination of the cell population required to inoculate milk to obtain a pH of 5.0 after 5.5 h at 32°C or 7 h at 25°C. High values thus indicate a low SAA.

Chemical analyses

Lactose content in the media was measured by an enzymatic method (ACLAB01, Boehringer). The contents in organic acids of the fermented media (citric, lactic, acetic and pyruvic acids) was determined by HPLC. Frozen samples (−18°C) were thawed at room temperature and diluted 1/10 with distilled water. One milliliter of TCA (36%) was added to the samples of fermented milk for the precipitation of milk proteins. The diluted samples were centrifuged at 3000×*g* for 15 min at 25°C and filtered (0.45-μm pore-size filters, nylon HVLP, Millipore). Compounds in the filtrates were separated on an HPX87H (Biorad) column maintained at 25°C, using a mobile phase of 0.008 N H_2SO_4 at a flow rate of 0.6 ml/min. A Waters photodiode array 996 monitor scanning from 200 to 400 nm every 1/15 s detected the peaks and enabled the confirmation of peak purity.

Aroma compounds

In addition to SAA, the ability of the starters to produce aroma compounds in milk was determined.

(1) Production. The test was carried with a starter that had been stored at 4°C for 24 h. The SAA test, carried out immediately after starter production, enabled the determination of the inoculation level required to reach pH 4.8 in 5.5 h at 32°C and 7 h at 25°C. Irradiated (5 kGy) skim milk was rehydrated (11%) and stored at 4°C for 16 h before inoculation. The milk was divided in 50-ml fractions and placed in four glass flasks by starter. Two flasks were incubated at 25°C and the two others at 32°C.

(2) Samples. When pH 4.8 was reached, the flasks were placed in ice and duplicate 2-g samples of fermented milk weighed and placed in 5-ml vials. One milliliter of 1 M H_2SO_4 was added to each sample, which was then sealed with a crimped cap and an aluminum capsule, and frozen (−18°C). The samples were thawed at room temperature and 0.75 g of ammonium sulfate was added to accelerate precipitation of proteins as well as to release volatile compounds [28], and the vials were then re-capped.

(3) Apparatus. Analyses of volatile compounds were performed on head space gasses in the vials. Parameters of the headspace sampler (Hewlett-Packard 19395A, Avondale, PA) were: heating temperature, 90°C; heating time 90 min; pressurization time, 15 s. Separation and detection were carried out on a Hewlett-Packard (Avondale, PA) 5890 series II gas chromatograph with an integrator (Hewlett-Packard 3396 series II) as well as an FID detector. The system was fitted with a DB-Wax column (Chromatographic Specialties, Brockville, ON, Canada; 30 m×0.32 mm, film thickness 0.5 μm). Operating parameters of the chromatograph were: injector and detector temperature, 250°C; H_2 flow rate in the column, 2.0 ml/min. The oven heating cycle was 35°C for 12 min, with a temperature increase of 10°C/min up to 150°C, followed by 150°C for 5 min. Acetaldehyde, acetone, ethanol and diacetyl were calibrated with external standards in skim milk (11%). The calibration curves were carried out with each standard (1 to 100 ppm). The internal standard used was 1-propanol.

Spectrophotometric analysis

The strains were characterized by automated spectrophotometry, with respect to optimum pH and temperature for growth, as well as sensitivity to phosphates. The automated spectrophotometric method described by Champagne *et al* [5] was used with some modifications.

(1) pH. The tests were carried out on the appropriate growth media, MRS broth for *La. rhamnosus* 9595M, M17 broth for lactococci and TJ broth (Oxoid) for *Le. cremoris*. The media were adjusted at pH 4.7, 5.0, 5.3, 5.6, 5.9, or 6.2 with 0.1 M NaOH before sterilization. The inoculating level was 0.01% and the incubation temperature was set to 28°C for 48 h.

(2) Temperature. The same cultures and media (adjusted to pH 6.0) as previously described were used. The inoculum level was 0.01% and temperature was maintained at 20, 24, 28 or 32°C for 48 h.

(3) Phosphate. Sensitivity to phosphates was carried out in a base medium composed of 0.5% Bacto Soytone (Difco), 0.25% Primatone (Sigma), 0.25% Tryptone (Difco), 0.25% yeast extract (Oxoid), 0.5% meat extract (Lab-lemco powder, Oxoid), 0.5% lactose (BDH), 0.05% ascorbic acid (Sigma, St. Louis, MO) and 0.25% $MgSO_4$z.ccirf;H_2O (Fisher Scientific). The phospate (K_2HPO_4 and KH_2PO_4; Anachemia, Ville St-Pierre, QC) levels tested were 0.0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0%. They were added to the base medium, the pH was adjusted at 6.0 (HCl, 0.1 M or NaOH, 0.1 N) and the mixture was autoclaved at 121°C, 15 min. The inoculum level was 0.01% and the temperature was 28°C for 48 h. The time needed to reach an optical density (OD) of 0.2 (600 nm) was determined.

Statistical analysis

The aim of this experiment was to assess the effect of two controlled factors, temperature and medium, and their interaction

to various response variables. The factors have two and four levels, respectively. Therefore to complete a replication of the experiment, a total of eight runs was necessary. There is, however, a constraint in the design and the analysis of this experiment; only two reactors were available. As a result, only two runs corresponding to a given combination of the two factors could be made per day. A design including blocks was chosen where blocks correspond to a day of the experiment. In this design a level of the temperature factor was randomly allocated to a day and the use of a balanced incomplete block design [6,40] was necessary to allocate the levels of the factor medium to reactors. The parameters of the chosen BIBD are: $t=4$, $k=2$, $r=3$, $b=6$. Where t is the number of levels of the factor medium, k is the number of available reactors (number of runs that can be made within a day), r is the number of times a treatment is tested over the whole experiment and b is the number of days. Three complete replications are necessary to ensure balance and were therefore carried out. This was done for each temperature level.

The statistical model reflecting the structure of these data is a split-plot design [27] in which the main plots are the days of the experiment to which a level of temperature is randomly allocated and subplots are the reactors to which a level of the medium factor is allocated according to an incomplete block design. Given the relatively complex structure of the data, a mixed model was used to perform univariate analyses of the data [29]. The data can be described by the linear model:

$$Y_{ijk} = \mu + \tau_i + \gamma_{ij} + \beta_k + \tau\beta_{ik} + \varepsilon_{ijk},$$

where the terms in the whole plots are: Y_{ijk}=the value of the response variable for temperature i, on repetition j, for medium k; μ=the overall mean; τ_i=the fixed effect of the temperature i; γ_{ij}=the random whole plot error effect. Terms in the subplots are: β_{ki}=the fixed effect of medium k, $\tau\beta_{ik}$=the fixed interaction effect of temperature i and medium k, ε_{ijk}=the residual effect defined as the random subplot error effect.

The mixed procedure of SAS was used [21] to analyze the data. For each response variable, an analysis of variance corresponding to the mixed model was done including the controlled factors and their interaction and an effect due to noise factor: reactors as sources of variation. Whenever a significant medium effect or interaction was detected, orthogonal contrasts were computed to determine where differences lie. The residual analysis was done for each ANOVA to ensure that the underlying hypothesis of the model was satisfied.

Results and discussion

Compatibility of the strains

Many lactic acid bacteria produce bacteriocins [18], and compatibility tests should be carried out before attempts are made to mix strains. A disk assay was carried out to detect the presence of inhibitory compounds that would affect the strains in the mixed culture. There were no inhibition zones when each culture was exposed to cell-free extracts of the three other strains. Thus, it was assumed that population dynamics would not be related to production of inhibitory compounds such as bacteriocins.

Effect of incubation temperature on bacterial populations in the starters

Temperature has a strong influence on bacterial population dynamics [23]. As an example, a temperature of 27°C was recommended by Hugenholtz [16] for *Le. cremoris* mixed cultures, because at this temperature the differences in growth rates between strains are minimal. Therefore, the effect of incubation temperature was the first to be examined.

Under the experimental conditions of this study, no significant effect of temperature on "total population" was recorded. But this is due to its lack of influence on *L. lactis* T1 (Table 2), which represented the highest portion of the starters (Figure 1). However, temperature had greater effects on the other strains, and the greatest influence was observed with populations of *La. rhamnosus* 9595M (Figure 1). The lactobacilli reached significantly higher populations when the starters were produced at 32°C (Table 2). This resulted in systematically higher *La. rhamnosus* 9595M contents (%) in starters produced at 32°C, compared to the cultures prepared at 22°C (Table 3). Of the four cultures used in this study, *La. rhamnosus* 9595M was the strain most stimulated by increasing the incubation temperature from 20 to 32°C (Figure 2). This at least partially explains the benefits of incubating the lactobacilli at 32°C.

Leuconostoc have shorter generation times at 22°C than at 32°C [9]. Thus it was expected that starters prepared at 22°C would have higher *Le. cremoris* CAF-500 counts than at 32°C. A tendency to obtain higher *Leuconostoc* populations at 22°C was indeed observed (Figure 1), but statistics reveal that the effect of temperature was not quite significant with *Le. cremoris* CAF-500 ($P=0.091$; Table 1). This could be related to our strain, which showed slower growth between 20 and 24°C than between 28 and

Table 2 Analysis of orthogonal contrasts on bacterial populations obtained in commercial media at the end of the fermentation. Values are the probability (P; $\alpha=0.05$) that there is no significant effect of the source of variation

Source of variation	*La. rhamnosus* 9595M	*Le. cremoris* CAF-500	*L. lactis* T1	*Le. cremoris* T2	Total
Temperature 22°C vs. 32°C	$P=0.005$	$P=0.091$	$P=0.298$	$P=0.058$	$P=0.158$
C1[a]: Milk vs. three others	$P=0.763$	$P<0.001$	$P<0.001$	$P<0.001$	$P=0.231$
C2[b]: M3 vs. (M1 and M2)	$P=0.001$	$P<0.001$	$P<0.001$	$P=0.023$	$P<0.001$
C3[c]: M1 vs. M2	$P=0.469$	$P=0.017$	$P=0.001$	$P<0.001$	$P=0.042$
Temperature×C1	$P=0.348$	$P=0.850$	$P<0.001$	$P<0.001$	$P=0.098$
Temperature×C2	$P=0.716$	$P=0.031$	$P=0.039$	$P<0.001$	$P=0.203$
Temperature×C3	$P=0.997$	$P=0.741$	$P=0.006$	$P<0.001$	$P=0.166$

[a]C1: contrast between skim milk and the three commercial media.
[b]C2: contrast between M3 (Marlac) medium and the two other commercial media (M1=MB Complete and M2=Thermolac).
[c]C3: contrast between M1 (MB complete) and M2 (Thermolac).

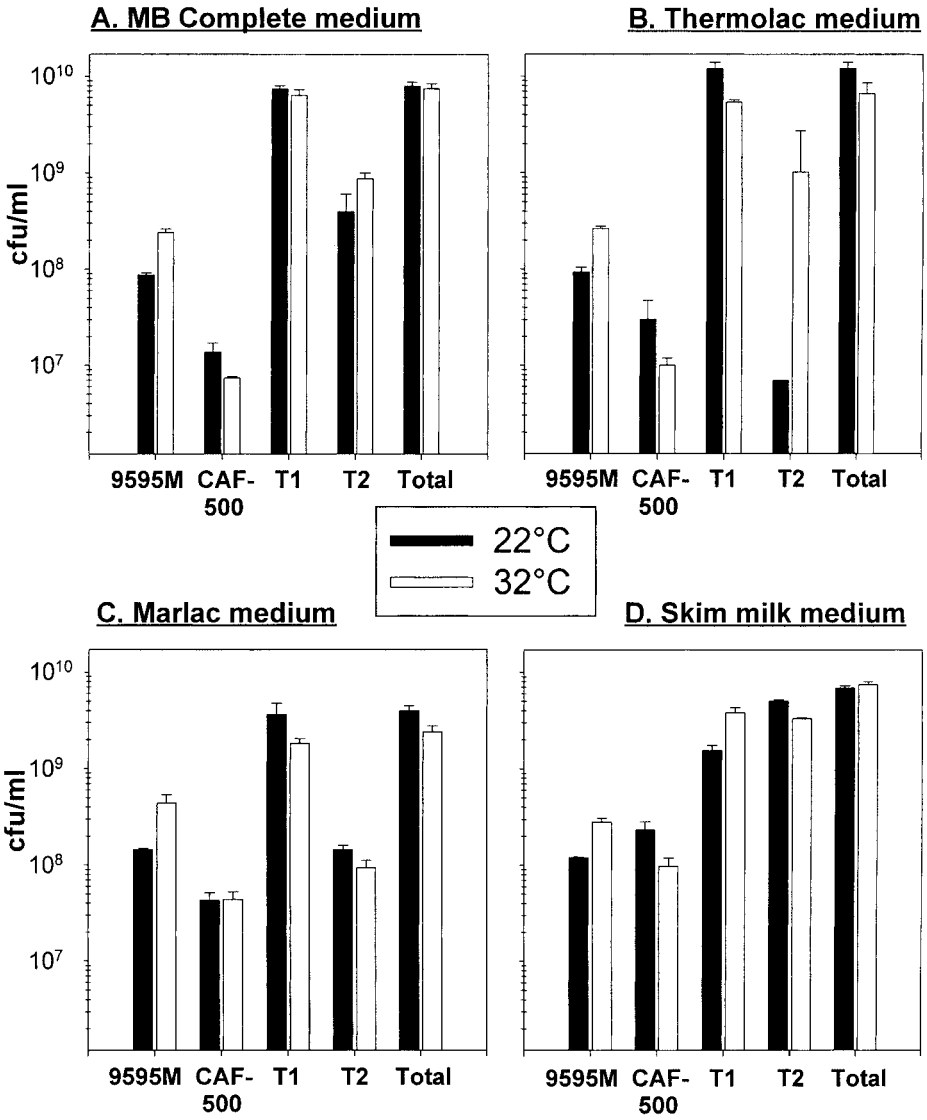

Figure 1 Effect of growth medium and incubation temperature on the post-incubation population of a mixed starter composed of *La. rhamnosus* 9595M, *Le. cremoris* CAF-500, *L. lactis* subsp. *lactis* T1 and *L. lactis* subsp. *cremoris* T2. Each culture was inoculated in the medium at 1×10^7 cfu/ml.

32°C (Figure 2). The limited effect of temperature on *Le. cremoris* CAF-500 could also be related to the presence of the lactococci. *Le. mesenteroides* does not reach as high populations in mixed cultures with *L. lactis*, as when they are grown as pure cultures [1]. This is related to the slower growth rates of *Leuconostoc* [1]. Therefore it could be argued that the beneficial effect of incubating at 22°C for *Le. cremoris* CAF-500 could have been reduced by the presence of lactococci.

Table 3 Content (%) of each strain constituent the different fresh mixed starter at the end of the fermentation

Media	Temperature (°C)	*La. rhamnosus* 9595M (%)	*Le. cremoris* CAF-500 (%)	*L. lactis* T1 (%)	*Le. cremoris* T2 (%)	Total (%)
MB Complete	22	1.1	0.2	93.7	5.0	100
	32	3.2	0.1	85.0	11.7	100
Thermolac	22	0.8	0.3	98.8	0.1	100
	32	3.9	0.2	80.6	15.3	100
Marlac	22	3.6	1.1	91.7	3.6	100
	32	18.2	1.8	76.1	3.9	100
Skim milk	22	1.7	3.4	22.4	72.5	100
	32	3.8	1.3	50.7	44.2	100

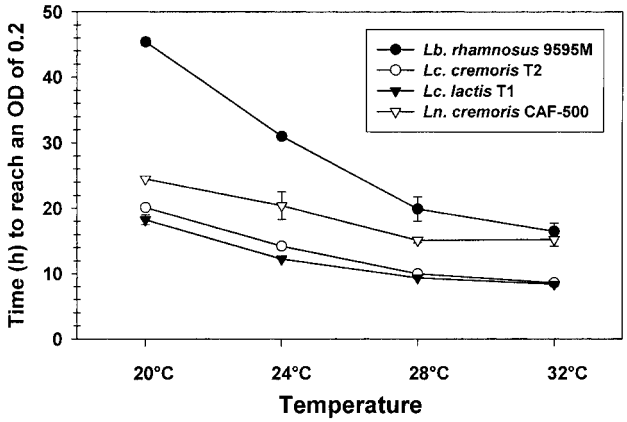

Figure 2 Effects of temperature on the growth rate of *La. rhamnosus* 9595M, *Le. cremoris* CAF-500, *L. lactis* subsp. *lactis* T1 and *L. lactis* subsp. *cremoris* T2. Data are the times required for each culture to reach an OD of 0.2 in an automated spectrophotometry unit.

The picture with the lactococci was more complex. There was a significant ($P<0.001$) interaction between temperature and growth medium for both strains. Thus, *Le. cremoris* T2 reached higher populations at 32°C if grown in MB complete and Thermolac, whereas in Marlac and skim milk, viable counts were higher at 22°C. It is noteworthy that the temperature–medium interaction (Table 2) occurred between high-phosphate and low-phosphate media (Table 1), which points to the potential involvement of phosphates in this occurrence. Other components of the media also seem to influence the temperature–medium interaction. As an example, temperature affects the growth of lactococci in milk differently than in the three commercial media (Table 2). In addition to the buffering agents, the MB complete, Thermolac and Marlac media differ from milk by the presence of whey and yeast extracts. It remains to be determined, however, in which fashion medium composition differentially affects the growth of the lactococci at 22 or 32°C.

Therefore, incubation temperature could be used to modify the composition of the starters in commercial media. As Table 2 shows, the starter contents in lactobacilli were proportionally higher if they are produced at 32°C, whereas the opposite tends to be the case with *Leuconostoc*.

Effect of growth on lactococci populations in the starters

The growth medium influences population dynamics [3,31]. This was also the case in this study, where populations of all strains were significantly ($P<0.01$) influenced by the growth medium. The total populations were highest in the media used under external pH control (Figure 1), which is typical when comparing with media used without external pH control [3].

The orthogonal contrast analyses were carried out between groups of media to detect some trends or global effects. The first comparison (C1, Table 2) was between milk and the whey-based media. The second (C2, Table 2) was between phosphated pH-control media and the medium buffered with carbohydrates and used without external pH control. The third (C3, Table 2) was between the two phosphated media used under external pH control.

The lactococci reached populations close to 10^{10} cfu/ml when grown under pH control, which is consistent with previous data [3]. A significant difference in lactococci populations was found between Marlac medium (internal pH control) and the two other commercial media (external pH control) (C2, Table 2). Marlac had less lactose than the other media (Table 1), presumably to prevent a pH drop under 5.0. This limits growth at and least partially explains the lower counts. The lower lactococci counts in Marlac explain the results of contrasts between milk and the three commercial media (C1; Table 2) and between Marlac and MB Complete/Thermolac media. There was also a significant difference for both lactococci between counts obtained in MB Complete and Thermolac (C3; Table 2), but no clear trend was noted because there was an interaction between the Thermolac–MB Complete contrast and temperature (Table 2). The interactions between temperature and the media, in contrast analyses between media, seem to be limited to the lactococci (Table 2).

Although the lactococci represented 50% of the total population at the beginning of the incubation, they composed between 80% and 99% of the total population following fermentation. In the phosphated media, *L. lactis* T1 dominated *Le. cremoris* T2, which confirms previous data [3]. It was postulated that *L. lactis* T1 could be favored over *Le. cremoris* T2 in commercial media because *Le. cremoris* is generally considered as more sensitive to phosphates than *L. lactis*. However, this may not be the case with these strains. The growth of both cultures was unaffected by the presence of up to 1% phosphates in a synthetic medium (Figure 3). Therefore, the reasons for domination of *L. lactis* T1 over *Le. cremoris* T2 in commercial media warrant further examination. Cheesemakers using starters grown on commercial media could find that the increased proportions of *L. lactis* could translate into faster acidification rates, but could also increase dangers of starter failure due to bacteriophage attack.

Effect of growth medium on Leuconostoc populations in the starters

The four cultures were each inoculated at 1.0×10^7 cfu/ml. Populations after fermentation suggest that there was no growth of *Leuconostoc* CAF 500 in phosphated media (Figure 1). The best medium for growth of *Leuconostoc* was milk, but populations of only 10^8 cfu/ml were reached. Although low, these values are typical of those found in milk starters [1].

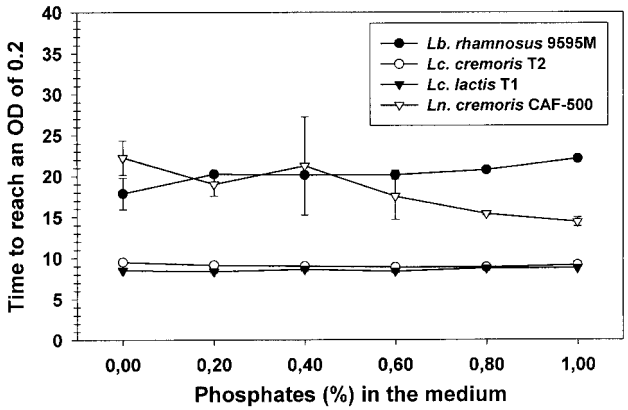

Figure 3 Effect of the phosphate content of the growth medium on the growth rate of *La. rhamnosus* 9595M, *Le. cremoris*. CAF-500, *L. lactis* subsp. *lactis* T1 and *L. lactis* subsp. *cremoris* T2. Data are the times required for each culture to reach an OD of 0.2 in an automated spectrophotometry unit.

The leuconostocs do not have high proteolytic activities [11] and cannot attain high populations in milk, which does not have significant amounts of free amino acids [1]. It was hoped that the presence of yeast extract in the commercial media would be favorable to development of the leuconostocs. Indeed, addition of yeast extracts to milk prevents domination of the lactococci, and that *Leuconostoc* populations in mixed starters reach the same level as in pure cultures [1]. In the commercial media used in this study, the benefits of yeast extract for growth were not apparent because the lowest *Leuconostoc* starter contents were observed in commercial media used under pH control. It could be argued that yeast extract also favored growth of lactococci. Because *Leuconostoc* has slower growth rates than *Lactococcus* [1,10], the latter would tend to gradually increase its proportion in a situation where there is no selective advantage for the leuconostocs. In addition to being slower, the leuconostocs do not seem to be as favored by fermentation under pH control as the lactococci [2]. Our results support this, but it is unexpected that fermentation under pH control does not seem to benefit *Leuconostoc*. Indeed, pH control requires medium agitation, which oxygenates the medium. Some lactococci are inhibited by aeration [33], which occurs during agitation, but *Leuconostoc* are reportedly favored by oxygen [32].

Leuconostoc are more sensitive to salt than the lactococci [12] and the high phosphate contents of MB Complete and Thermolac might act as inhibitors. Therefore, the sensitivity of *Le. cremoris* CAF 500 to phosphates was examined in a defined medium. This strain's growth was not inhibited by phosphate content up to 1% (Figure 3). On the contrary, phosphates promoted growth suggesting that the low *Leuconostoc* populations of the phosphated media are not related to phosphate content *per se*. One possibility of the inhibitory effect of phosphates is their binding of mineral growth factors, such as Mn, during pasteurization of the medium. This remains to be examined.

Citrate and Mn are promoters of the growth of *Leuconostoc* [1,7,8,35]. Citrates are generally not added in starter media used for the growth of cheddar or mozzarella starters, because they could be at the origin of gas production. It is understandable that the citrate content of the commercial media is lower than that found in the milk (Table 1), and this could explain the higher *Leuconostoc* content of the starters produced in the milk medium (Table 3). Citrate was used in all media, and could not be detected at the end of the fermentation. Thus, citrate was a substrate and was not in excess. Additionally, all media were poor in Mn (Table 1). Bellengier *et al* [1] reported a strong *Leuconostoc* growth stimulation in milk following the addition of Mg and Mn, but none of the media were supplemented with these minerals.

Therefore, cheesemakers who wish to use MB Complete and Thermolac for production of *Leuconostoc*-containing mixed starters should expect lower contents of *Leuconostoc* than when they are milk-grown in a traditional fashion. Indeed, *Leuconostoc* reportedly constitute 1% to 20% of the population in mixed cultures [2,39], and the values obtained in this study for MB Complete and Thermolac were well under 1% (Table 3).

Effect of growth medium on Lactobacillus populations in the starters

The growth of *La. rhamnosus* 9595M was mainly influenced by temperature, but the growth media also had a significant (P=0.012) effect. The contrast analysis between Marlac and the phosphated media was also significant (C2; Table 2). The lactobacilli did not grow well in MB Complete, which has the highest phosphate content. Lactobacilli do not have a high tolerance for phosphates [41], and the 9595M strain showed this sensitivity (Figure 3). Populations in Marlac were proportionally the highest in lactobacilli. This could be the result of the stimulatory action of CO_2 on the lactobacilli [13], or due to the fact that Marlac was used without pH control. The lactobacilli generally tolerate acid environments better than the lactococci and *Leuconostoc.* Indeed, the growth of *La. rhamnosus* 9595M was less affected by pH values between 4.7 and 6.2 than were *Le. cremoris* T2 or *Le. cremoris* CAF 500 (Figure 4). This study suggests that Marlac is worth considering as a starter medium for production of lactobacilli-containing cultures but total populations were lower in Marlac (Figure 1) than with the other commercial media.

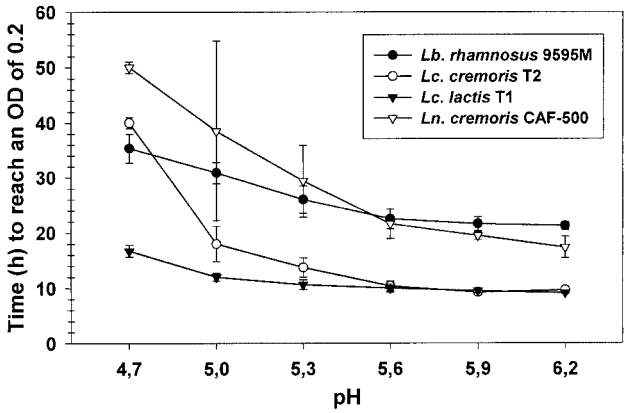

Figure 4 Effect of pH of the growth medium on the growth rate of *La. rhamnosus* 9595M, *Le. cremoris* CAF-500, *L. lactis* subsp. *lactis* T1 and *L. lactis* subsp. *cremoris* T2. Data are the times required for each culture to reach an OD of 0.2 in an automated spectrophotometry unit.

Effect of starter production temperature on SAA

For the fermented milks industry, the acidifying activity is the most important feature of the culture. This property will be influenced by bacterial population in the starter, as well as by the SAA of the cells.

Fermentation time is an important parameter in industry operations; thus, the test carried out to evaluate SAA determined the number of cells necessary to inoculate milk and reach a pH of 5.0 in a given time. The higher the numbers, the lower the SAA. The methodology used to conduct the SAA tests enabled the subsequent calculation of the inoculation level required to carry out the fermentation to pH 5.0 in 5.5 h (32°C) or 7 h (25°C). The calculations were done according to the formula: Inoculation rate (%) = (SAA÷population in starter) × 100.

When the SAA test was conducted at 25°C, the starters prepared at 32°C were more active than those cultured at 22°C (Table 4). This did not automatically translate into lower inoculation levels, because starter populations were sometimes higher when they were prepared at 22°C.

The reason for the lower SAA at 25°C of the starters produced at 22°C is not clear. The higher content of nonacidifying *Leuconostoc* cells in the 22°C-grown starter could be responsible for this effect. However, *Leuconostoc* cells never represented more than 4% of the starter population, which excludes any significant

Table 4 Results of the SAAs are the populations (in log scale) required to inoculate milk to obtain a pH of 5.0 after a given incubation time (5.5 h at 32°C and 7 h at 25°C)

Media	Temperature (°C)	Log_{10} of counts at			
		SAA 25°C	Inoculation level 25°C	SAA 32°C	Inoculation level 32°C
MB Complete	22	8.43 (0.09)	3.48 (0.63)	8.08 (0.06)	1.52 (0.09)
	32	8.21 (0.07)	2.13 (0.09)	8.00 (0.07)	1.34 (0.07)
Thermolac	22	8.27 (0.16)	1.58 (0.28)	7.89 (0.17)	0.67 (0.14)
	32	8.16 (0.09)	1.97 (0.06)	7.99 (0.06)	1.34 (0.16)
Marlac	22	7.97 (0.09)	2.39 (0.23)	7.63 (0.10)	1.09 (0.15)
	32	7.85 (0.07)	3.06 (0.09)	7.61 (0.08)	1.78 (0.08)
Skim milk	22	8.23 (0.06)	2.51 (0.26)	7.95 (0.08)	1.33 (0.16)
	32	8.18 (0.05)	2.01 (0.16)	7.94 (0.04)	1.17 (0.09)

Taking into account the total populations in the starter, the inoculation levels (% v/v) required to achieve the acidification rate were calculated. Values in parentheses are standard errors of means.

effect of this species in the acidification tests. The cultures grown at 32°C had higher contents of lactobacilli, which suggests that SAA could be affected. If this were the case, however, the SAA carried out at 32°C should also reflect this contribution of the lactobacilli, which was not observed (Table 4). Further work is needed to determine if changes in SAA of mixed cultures due to growth temperature are more related to changes in ratios or to effects of temperature *per se*.

Effect of growth medium on SAA of the starters

There was a significant ($P=0.02$) effect of growth medium on the SAA of the cultures on activity tests conducted at both 25 and 32°C. Cultures grown on Marlac had the highest SAA value, whereas those grown on MB Complete had the lowest. Starter production on Marlac was carried out without pH control. The experimental plan designed for these assays did not enable us to determine if the higher SAA of the Marlac-grown cells were related to medium composition or the absence of external pH control. The SAA of cells grown under external pH in commercial media have lower levels than those allowed to acidify the medium [3].

Statistical analyses also show that the calculated inoculation levels were significantly influenced by media. On the average, lower inoculation levels would be required with starters produced on Thermolac (Table 4). Although Thermolac-grown cells had inferior SAA than Marlac-grown cells, the higher populations obtained in Thermolac more than compensated. It is doubtful that population ratios were responsible for these results. The strain ratios in Thermolac medium were similar to those in the MB complete (Table 2).

Secondary metabolites

A second series of fermentations was carried out to determine the activities of the starters. In this instance, the fermented milks were analysed for the presence of secondary metabolites (Table 5). Diacetyl and acetaldehyde in particular contribute to the flavour of the fermented dairy products [19,20]. Thus, the test was designed to provide insight on the aroma-producing activities of the starters.

Overall, more ethanol and diacetyl were detected in the fermented milks when the starters used to inoculate them were produced at 22°C (Table 5). It is well established that *Leuconostoc* produces these metabolites [8]. Since the *Leuconostoc* content of

Table 5 Aroma compounds in milks fermented at 25°C or 32°C until a pH of 4.8 was reached, and which were inoculated with the mixed starters produced at either 22 or 32°C

Media	Temperature of production of the starter (°C)	Milk fermentation temperature	Acetaldehyde (ppm)	Acetone (ppm)	Ethanol (ppm)	Diacetyl (ppm)
MB Complete	22	25	0.43	0.59	41.6	1.42
		32	1.04	0.58	35.2	0.59
	32	25	2.32	0.61	20.5	0.42
		32	3.16	0.61	24.1	0.60
Thermolac	22	25	0.57	0.59	46.4	1.32
		32	1.06	0.57	40.7	0.87
	32	25	2.05	0.69	21.5	1.10
		32	3.37	0.59	22.3	0.94
Marlac	22	25	0.51	0.55	54.7	1.01
		32	1.09	0.54	42.7	0.34
	32	25	0.67	0.61	29.3	1.07
		32	1.96	0.61	27.6	0.78
Skim milk	22	25	0.51	0.59	43.2	1.09
		32	0.86	0.69	32.1	0.96
	32	25	0.56	0.60	33.8	0.31
		32	1.60	0.61	31.1	1.09

the starters tended to be highest at 22°C, the *Leuconostoc* population of the starter could have a noticeable effect. *Leuconostoc* are also reputed for assimilating acetaldehyde [20,22]; the fact that acetaldehyde content of the fermented milks was lowest when starters were grown at 22°C adds to the potential effect of *Leuconostoc* content in the starters.

The lower content of acetaldehyde in products inoculated with starters produced at 22°C, compared to those obtained from starters grown at 32°C, could also be related to the lactobacilli content. Several lactobacilli produce acetaldehyde [14]. Thus, the combined effects of lactobacilli and leuconostoc populations in the starters could account for acetaldehyde contents of the fermen-ted products.

The secondary metabolite data suggest that the shifts in diacetyl, ethanol and acetaldehyde contents of fermented milks could be related to *Leuconostoc* and *Lactobacillus* in the starter cultures. To support these assumptions, metabolites were analysed in the pure cultures. Both lactococci produced ethanol (19 to 27 ppm) and acetaldehyde (0.7 to 2.1 ppm). Furthermore, the lactobacilli produced 1.4 ppm of diacetyl in milk. Therefore the lactococci ratios in the starters could also contribute to production of the secondary metabolites.

Conclusion

It is possible to produce mixed starters of *Lactococcus, Leuconostoc* and *Lactobacillus* in commercial media. However, the media do not seem designed to promote the growth of *Leuconostoc,* and the lactococci became the dominant species. *Leuconostoc* and *Lactobacillus* starter populations obtained in the commercial media or under pH control are sufficiently high to influence subsequent production of secondary metabolites. Nevertheless, if production managers require higher counts in the nonlactococci fraction of the starter, incubation temperature can be used to promote either lactobacilli or leuconostoc populations. Attempts are under way to determine if modifications to the media or of the pH-control parameters can provide further means of adjusting the population ratios of the starters.

Acknowledgements

The authors thank the Natural Sciences and Engineering Research Council of Canada (Research Partnership Program-Research Network on Lactic Acid Bacteria), Agriculture and Agri-Food Canada, Novalait Inc., Dairy Farmers of Canada and Institut Rosell Inc. for financial support. Gratitude is also expressed to Nancy Gardner, Pierre Ward and Lorraine Charlebois for scientific and technical support.

References

1 Bellengier P, J Richard and C Foucaud. 1997. Associative growth of *Lactococcus lactis* and *Leuconostoc mesenteroides* strains in milk. *J Dairy Sci* 80: 1520–1527.
2 Boquien CY, G Corrieu and MJ Desmazeaud. 1988. Effect of fermentation conditions on growth of *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091 in pure and mixed cultures. *Appl Environ Microbiol* 54 (10): 2527–2531.
3 Champagne CP, M Piette and D St-Gelais. 1995. Characteristics of lactococci cultures produced on commercial media. *J Ind Microbiol* 15: 472–479.
4 Champagne CP, D St-Gelais and P Audet. 1996. Starters produced on whey protein concentrates. *Milchwissenschaft* 51 (10): 561–564.
5 Champagne CP, H Gaudreau, J Conway, N Chartier and E Fonchy. 1999. Evaluation of yeast extracts as growth media supplements of lactococci and lactobacilli using automated spectrophotometry. *J Gen Appl Microbiol* 45: 17–21.
6 Cochran WG and GM Cox. 1957. Experimental Designs. Wiley, New York.
7 Cogan TM. 1987. Co-metabolism of citrate and glucose by *Leuconostoc* spp.: effects on growth, substrates and products. *J Appl Bacteriol* 63 (6): 551–558.
8 Cogan TM and KN Jordan. 1994. Metabolism of *Leuconostoc* bacteria. *J Dairy Sci* 77: 2704–2717.
9 Cooper RK and EB Collins. 1978. Influences of temperature on growth of *Leuconostoc cremoris*. *J Dairy Sci* 61: 1085–1088.
10 Demirci Y and D Hemme. 1994. Growth of *Leuconostoc mesenteroides* strains isolated from French raw milk cheeses in reference milk. *Milchwissenschaft* 49 (9): 483–485.
11 Demirci Y and D Hemme. 1995. Acidification test based on the use of biomass for screening of *Leuconostoc*. Application to *Ln. mesenteroides* strains isolated from French raw milk cheeses. *J Dairy Res* 62: 521–527.
12 Devoyod JJ and F Poullain. 1998. Les Leuconostocs. Propriétés: leur rôle en technologie laitière. *Lait* 68 (3): 249–280.
13 Driessen FM, F Kingma and J Stadhouders. 1982. Evidence that *Lactobacillus bulgaricus* in yogurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. *Neth Milk Dairy J* 36 (2): 135–144.
14 Gonzales S, VM De Ambrosini, MM De Nadra, AP De Ruiz Holgado and G Oliver. 1994. Acetaldehyde production by starins used as probiotics in fermented milk. *J Food Prot* 57 (5): 436–440.
15 Harvey RJ. 1965. Damage to *Streptococcus lactis* resulting from growth at low pH. *J Bacteriol* 90: 1330.
16 Hugenholtz J. 1986. Population dynamics of mixed starter cultures. *Neth Milk Dairy J* 40: 129–140.
17 Jonas LL, CA Ernstrom and GH Richardson. 1977. Major savings realized in the production of bulk lactic culture with whey-based phage inhibitory medium provided with pH-control. *Cult Dairy Prod J* 12 (2): 12–14.
18 Klaenhammer TR. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70 (3): 337–349.
19 Libudzisz Z and J Pajek-Bilska. 1980. Characteristics of mixed-strain starters of *Streptococus cremoris* and *Leuconostoc cremoris*. *Acta Aliment Pol* 6 (4): 259–267.
20 Libudzisz Z, A Piatkiewicz, H Oberman and M Lubnauer. 1993. Heterogeneity of the physiological activity of *Lactococcus* and *Leuconostoc* sp. strains. *Acta Microbiol Pol* 42 (2): 181–192.
21 Littell RC, GA Milliken, WW Stroup and RD Wolfinger. 1996. SAS System for Mixed Models. SAS Institute, Cary, NC. 633 pp.
22 Liu SQ, RV Asmundson, R Holland and VL Crow. 1997. Acetaldehyde metabolism by *Leuconostoc mesenteroides* subsp. *cremoris* under stress conditions. *Int Dairy J* 7: 175–183.
23 Lucas S and J Reyrolle. 1989. Étude d'un lot de ferments lactiques mésophiles. Équilibre des flores au cours de la première étape de la fabrication de lavain. *Lait* 69 (2): 121–130.
24 Mayeux JV, WE Sandine and PR Elliker. 1962. A selective medium for detecting *Leuconostoc* organisms in mixed-strain starter. *J Dairy Sci* 45: 655–656.
25 McDonald IJ, B Reiter and PL Rogers. 1973. Growth of *Streptococcus cremoris* and *Streptococcus lactis* in chemostat. Production of cells and survival of bacteria during frozen storage. *Can J Microbiol* 19: 1285.
26 Mermelstein NH. 1982. Advanced bulk starter medium improves fermentation processes. *Food Technol* 36 (8): 69–76.
27 Montgomery DC. 1991. The Design and Analysis of Experiments. 3rd ed. Wiley, New York.
28 Murti TW, C Bouillanne, M Landon and MJ Desmazeaud. 1993. Bacterial growth and volatile compounds in yoghurt-type products from soymilk containing *Bifidobacterium* ssp. *J Food Sci* 58 (1): 153–157.
29 Parrish RS and Go Ware. 1989. Analysis of a split-plot experiment using mixed model equations: a forest site preparation study. Applications of mixed models in agriculture and related disciplines,

Southern co-operative series bulletin No. 343. Louisiana Agricultural Staion, Baton Rouge, LA, pp. 155–173.
30 Perdigon G, S Alvarez, M Rachid, G Aguero and N Gobbato. 1995. Immune system stimulation by probiotics. *J Dairy Sci* 78: 1597–1606.
31 Petterson HE. 1975. Studies on batch production of bacterial concentrates from mixed species lactic starters. *Appl Microbiol* 29 (2): 133–140.
32 Plihon F, P Taillandier and P Strehaiano. 1995. Oxygen effect on batch cultures of *Leuconostoc mesenteroides*: relationship between oxygen uptake, growth and end-products. *Appl Microbiol Biotechnol* 43: 117–122.
33 Shekar S and GS Bhat. 1983. Influence of dissolved oxygen on acid production in buffalo milk by lactic cultures. *J Food Prot* 46 (4): 321–324.
34 Stanley G. 1977. The manufacture of starters by batch fermentation and centrifugation to produce concentrates. *J Soc Dairy Technol* 31 (1): 36–39.
35 Starrenburg MJC and J hugenholtz. 1991. Citrate fermentation *Lactococcus* and *Leuconostoc* spp. *Appl Environ Microbiol* 57 (12): 3535–3540.
36 Thunell RK. 1988. pH-controlled starter: a decade reviewed. *Cult Dairy Prod J* 23: 10–16.
37 Turner N, WE Sandine, PR Elliker and EA Day. 1963. Use of tetrazolium dyes in an agar medium for differentiation of *Streptococus lactis* and *Streptococcus cremoris*. *J Dairy Sci* 46: 380–385.
38 Ustunol Z, CL Hicks and J O'Leary. 1986. Effect o commercial internal pH control media on cheese yield, starter culture growth, and activity. *J Dairy Sci* 69 (1): 15–21.
39 Vedamuthu ER. 1994. The dairy *Leuconostoc*: use in dairy products. *J Dairy Sci* 77 (9): 2725–2737.
40 Williams ER and AC Matheson. 1996. Experimental Design and Analysis for Use in Tree Improvement. CSIRO, Australia.
41 Wright CT and TR Klaenhammer. 1984. Phosphated milk adversely affects growth, cellular, morphology and fermentative ability of *Lactobacillus bulgaricus*. *J Dairy Sci* 67: 44–51.