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Characteristics of lactococci cultures produced in commercial media

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Strains of *Lactococcus lactis* ssp *lactis* and *L. lactis* ssp *cremoris* were propagated on milk, three commercial highly buffered media (HB media), and four commercial media designed for external pH control (EC media). With milk and HB media, fermentation was allowed to proceed until a pH of 4.9 was reached. With EC media, pH was maintained at 6.0 with 5 N NH_4OH. The cultures were analyzed for chain length, viable population, specific acidifying activity (SAA) and specific proteolytic activity (SPA). The starters were stored at 4° C for 3 days, and analyses for chain length, viable population and SAA were repeated. It was more difficult to standardize medium composition with the rehydrated commercial blends, as their titratable acidities had greater proportional variations than milk. As a rule, chain length was longer in fresh cultures than in the stored starters, and *L. lactis* spp *cremoris* cultures had longer chains than *L. lactis* ssp *lactis*. All commercial media produced starters with total populations at least as high as that obtained in milk. With the EC media, populations could be five times greater than with milk; increases were less important in HB media. The increase in population in EC and HB media was more marked with *L. lactis* ssp *lactis* than for *L. lactis* ssp *cremoris* strains. Storage at 4° C for 3 days did not significantly reduce *L. lactis* populations, but mortality (up to 70%) was observed with *L. lactis* ssp *cremoris*. The overall SAA of *L. lactis* ssp *lactis* cultures in EC media was 35% lower than milk- or HB media-grown starters, but the greater populations reached in EC media enabled a significant reduction in inoculation rate. Some statistically significant correlations were obtained between SAA and SPA (positive) as well as with chain length (negative), but the coefficients of determination were generally very low. The drop in pH during storage at 4° C was less with HB media than in milk, and was in relation to their buffering capacity.

Keywords: starters; activity; population; proteolysis

Introduction

Control of pH enables extended growth of the lactic acid bacteria [13, 20] and higher populations can be obtained [3, 30]. Although milk still is the preferred substrate for the preparation of starters at dairy plants in Europe, commercial whey or milk-based media with some form of pH control are frequently used in North American cheese plants [32]. Some formulations are highly buffered and are occasionally called internal pH control media; in this study they will be referred to as highly buffered (HB) media. When an alkali solution is added to the fermenting medium in order to maintain constant pH, the system is termed 'external pH control' (EC media). The same medium can serve in both HB and EC systems, but they are then adjusted at different solids levels.

Studies on starter production in pH-controlled media are limited to either HB [18, 34, 35] or EC media [1, 4, 22, 27–29]. In some studies, only population levels [4], acidifying activity [2, 13, 27, 29] or inoculation rates [1] are provided. In many instances, activity tests are presented with population levels [13, 18, 22, 28, 30, 31, 33], but it is difficult to establish acidifying activities for a given biomass (specific acidifying activity). To our knowledge no comprehensive study has been published on commercial media where both HB and EC media are compared for biomass production, and where specific acidifying activity is taken into account for the establishment of inoculation rates. Although there is some data on the effect of pH control on chain length of the cultures [31] and their proteolytic activity [27, 28], there is no comprehensive study on the interactions between these parameters. Such was the aim of this work.

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Materials and methods

Biological material

Lactococcus lactis ssp *lactis* (*L. lactis*) AB and AD as well as *Lactococcus lactis* ssp *cremoris* (*L. cremoris*) AC and AR were obtained from Rhône-Poulenc (Madison, WI, USA) and Sanofi (Waukesha, WI, USA). The frozen cultures were stored in liquid nitrogen. Inocula were prepared by inoculating 0.5 ml of the frozen culture in 100 ml of 11% solids 'low-heat' rehydrated skim milk powder, previously sterilized at 110° C for 10 min, and incubated at 21° C until a pH of 4.7–4.9 was reached.

Culture media

Commercial culture media were obtained from Rhône-Poulenc (Madison, WI, USA), Sanofi (Waukesha, WI, USA), Hansen's (Madison, WI, USA) and Saputo (St Hyacinthe, Quebec, Canada). Ingredients of the media are listed in Table 1. In attempting to make the study on comparable products, all media were milk-based (Table 1).

Table 1 Ingredients of the media

Ingredient	Medium						
	HB1	HB2	HB3	EC1	EC2	EC3	EC4
Skim milk	+	+	+	+	+	+	+
Whey	−	−	−	+	−	−	+
Phosphates	+	+	+	+	+	+	+
Citrates	−	−	−	−	−	−	+
Yeast extract	+	−	+	+	+	+	+
Carbohydrates[1]	+	+	+	+	+	+	+
Modified milk	−	+	−	−	−	−	−
Anti-caking agents	+	+	−	−	+	+	−

[1] Dextrose, lactose and/or maltodextrins

The media were rehydrated to the solids level recommended by the manufacturer, which ranged from 9 to 11.8% in the HB media and 7 to 7.9% in the EC media. The titratable acidities were always determined, prior to heat treatment, so as to ascertain that they fell in the recommended ranges of the manufacturers. 'Low-heat' skim milk (11% solids) served as control. All media were heated at 88° C for 40 min prior to inoculation. Two different bags of each commercial starter medium were purchased. In order to reduce the potential effect of product sample on our results, each fermentation medium was prepared by combining the powders of each bag at an equal ratio.

The milk powder used for acidifying activity tests was irradiated at 5 kGy prior to rehydration in order to lower bacterial counts. Thus, contaminants would not interfere with the specific acidifying activity determinations. Preliminary tests showed that this 5-kGy treatment did not influence the starters' growth, confirming results obtained by Chamba and Prost [5].

Fermentations

Fermentations were carried out in 7-L Chemap (α-Laval, Männendorf, Switzerland) or Bioengineering (Wald, Switzerland) units controlled by a Bio-Génie (Quebec City, Quebec, Canada) software system. Each medium was inoculated (1% vol/vol) with a fresh milk-grown culture, and incubated at 24° C. With milk and HB media, the fermentation was allowed to proceed until a pH of 4.95 was reached. Cooling of the starter was immediately started at pH 4.95, irrespective of the fermentation time, so that the final pH of the cooled starter was 4.90. With EC media, pH was maintained at 6.0 with periodic additions of 5 N NH_4OH. Agitation was maintained at 50 rpm throughout the fermentations. So as to obtain starters having similar physiological states, preliminary fermentations were conducted with the four EC media in order to determine the appropriate fermentation times. The starters were cooled when it was estimated that neutralization had stopped or had greatly reduced. Samples of fresh cultures were taken once they had been cooled to 4° C, and a portion was placed in bottles for storage at 4° C during 3 days.

Analyses

Viable counts were obtained on Petrifilm (3M, St Paul, MN, USA) since preliminary studies showed that readings were as high as with M-17 agar [8].

Microscopic counts were performed with a field method [25] using methylene blue. Starters were all diluted 1 : 10 in 1% rehydrated skim milk, because milk-containing samples showed better adherence to the slides than did the formulated media. Since lactic starters produce chains of various lengths, both chain counts and average chain length (number of cells per chain) were determined. Results of microscopic examination of the samples will thus be referred to as direct microscopic chain counts (DMCC). The DMCC could be compared to viable counts to estimate viability of the culture. The total bacterial population was estimated by multiplying the viable count by the average chain length.

Specific acidifying activities (SAA) were determined by inoculating various levels of the starter (typically from 0.1 to 5% vol/vol) in 9% rehydrated 'low temp' irradiated skim milk and incubating the cultures for 5 h under the following time-temperature profile: 1 h at 32° C, gradual increase to 38° C during 30 min, gradual decrease to 37° C during 30 min, 2 h 10 min at 37° C, and a gradual decrease to 32° C during 40 min. This simulated a Cheddar making process. A programmable water bath (Lauda Model RK20, Königshofen, Germany) served this purpose. The irradiated milk powder used for the test was rehydrated and incubated at 30° C for 30 min [12], but not sterilized so as to more closely reflect cheese making conditions where milk is only pasteurized. Following the 5-h incubation, pH was determined in the various samples. Once the viable counts were obtained, it was possible to plot the 'inoculation level – 5 h pH' curve and determine the starter population needed to be inoculated so that a pH of 5.2 could be reached in 5 h (Figure 1). Thus, specific activity tests will be reported in this fashion; it was considered that the higher the readings, the lower the specific acidifying activities.

Proteolytic activities were carried out as described in Champagne *et al* [6] which was based on the spectrophotometric method of Church *et al* [10]. The tetracycline-containing milk was inoculated at a level of 10^7 CFU ml^{-1} (as determined by DMCC) incubated 24 h at 30° C and analyzed for the hydrolysis products. The calibration curve was obtained with glutamic acid as the standard. Hence, results of the specific proteolysis activity (SPA) are expressed as 'equivalent glutamic acid' and are based on the activity of 10^7 cells over 24 h at 30° C.

Statistical analyses were conducted using SAS (Cary,

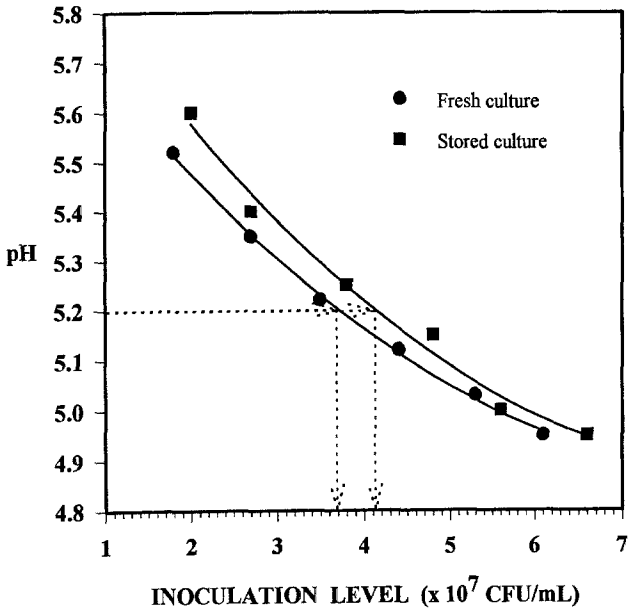

Figure 1 Typical acidification curve used to evaluate the specific acidifying activity of lactococci. pH reached after a 5-h incubation, under a Cheddar-making temperature profile, were plotted against corresponding inoculation levels. Such curves were used to determine the exact inoculation level required to acidify milk at pH 5.2 in 5 h

NC, USA) software. Multiple comparisons were performed on least squares means (series of *t*-tests). To stabilize variance, analyses on the data of 'total populations' and 'SAA' were carried out on their $\log_{10}$ transformations. At least three independent fermentations were conducted with each strain and medium.

Results and discussion

Medium preparation

Manufacturers recommend that the titratable acidity (TA) of the medium be determined, prior to pasteurization, in order to ascertain if rehydration was carried out to obtain the recommended solids level. Although the powders were taken from two separate bags in the preparation of each medium, and precision balances were used in weighing the powders, substantial variations were observed in TA (Table 2). On a relative scale, the standard error on the means of the commercial media's TA were two to three times greater than with milk. This was common for all manufacturers. It was not determined if this variation was related to uneven distribution of ingredients in the bag or difficulties in obtaining a representative sample. These results suggest that dairy plants should expect greater variations in medium composition with commercial blends than with milk, particularly if, as was the situation in this study, only a portion of a bag is used. All commercial media showed similar standard deviations, on a relative scale. This could influence starter properties [7]. As an example, phosphate content will influence phage protection properties [1] and morphological properties of lactobacilli [37].

Table 2 Variations in titratable acidities of the media prior to heat treatment

Medium[1]	Titratable acidity (°D)	Standard error of mean
Milk	19	0.3
HB1	71	2.9
HB2	87	2.4
HB3	79	3.6
EC1	52	1.5
EC2	55	1.6
EC3	53	1.3
EC4	48	1.5

[1] HB = highly buffered media, and EC = media with external pH control

Chain length

As a rule, chain length (number of cells per chain) was longer in *L. cremoris* than *L. lactis* cultures (Table 3). Moreover, fresh cultures had higher chain lengths than stored starters, especially with *L. cremoris*. Although in some instances there were significant effects of media on chain length of fresh cultures, this effect varied between strains. A somewhat different picture emerged with the stored cultures. With three strains (AB, AD, AM), chain length of the 3-day-old milk cultures was significantly higher than those produced in three HB media. There thus appeared to be greater chain breakage during storage with the highly buffered media than with milk-grown cultures.

Uneven chain length modification as a function of the medium has two practical implications. First, if a deliberate step is not carried out to break chains during analysis for viable plate counts (glass beads or other), an erroneous increase in viable population will be noted during storage. Secondly, chain length may reflect activity in some instances; the relationship between chain length and specific acidifying activity will be discussed further.

Direct microscopic chain counts

The DMCC were performed with two objectives. The first was to evaluate the mortality rate (chains were counted in DMCC so as to parallel CFU). The second was to note the average chain length in order to calculate the total population of the sample (CFU × chain length).

Mortality occurred during storage (Table 4). The CFU/DMCC ratios after three days of storage were slightly higher in the cultures prepared under external pH control than those stored in milk or the HB media. The HB media were not better than milk in preventing cell mortality. The claim by media suppliers that EC media offer greater protection during storage than conventional milk-grown starters [34, 35] obtains some support with these results. The pH of the EC-grown starters at the beginning of storage was 6.0, while that of the milk and HB-grown cultures were at 4.9. Although the detrimental effect on cell viability and activity of exposure to pH values lower than 5.0 is well established in milk [15, 26] this is the first report of EC-HB media comparisons. It is also noteworthy that the detrimental effect of storage on viability was mostly related to mortality in *L. cremoris* cultures (Table 4).

With *L. lactis*, there were no important differences between DMCC and plate counts for fresh or stored cultures (Table 4). No chain breakage thus occurred during shaking of the dilution bottles required for plate count analyses. This leads us to believe that the method used to

Table 3 Effect of growth medium, and storage (three days at 4° C) on chain length of *Lactococcus lactis* ssp *lactis* AB and AD, as well as *Lactococcus lactis* spp *cremoris* AM and AR

Medium[1]	Fresh culture				Stored culture			
	L. lactis		*L. cremoris*		*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR	AB	AD	AM	AR
Milk	1.7 a	1.8 a	4.1 ab	2.9 ab	1.6 a	1.8 a	3.3 a	2.2 a
HB1	1.5 ab	1.7 a	5.1 b	1.9 a	1.4 ab	1.6 a	3.2 ac	1.8 a
HB2	1.5 ab	1.7 a	2.8 ab	3.0 ab	1.5 ab	1.7 a	1.8 b	2.4 a
HB3	1.5 ab	1.7 a	2.7 ab	6.2 b	1.4 ab	1.6 a	1.9 bc	4.8 ab
EC1	1.6 ab	1.7 a	3.5 ab	4.8 ab	1.6 ab	1.6 a	2.8 abc	3.4 ab
EC2	1.5 ab	1.8 a	5.5 b	6.0 b	1.3 ab	1.6 a	3.5 a	6.0 b
EC3	1.4 ab	1.6 a	2.0 a	4.7 ab	1.2 b	1.7 a	1.3 b	4.5 ab
EC4	1.3 b	1.7 a	1.9 a	2.4 a	1.4 ab	1.6 a	1.3 b	2.2 a

[1] HB = highly buffered media, and EC = media with external pH control
abc: values in a given column that are followed by the same letter are not significantly different ($P>0.05$)

Table 4 Comparison between direct microscopic chain counts (DMCC) and viable counts (CFU) of various *Lactococcus lactis* ssp *lactis* and *Lactococcus lactis* ssp *cremoris* starters. Results are expressed as CFU/DMCC ratios: a value of 1.0 signifies that counts are identical

Group	Fresh culture	Stored culture
Milk and HB cultures[1]	1.01	0.72
EC cultures[1]	0.92	0.85
L. lactis AB[2]	1.01	1.04
L. lactis AD[2]	0.98	1.05
L. cremoris AM[2]	1.09	0.33
L. cremoris AM[2]	0.80	0.66

[1] Combination of *L. lactis* and *L. cremoris* cultures. HB = highly buffered media, and EC = media with external pH control
[2] Average values for all media

estimate the total viable population (CFU × average chain length) was adequate for *L. lactis*.

With *L. cremoris* AM, the DMCC count was lower than that of CFU (1.09, Table 4), which suggests that some chain breakage did occur in the steps leading to viable count (CFU ml^{-1}) determinations. The total population counts reported with *L. cremoris* AM (Table 5) may be slightly overestimated. The approach taken in this study to determine total populations thus seems very well adapted for *L. lactis* cultures. The correction is not as precise when applied to *L. cremoris* AM, and this should be taken into account when examining the following results. With *L. cremoris* strains, more precise total population determinations might preferably be obtained by conducting viable counts and chain length measurements on samples that have been homogenized.

Table 5 Effect of growth medium on the total cell populations of *Lactococcus lactis* ssp *lactis* AB and AD, as well as *Lactococcus lactis* ssp *cremoris* AM and AR fresh cultures

Medium[1]	*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR
Milk	5.8×10^9 c	5.6×10^9 b	2.7×10^9 b	2.3×10^9 b
HB1	9.1×10^9 bc	9.3×10^9 ab	3.1×10^9 ab	2.4×10^9 b
HB2	7.9×10^9 bc	6.1×10^9 b	2.7×10^9 b	2.7×10^9 ab
HB3	1.2×10^{10} ab	7.0×10^9 ab	3.4×10^9 ab	2.9×10^9 ab
EC1	2.1×10^{10} a	1.5×10^{10} ab	2.0×10^{10} c	5.7×10^9 c
EC2	3.4×10^{10} a	3.8×10^{10} c	1.2×10^{10} ac	5.3×10^9 ac
EC3	3.2×10^{10} a	2.0×10^{10} abc	1.3×10^{10} ac	4.8×10^9 ac
EC4	3.0×10^{10} a	2.8×10^{10} ac	3.9×10^9 ac	4.0×10^9 abc

[1] HB = highly buffered media, and EC = media with external pH control
abcd: values in the same column that are followed by the same letter are not significantly different ($P>0.05$)

Total viable populations

Total viable populations (viable plate count × chain length) of *L. lactis* starters were higher than those of *L. cremoris* (Table 5). With all strains, growth conditions had significant effects on bacterial populations. None of the HB or EC media ever gave lower cell counts than those found in milk (Table 5), which confirms the effectiveness of commercial media.

There were some exceptions but, as a rule, media used in conjunction with external pH control (EC1–EC4) gave higher populations than milk or buffered media (HB1–HB3). On the average, there were between 4.5 and 5 times more cells in EC media cultured with *L. lactis* strains. The effect of external pH control was not so marked with *L. cremoris* AR, as a population obtained in EC media were only twice those obtained in milk and HB media.

When combining results of *L. lactis* cultures, significantly ($P<0.05$) higher populations were obtained in HB media than in milk. This was not the case with the *L. cremoris* strains where cultures obtained in HB media or milk gave similar populations. The beneficial effect of internal buffering was therefore more marked with *L. lactis*. Close examination of published data reveals that this observation is in agreement with other studies where the effect of species was examined. A previous report evaluating the beneficial effect of phage inhibitory media on nine *L. lactis* and 25 *L. cremoris* strains, found that 11 strains had lower efficiency when prepared in the phosphated media than when prepared in non-fat dry milk: all were of *L. cremoris* [14].

Storage for 3 days at 4° C did not significantly reduce

viable *L. lactis* populations (data for stored cultures not shown). *L. cremoris* was more sensitive, however, and viable populations of the stored cultures were, on the average, 30% lower than the fresh starters. These results basically confirmed data of CFU/DMCC (Table 4). It was found in this study that mortality upon storage was more affected by strain than by medium composition.

Not considering potential population dynamics, the data on viable populations of fresh and stored cultures suggest that *L. lactis/L. cremoris* strain ratios would tend to be higher in the commercial media than in milk. High phosphate content as well as low availability of calcium and magnesium, may be involved [36, 37]. This potential shift in ratios might be of concern to manufacturers of aged Cheddar cheese. Although all strains have the potential of producing bitter peptides [16], *L. lactis* cultures are reputed to be more often involved in generating bitterness in Cheddar than *L. cremoris* strains [11, 21, 23, 24]. On the positive side, *L. lactis* cultures are often more rapid milk acidifiers than *L. cremoris* starters. A shift towards *L. lactis* could shorten manufacturing time. This would be advantageous for the manufacture of fresh/mild or processed cheeses, but would require good manufacturing practices in order to prevent overacidification or bitterness.

Specific acidifying activities (SAA)

When compared individually, media did not have a significant effect on SAA of *L. lactis* strains (Table 6). However, in overall comparisons between HB and EC media, fresh *L. lactis* cultures had 35% more SAA when prepared on milk and HB media than when prepared in EC media. This was also the case with the stored cultures of *L. lactis*, but the difference was less (25%, data not shown). The smaller difference in SAA level in stored starters appears related to activity losses during storage. Indeed, with *L. lactis*, a significant loss in SAA was observed with overall values of cultures produced in milk and HB media, which was not the case with the four EC overall values. With *L. cremoris* strains, no important differences were seen between HB- and EC-grown cells. However, SAA losses were greater with HB media than with EC cultures. Thus, in addition to slightly reducing mortality during storage, production under external pH control prevented SAA losses.

Chain length and acidifying activity

Growth conditions can affect morphology and chain length [36, 37]. The potential relationship between chain length and SAA was thus examined. Some significant relationships were found with *L. cremoris*. In most cases the coefficients of regression were very low (around 0.40). Correlations between chain length and SAA were generally poor with *L. lactis* strains. Since *L. cremoris* strains produced longer chains than *L. lactis* (Table 3), it is understandable that correlations between chain length and activity be more frequent with the former.

In all instances where correlations were significant, the longer the chains, the more cells had to be inoculated to obtain a given acidification (which testifies to reduced SAA). These results seem to be in agreement with those of Stanley [31] who observed that 'fermenter cultures' grown under pH control had longer chain lengths than conventional milk-grown starters, and also occasionally had lower acidifying activities.

Thus, chain length can only be considered to be a very limited indication of SAA, and principally with *L. cremoris* starters.

Inoculation level

In order to select the most cost-effective medium, one must know what inoculation rate is required for a given strain/medium combination. Predictions of inoculation rate were made by taking into account the population of the starter (Table 5) and the corresponding SAA of the cells (Table 6). The values reported (Table 7) are those of the estimated inoculation rate required (%) to acidify milk to pH 5.2 in 5 h, in a simulated Cheddar temperature profile.

With all strains, external pH control would enable much lower inoculation rates (Table 7). Higher inoculation rates would be required with *L. cremoris* starters than for *L. lactis*. On the average, the calculated inoculation rates with EC-grown cultures were estimated at half those required for milk/HB cultures (Table 7), which basically confirms the suppliers' claim of 50% reductions in starter requirements with such media. All instances where lower inoculation rates are estimated were related to population increases rather than higher SAA *per se*. It must be kept in mind that EC-grown cultures may have up to five times the population levels of milk starters (Table 5). The reason why the inoculation rates of EC cultures are not reduced by a proportional five-fold factor was related to their lower SAA.

Higher inoculation rates were generally required with the stored cultures. This was more influenced by strain than by medium. An increase in 10% of inoculation level was required of stored *L. lactis* cultures, and this small increase was not judged to be significant ($P = 0.26$). Storage of *L. cremoris* starters, on the other hand, generated a need for a 40% increase in inoculation rate of the culture and this difference was significant ($P = 0.0001$). As previously demonstrated, this was a reflection of greater mortality of *L. cremoris* during storage as well as the reduced SAA of the surviving cells, particularly in milk/HB media.

Table 6 Effect of growth medium on the specific acidifying activities of *Lactococcus lactis* ssp *lactis* AB and AD, as well as *Lactococcus lactis* ssp *cremoris* AM and AR fresh cultures. Values are inoculation level required to obtain a pH of 5.2 in 5 h in the activity test

Medium[1]	*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR
Milk	3.2×10^7 a	1.0×10^8 a	9.0×10^7 a	4.2×10^7 ab
HB1	3.3×10^7 a	1.3×10^8 a	6.6×10^7 a	3.5×10^7 a
HB2	4.1×10^7 a	1.1×10^8 a	6.3×10^7 a	4.0×10^7 ab
HB3	4.8×10^7 a	6.5×10^7 a	5.0×10^7 a	4.8×10^7 ab
EC1	5.4×10^7 a	1.3×10^8 a	7.3×10^7 a	8.1×10^7 b
EC2	5.9×10^7 a	1.7×10^8 a	8.6×10^7 a	5.7×10^7 ab
EC3	5.3×10^7 a	1.6×10^8 a	3.3×10^7 a	4.9×10^7 ab
EC4	4.0×10^7 a	1.2×10^8 a	3.8×10^7 a	3.3×10^7 a

[1] HB = highly buffered media, and EC = media with external pH control
ab: values in the same column that are followed by the same letter are not significantly different ($P>0.05$)

Table 7 Inoculation rate (%, vol/vol) of the fresh cultures required to acidify milk to pH 5.2 in 5 h, in a simulated Cheddar cheese temperature profile

Medium	Fresh culture				Stored culture			
	L. lactis		*L. cremoris*		*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR	AB	AD	AM	AR
Milk	0.55 d	1.84 b	2.78 b	1.81 b	0.73 b	2.34 b	3.74 b	1.95 bc
HB1	0.36 bc	1.41 b	2.26 b	1.47 ab	0.46 bc	1.09 ab	3.43 b	1.37 bcd
HB2	0.52 bd	1.81 b	2.46 b	1.78 ab	0.50 bc	2.09 b	4.00 b	2.29 bc
HB3	0.40 b	1.18 ab	1.48 ab	1.73 ab	0.53 bc	1.27 ab	2.83 ab	2.52 b
EC1	0.26 ce	0.87 ab	0.37 a	1.43 ab	0.27 ac	1.06 ab	0.74 a	1.45 bcd
EC2	0.18 ae	0.63 a	1.18 ab	1.09 ab	0.17 a	0.66 ab	2.48 ab	1.31 acd
EC3	0.17 ae	0.79 ab	0.26 a	1.02 ab	0.17 a	0.42 a	0.61 a	1.08 ad
EC4	0.14 a	0.63 a	0.99 ab	0.85 a	0.19 a	0.60 ab	1.30 ab	0.67 a

HB = highly buffered media, and EC = media with external pH control
abcdef: values in a given column that are followed by the same letter are not significantly different ($P>0.05$)

The effect of production media (HB vs EC) on the increase in inoculation level generated by storage was not as marked as with the strains. Stored cultures produced under external pH control (EC) required an average increase of 17%, while storage of starters produced in milk or highly buffered media (HB) generated a 23% increase in inoculation requirement.

Specific proteolytic activity

The growth medium affects the proteolytic activity of the resulting cells [27]. It was thus investigated if production in EC and HB media modified the proteolytic activity of the cultures. In SPA determinations, bacterial growth was prevented by the addition of tetracycline as an inhibitor of protein synthesis. Consequently, the population did not increase throughout the SPA incubation and no acidification was recorded. This technique has the advantage of preventing amino acid assimilation which would occur during bacterial growth, and was considered to represent a true picture of the hydrolysis by the cell wall proteases. In classical methods where cell growth is allowed, amino acid content of the milk at the end of incubation is not only a reflection of proteolysis activity, but also of the level of amino acid assimilation.

SPA readings were not high (Table 8). The technique employed, where bacterial multiplication was prevented, explains this situation. The inoculation level used (10^7 CFU ml^{-1}) was sufficient with a previous study with *Lactobacillus bulgaricus* [6], but could have been increased with the lactococci.

L. cremoris strains demonstrated about twice the SPA as did *L. lactis* cultures (Table 8). Therefore, the appearance of higher activity of *L. lactis* starters, evidenced by the lower inoculation rates required for Cheddar production (Table 7), were not related to higher proteolytic activity. It is rather a reflection of higher populations reached in *L. lactis* cultures (Table 5). These data also help understand phenomena related to bitterness. Some *L. cremoris* strains generate bitterness [11], and the strains used in this study were indeed more proteolytic than *L. lactis* cultures. Lowry *et al* [23, 24] suggested that the number of cells in the product is a critical factor in the development of bitterness.

Table 8 Effect of growth medium on the specific proteolytic activities (equivalent of ppm glutamic acid liberated) of *Lactococcus lactis* ssp *lactis* AB and AD, and *Lactococcus lactis* ssp *cremoris* AM and AR

Medium[1]	*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR
Milk	2.61 bc	2.34 a	4.05 a	8.20 a
HB1	1.89 b	2.40 a	5.85 a	5.22 a
HB2	5.85 a	4.59 a	5.13 a	8.20 a
HB3	2.25 bc	3.60 a	4.80 a	9.45 ab
EC1	3.15 ab	4.50 a	5.40 a	3.51 a
EC2	4.59 ab	5.58 a	9.72 a	6.66 a
EC3	4.77 ab	3.87 a	6.66 a	6.21 a
EC4	5.22 ac	4.77 a	12.69 a	18.72 b

[1] HB = highly buffered media, and EC = media with external pH control
abcd: values in the same column that are followed by the same letter are not significantly different ($P>0.05$)

Although *L. lactis* cultures are not necessarily more proteolytic that *L. cremoris* (Table 8), higher populations of *L. lactis* were obtained in the commercial media (Table 5). As stated previously, inoculation rates with EC starters can be reduced by half that required with milk-grown cultures. However, this study shows that population increases can be five-fold in the commercial EC media (Table 5). Thus, by reducing the inoculation level only by half, a greater number of cells are being added to the milk. Logically, a higher initial number of cells in the milk would translate in a larger number of cells in the product, increasing chances for the development of bitterness. However, results from industrial trials do not always confirm this hypothesis (data not shown), and specific population analyses in the product would be useful in the investigation of a bitterness accident.

There was no significant effect of pH control on the SPA of the resulting cultures. The commercial media contain protein hydrolysates and/or yeast extract which supplement the milk ingredients in free amino acids or peptides. The expected decrease in SPA of cells produced on commercial media, due to a regulatory action of the free amino acids [17, 19], was not obtained. Although the commercial media contained whey, all the formulations used in this study also

included skim milk. It remains to be determined if the presence of caseins prevents the regulatory action of free amino acids or if the concentration of free amino acids in the media was adjusted by the manufacturers to prevent the repression of protease synthesis.

In addition to comparisons between species, the relationship between SAA and SPA in a given strain was examined. With two strains (AR and AD), there were instances of correlations between SAA and SPA that were determined to be statistically significant, but r^2 values were very low (0.2–0.4). Therefore, this study did not find conclusive evidence linking original SPA of the cultures with their subsequent SAA.

pH drop during storage

Acidification continued during storage at 4° C for 3 days (Table 9). In milk and HB media, where pH was at 4.9 at the beginning of storage, post-fermentation acidification wsa generally greater with *L. lactis* than with *L. cremoris* cultures. This confirms the general belief that *L. lactis* is more acidotolerant than *L. cremoris* [9]. In milk and HB media, post-fermentation acidification was related to buffering capacity of the media (Tables 1 and 8).

In EC media, lactose should be depleted at the end of the fermentation, and thus post acidification should be minimal. With the exception of medium EC1, this is what occurred (Table 9). Growth in low-lactose environments changes the physiology of lactic cultures, particularly lactate dehydrogenase activity. However, no significant effect on the SAA activity was recorded when EC1-grown cultures were compared to those obtained on the three other EC media (Table 6).

Conclusion

In this study a comprehensive evaluation of media and pH control techniques was attempted in which not only starter populations are recorded but specific activities are examined, all taking into account chain length of the cultures. A method is proposed that enabled a quantification of differences in SAA which is difficult to obtain with Δ pH readings that are on a logarithm scale and are influenced by buffering properties.

Table 9 pH of *Lactococcus lactis* ssp *lactis* AB and AD, and *Lactococcus lactis* ssp *cremoris* AM and AR after three days of storage at 4° C; the pH of fresh cultures in milk and HB1–HB3 was 4.9; the pH of fresh cultures in EC1–EC4 was 6.0

Medium[1]	*L. lactis*		*L. cremoris*	
	AB	AD	AM	AR
Milk	4.48	4.51	4.57	4.53
HB1	4.74	4.65	4.86	4.93
HB2	4.78	4.69	4.67	4.78
HB3	4.89	4.72	4.91	4.91
EC1	5.40	5.56	5.46	5.68
EC2	6.12	6.04	5.11	5.80
EC3	6.15	5.98	6.02	5.71
EC4	6.02	6.21	6.04	5.97

[1] HB = highly buffered media, and EC = media with external pH control

It is clear that external pH control generates higher starter populations and reduces significantly the required inoculation rate, even though such cultures have a slightly lower specific acidifying activity. The assumption that better stability should be found in cultures stored at high pH was confirmed in this study. Production in EC media reduced both cell mortality and SAA losses. The HB media did help reduce post-acidification, but did not significantly reduce activity losses. It must be stressed that the protective effect of EC media on culture stability during storage is limited, and certain strains (mostly *L. lactis*) are simply better suited to storage. Overall, the benefits of the commercial media seem to be greater with *L. lactis* strains. Thus, if appropriate strains of *L. cremoris* are not selected in a given medium, it can be feared that the strain ratio of a mixture of *L. lactis* and *L. cremoris* would shift in favour of *L. lactis* and impact on the vat performance of the starter (faster acidification, less effect of cooking temperature and salting on acidification) as well as the properties of the cheese (potential bitterness) [9]. This study suggests that there may still be a need for continued *L. cremoris* strain isolation, on the basis of growth and activity in these media.

It is noteworthy to mention that no clear effectiveness association could be made between commercial origin of the strain and that of the growth medium. If a cheesemaking plant has a very limited rotation of strains designed for external pH control, it may be worthwhile to test various media to determine the most appropriate, and least expensive, for their specific cultures.

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