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Original article

Characterisation of lactic starters based on acidification and reduction activities

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Abstract – The acidification and reduction activities of lactic starters have been followed by continuous measurement of the pH and the Eh during the course of milk fermentation. These measurements allowed the calculation of the maximum acidification and reduction rates, and the time, pH and Eh at which these values occurred. Nine strains of *Lactococcus* sp., 6 strains of *Streptococcus thermophilus* and 5 strains of *Lactobacillus helveticus* were studied. In general, the maximum reduction rate of the lactococci was six-fold higher than those of the streptococci and lactobacilli. On the other hand, the streptococci and the lactobacilli acidified with a higher maximum acidifying rate than those of lactococci. Consequently, it was observed that all the cultures with lactococci reached their final Eh before the end of the lactic acid fermentation, while acidifications with the streptococci or the lactobacilli finished before the end of the reduction phase. A principal components analysis clearly differentiates the three species on the basis of their aptitudes for acidification and reduction. This new approach might be used to select adequate starters for the manufacture of fermented dairy products.

Acidification / reduction / *Lactococcus* / *Streptococcus thermophilus* / *Lactobacillus helveticus*

Résumé – **Caractérisation de levains lactiques sur la base de leurs activités acidifiantes et réductrices.** Le suivi de l'acidification et de la réduction du lait par des levains lactiques a été réalisé par l'enregistrement en continu du pH et du Eh. Les valeurs mesurées ont permis de calculer les vitesses maximales d'acidification et de réduction, et le temps, le pH et le Eh auxquels ces maxima sont obtenus. L'étude a porté sur 9 souches de *Lactococcus* sp., 6 souches de *Streptococcus thermophilus*, et 5 souches de *Lactobacillus helveticus*. D'une manière générale, la vitesse maximale de réduction des lactocoques était six fois plus grande que celle des streptocoques et des lactobacilles. Par contre, les streptocoques et lactobacilles acidifiaient avec une vitesse maximale supérieure à celle des lactocoques. En conséquence, toutes les cultures avec les lactocoques atteignaient leur Eh final avant la fin de la fermentation lactique, alors que l'acidification avec les streptocoques et les lactobacilles se

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terminait avant la fin de la phase de réduction. L'analyse en composante principale différencie clairement les 3 espèces sur la base de leurs aptitudes acidifiantes et réductrices. Ces nouveaux paramètres pourraient être associés à ceux classiquement utilisés pour sélectionner les levains les plus adéquats pour la fabrication de produits laitiers fermentés.

Acidification / réduction / *Lactococcus / Streptococcus thermophilus / Lactobacillus helveticus*

1. INTRODUCTION

The activity of lactic starters is of great importance in the processes used for the manufacture of fermented dairy products. These micro-organisms interact with their physico-chemical environment and alter its characteristics in a very complex manner. The oxido-reduction potential (Eh), as pH or temperature, is an important parameter of the physico-chemical environment. It contributes to the microbial quality of fermented dairy products [4–6, 9, 14], and creates the conditions necessary for a balanced flavour development [17, 18, 20]. It may control redox reactions but also protects flavour compounds from oxidation. Kristoffersen et al. [17] related Eh changes in cheese to active sulfhydryl groups, indicating a chemical relationship with flavour changes. Sulphur containing compounds may be produced by lactic acid bacteria [10] and they participate in the creation of the aroma in cheese [21]. Urbach [27] reviewed the contribution of both -SH groups and Eh to the flavour of dairy products. The author assumed that the low Eh value in good quality cheeses is an indicator of anaerobic conditions required for the flavour forming reactions.

The lactic starters are able to decrease the Eh during milk fermentation [12, 16, 25], but while the kinetics of acidification have been well studied, the kinetics of reduction have not yet been characterised. There are several methods available, such as pH or impedance measurements, to study the kinetics of acidification by lactic starters [3, 7, 8, 19, 22, 26]. The method of Spinnler and Corrieu [26] involves the calculation of characteristic kinetic parameters from the measurement of pH decrease [1, 2, 11, 23, 28]. Picque et al. [23] observed that among all the parameters that can be calculated, the best parameters were the maximum acidification rate (V_m), and the time (T_m) and pH (pH_m) at which V_m occurred.

In this study this method was adapted to characterise both the acidification and the reduction capacity of thermophilic lactic starters (*Lactobacillus helveticus* and *Streptococcus thermophilus*) and mesophilic lactic starters (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* and *Lactococcus lactis* ssp. *cremoris*).

2. MATERIALS AND METHODS

2.1. Bacterial strains

Lactobacillus helveticus L116, L122, L124, L72, L99, L54, *Streptococcus thermophilus* S3, S18, S79, S107, S119, *Lactococcus lactis* ssp. *lactis* SRTA2001, SRTA2004 and SRTA2056 were isolated during Comté cheese manufacture. *Lactococcus lactis* ssp. *lactis* SL01, SL03, SL04, *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* SD17, SD18 and *Lactococcus lactis* ssp. *cremoris* SC09 were obtained from the ARILAIT collection. A stock cell suspension of each strain was kept frozen at –80 °C.

2.2. Media and culture conditions

Cells of the stock suspension were inoculated in 10 mL MRS lactose medium for lactobacilli and lactococci, and in 10 mL M17 lactose medium for streptococci. The cultures were incubated overnight, and

used to inoculate 100 mL of their respective medium. The flasks were incubated in static conditions, and the optical density at 575 nm was followed (Novaspec 4049, LKB, Cambridge, UK). At an optical density of 1 unit, 1.4 mL of the culture was used to inoculate 275 mL of milk. Sterile skim milk (Candia, Lyon, France) was used in order to avoid medium modifications during the time of the study. The cultures were conducted in triplicate in Erlenmeyer flasks. The temperatures for pre-cultures and cultures were 42 °C for lactobacilli and streptococci, and 26 °C for lactococci.

2.3. Data acquisition and treatment

A multi-channel pH-meter/redox-meter Consort P507 (Bioblock Scientific, Ilkirsh, France) was used to follow simultaneously the pH and the measured oxido-reduction potential (Em, mV) values of the milk. Combined pH electrodes (Inlab 407, Metler-Toledo SARL, Paris, France) and redox electrodes (PT4805-DKK-S8/120, Mettler-Toledo SARL, Paris, France) were connected to the Consort P507. pH electrodes were standardised using two buffers (pH 4.0 and pH 7.0) and cleaned, after each run, using a pepsin/HCL solution (Poly Labo, Paris, France). The redox electrodes were polished with a fine alumina powder (Aluminium oxyde, Prolabo, Lyon, France) to restore the platinum surface [13]. In order to avoid interference caused by atmospheric oxygen on the redox measurement, the cultures were carried out under static conditions. It had previously been verified that this method did not change the acidification profiles. Software, run on a PC, automatically registered the pH and the oxido-reduction potential (Em), and converted the Em into Eh values, according to the standard electrode value (Er) at the temperature of fermentation (Eh = Em + Er).

The pH and Eh values of three different experiments for each strain were mathematically treated to calculate the acidification rate (dpH/dt, pH unit/h) and the reduction rate (dEh/dt, mV/h). They allowed determination of the maximum acidification rate (V^a_m, pH unit/h) and the maximum reduction rate (V^r_m, mV/h), the time at which these maximum rates occurred, respectively T^a_m (h) and T^r_m (h), and the pH and Eh at which these maximum rates occurred, respectively pH^a_m (pH unit), Eh^a_m (mV) and pH^r_m (pH unit), Eh^r_m (mV).

The Eh values were dependent on pH according to the following equations:

$$Eh = Eo + \frac{\Re T}{n\Im} \ln \frac{[ox][H^+]^m}{[red]}$$

$$Eh = Eo + \frac{2.303\Re T}{n\Im} \log \frac{[ox]}{[red]} - \frac{2.303m\Re T}{n\Im} pH$$

at 26 °C:

$$Eh\ (mV) = Eo + \frac{59}{n} \log \frac{[ox]}{[red]} - \frac{59m}{n} pH$$

at 42 °C:

$$Eh\ (mV) = Eo + \frac{62}{n} \log \frac{[ox]}{[red]} - \frac{62m}{n} pH$$

Eo is the standard redox potential (V), $\Re$ is the gas constant (8.31 $J \cdot K^{-1} \cdot mol^{-1}$), T is the temperature (K), $\Im$ is the Faraday constant (96 485 $C \cdot mol^{-1}$), *n* and *m* are respectively the number of electrons and protons involved in the redox reaction. If the reactants are ionised, the dissociated protons must also be taken into account in the Eh calculation. For a simple redox system, these mathematical relations allow calculation of the pH dependence of Eh. For milk medium, there are unknown redox systems, therefore the relation between Eh and pH must be experimentally determined [13]. For the sterilised skim milk used in this study, we measured an Eh variation of 38 mV/pH units at 26 °C and 40 mV/pH units at 42 °C. These coefficients allowed the calculation of the Eh7 value, a redox potential which is corrected for pH:

at 26 °C: Eh7 = Eh – 38 (7 – pH)
at 42 °C: Eh7 = Eh – 40 (7 – pH)

The measurement of dissolved oxygen concentration during the course of milk fermentation used oxygen sensors (InPro6100/120/T/N, Metler-Toledo SARL, Paris, France), and a BIOSTAT® Q multiple fermentor unit (B. Braun Biotech International, Melsungen, Deutschland).

The values of V^a_m, V^r_m, T^a_m, T^r_m, pH^a_m and pH^r_m were analysed using a Principal Components Analysis (PCA). This statistical multivariate procedure and the variance analysis were performed using the SAS software [24].

3. RESULTS AND DISCUSSION

The evolution throughout time of pH, Eh, Eh7, the acidification rate and the reduction rate are presented in Figure 1 for lactococci, as an example. The initial pH and Eh values were respectively 6.52 ± 0.07 and 233 ± 30 mV (n = 50), and the fermentations were stopped at the end of the acidification step. The starter activity was observed 2–3 h after the milk was inoculated.

3.1. Acidification activity

Within each species the parameters V^a_m, T^a_m, and pH^a_m for protease positive strains (prt^+) showed a lower standard deviation (Tab. I) than those calculated previously [23]. The ratio between the maximum and the minimum value of kinetic parameters, within each species, ranged from 1.3 to 1.4 for V^a_m and from 1.4 to 1.6 for T^a_m, whereas a ratio ranging from 2.2 to 3.9 for V^a_m and from 1.4 to 4.2 for T^a_m, was obtained previously [23]. These differences may be explained by strain diversity within the same species, and because a majority of strains in the present study are adapted to the same dairy process (the manufacture of Comté cheese). In addition, for mesophilic bacteria previous results [23] summarised the

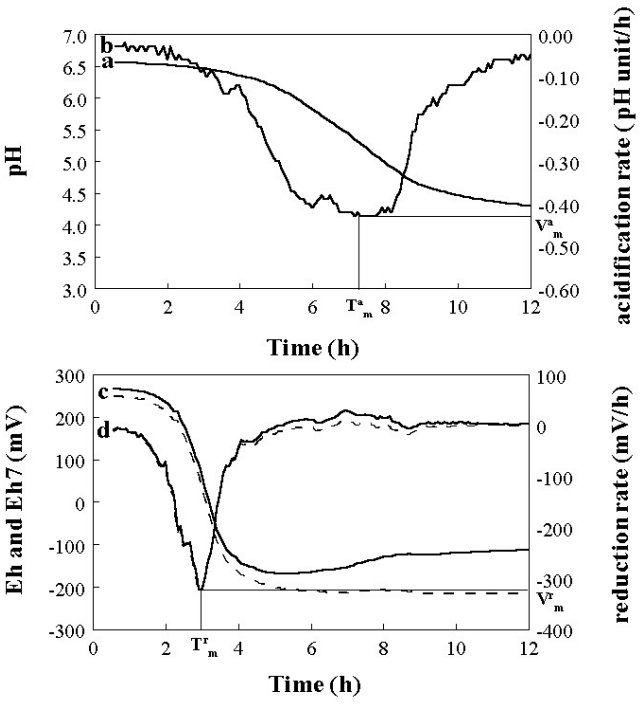

Figure 1. Typical curves of pH (a), acidification rate (b), Eh (c), Eh7 (c, dashed lines), and reduction rates (d) of *Lactococcus* sp. prt^+ (T = 26 °C) in sterilised skim milk.

Table I. Mean values of the kinetic parameters calculated from acidification and reduction rates of *Lactococcus* sp. prt$^+$ (n = 9, T = 26 °C), *Lactococcus* sp. prt$^-$ (n = 8, T = 26 °C), *Streptococcus thermophilus* (n = 5, T = 42 °C) and *Lactobacillus helveticus* (n = 6, T = 42 °C) in sterilised skim milk.

	Final pH	V^a_m (pH unit/h)	T^a_m (h)	pH^a_m	Eh^a_m (mV)	V^r_m (mH/h)	T^r_m (h)	pH^r_m	Eh^r_m (mV)
Lactococcus sp. (Prt$^+$) *(SD)* (grouping with n = 9)	4.43^a *(0.16)*	–0.38^a *(0.04)*	7.8^a *(0.76)*	5.27^a *(0.08)*	–144^a *(6)*	–316^a *(20)*	3.04^a *(0.27)*	6.42^a *(0.12)*	0^a *(20)*
Lactococcus sp. (Prt$^-$) *(SD)* (grouping with n = 8)	5.43^b *(0.08)*	–0.18^b *(0.03)*	5.85^b *(0.72)*	6.09^b *(0.17)*	–95^a *(62)*	–292^a *(105)*	3.54^a *(0.80)*	6.36^a *(0.13)*	51^a *(67)*
Streptococcus thermophilus (SD) (grouping with n = 6)	4.66^c *(0.10)*	–0.55^c *(0.06)*	3.3^c *(0.4)*	6.16^b *(0.05)*	155^b *(15)*	–46^b *(5)*	4.18^a *(1.26)*	5.78^b *(0.26)*	133^b *(19)*
Lactobacillus helveticus (SD) (grouping with n = 5)	3.42^d *(0.09)*	–0.53^c *(0.04)*	9.17^d *(1.03)*	4.37^c *(0.1)*	152^b *(4.6)*	–62^b *(3)*	7.62^b *(1.05)*	5.08^c *(0.06)*	174^c *(7)*

$^{a, b, c}$ Results of Student-Newman-Keuls' test at $P < 0.05$. Values in a given column with the same letter are not significantly different.

data of several species, *Lactococcus*, *Leuconostoc* and *Lactobacillus*, which may increase standard deviation. Finally, we observed, with protease negative variants (prt^-) of *Lactococcus* sp., a 53% and 25% decrease for V^a_m and T^a_m, respectively, and a 16% increase for pH^a_m (Tab. I) compared to prt^+ strains. Therefore, if the difference in proteolytic activity is not taken into account, the standard deviation may be significantly increased. Casalta et al. [2] differentiated $prot^+$ and $prot^-$ strains of *Lactococcus lactis*. They calculated acidifying activities for the prt^+ strains with a CV of 10.6% (n = 12), which is very close to the CV calculated in our study.

The V^a_m of lactococci prt^+ represents approximately 69% of the V^a_m of thermophilic starters. This observation is consistent with previous studies [23], and may also be the result of a suboptimal temperature of incubation for the lactococci. The V^a_m of lactobacilli and streptococci were not statistically different, but they were obtained earlier and at a much higher pH for streptococci than for lactobacilli (see values of T^a_m and pH^a_m) which is in agreement with previous studies [1, 23]. For the streptococci, there are two different phases during pH decrease which may be related to the urease activity in this species [15, 23, 26]. In general, the lower the pH^a_m the better the milk acidification, the final pH value being 10–30% lower than pH^a_m. It may be assumed that when the culture reached pH^a_m the conditions were optimal for high metabolic activity together with a sufficient cell density. The lactococci demonstrated a capacity to acidify milk statistically different from streptococci, while *Lactobacillus* was the strongest acidifying species.

3.2. Reduction activity

For lactococci, the acidification and reduction activities were clearly differentiated and distributed during the course of milk fermentation (Fig. 1). The pH modified the kinetics of Eh decrease during the course of milk fermentation (see Sect. 2). The evolution through time of Eh7 explained the increase of Eh after 5 h of fermentation by an indirect pH effect. The lactococci decreased Eh by 377 mV before reaching their maximum acidification rate, while for thermophilic strains the Eh decreased slowly (about 80 mV) and reached V^a_m together with V^r_m. For the latter mentioned bacteria, it may be assumed that related enzymatic activities were involved in both acidification and reduction maximums. The V^r_m of lactococci is five- to seven-fold higher than those of thermophilic starters which have similar values of V^r_m, even if the streptococci reduced before the lactobacilli. The higher reduction activity for lactococci than for *Streptococcus thermophilus* and *Lactobacillus helveticus* was conserved in the panel of prt^- strains, with the highest variability for the latter. The dissolved oxygen concentration measured during the course of milk fermentation reached 0% after 4–5 h for lactococci, while for thermophilic bacteria, dissolved oxygen was still present at the end of the acidification phase (13–14 h) (data not shown). As a probable consequence, while for lactococci Eh decrease stopped before 6 h, for *Streptococcus thermophilus* and *Lactobacillus helveticus* after the V^r_m peak, a low but constant reduction activity was maintained and allowed the acidification to finish before the end of the reduction phase.

3.3. Multivariate factorial analysis

In order to study relationships between the kinetics of acidification and reduction of lactococci, streptococci and lactobacilli, a principal components analysis was applied using the original variables V^a_m, V^r_m, T^a_m, T^r_m, pH^a_m and pH^r_m (Fig. 2). There are three distinct groups of strains corresponding to the three species: *Streptococcus thermophilus*, *Lactobacillus helveticus* and

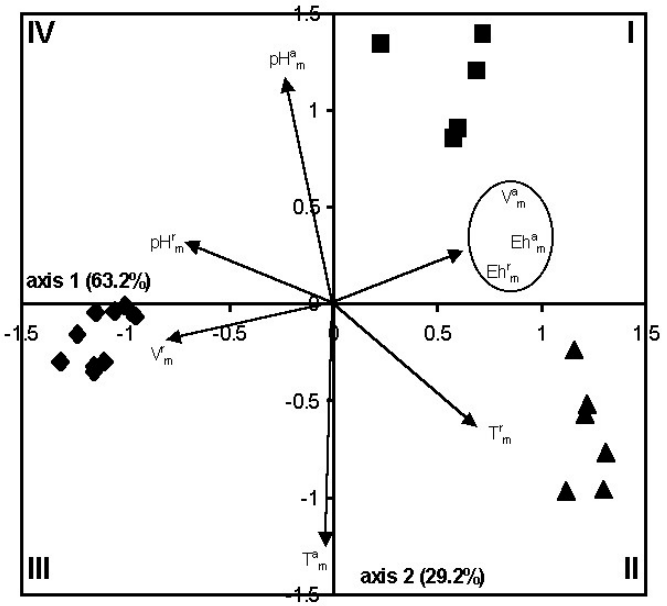

Figure 2. Results of the principal components analysis: loading plot and score plot of the first two components (axis 1 and axis 2). Symbols: ◆ *Lactococcus* sp. prt$^+$, ■ *Streptococcus thermophilus*, ▲ *Lactobacillus helveticus*.

Lactococcus sp. Since the lactococci were concentrated in quadrant III (negative values of axis 1), they are associated with an acidification and a reduction activity at high values of pH and low values of Eh. In addition, while their reduction activity was faster than those of streptococci and lactobacilli, they acidified at a lower rate. The streptococci were placed in quadrant I while the lactobacilli were placed in quadrant II, thus these two species were mainly differentiated by the time and the pH at which they acidified and reduced the medium (axis 2).

4. CONCLUSION

The manufacture of high quality fermented products demands attention to all the parameters that may affect starter culture metabolism and product characteristics. The method described in this study allows quantification of the reduction activity of lactic starters in association with the classical measurement of their acidification activity. Eh modifications may participate both in the microbial quality (non-development of spoilage micro-organisms, strain equilibriums in mixed cultures, biological activity of non-starter lactic acid bacteria), and the sensorial quality (synthesis and/or stability of aroma compounds) of cheeses and fermented milks. The input of the reduction activity, in addition to acidification activity, proteolysis products, aromatic compounds or fermented carbohydrate profiles, may allow a good description of lactic starters. It might be used to characterise their technological properties and to propose adequate starters for the different processes in the dairy industry.

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REFERENCES

[1] Béal C., Corrieu G., Viability and acidification activity of pure and mixed starters of *Streptococcus salivarius* ssp. *thermophilus* 404 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 398 at the different steps of their production, Lebensm. Wiss. Technol. 27 (1994) 86–92.

[2] Casalta E., Vassal Y., Desmazeaud M.J., Casabianca F., Comparaison de l'activité acidifiante de souches de *Lactococcus lactis* isolées de lait et de fromage de Corse, Lebensm. Wiss. Technol. 28 (1995) 291–299.

[3] Champagne C., St-Gelais D., de Candolle A., Acidification rates and population ratios of lactic starters in carbonated milk, Lebensm. Wiss. Technol. 31 (1998) 100–106.

[4] Dave R., Shah N., Effect of cysteine on the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures, Int. Dairy J. 7 (1997) 537–545.

[5] Dave R., Shah N., Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures, Int. Dairy J. 7 (1997) 435–443.

[6] Dave R.I., Shah N.P., Ingredient supplementation effects on viability of probiotic bacteria in yogurt, J. Dairy Sci. 81 (1998) 2804–2816.

[7] De Brabandere A.G., de Baerdemaeker J.G., Effects of process conditions on the pH development during yogurt fermentation, J. Food Eng. 41 (1999) 221–227.

[8] Demarigny Y., Juillard V., Deschamps N., Richard J., Comparaison, sur le plan pratique, de 3 modèles d'étude de la cinétique d'abaissement du pH du lait cultivé par *Lactococcus lactis*. Proposition du concept "Vmar", Lait 74 (1994) 23–32.

[9] Desmazeaud M., Principaux paramètres de l'écologie microbienne de produits laitiers fermentés, Microbiol. Aliments Nutr. 15 (1997) 99–114.

[10] Dias B., Weimer B., Conversion of methionine to thiols by lactococci, lactobacilli, and brevibacteria, Appl. Environ. Microbiol. 64 (1998) 3320–3326.

[11] Fonseca F., Béal C., Corrieu G., Method for quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage, J. Dairy Res. 67 (2000) 63–80.

[12] Galesloot T.E., Kooy J.S., De redoxpotentiaal van zuursel en met zuursel geente melk, Neth. Milk Dairy J. 14 (1960) 1–22.

[13] Jacob H.E., Redox potential, Methods Microbiol. 2 (1970) 91–123.

[14] Jönsson H., Pettersson H.-E., Studies on the citric acid fermentation in lactic starter cultures with special interest in alpha-aceto-lactic acid. 2. Metabolic studies, Milchwissenschaft 32 (1977) 587–594.

[15] Juillard V., Desmazeaud M.J., Spinnler H.E., Demonstration of urease activity in *Streptococcus thermophilus*, Can. J. Microbiol. 34 (1988) 818–822.

[16] Keen A.R., Growth studies on the lactic streptococci. IV. Some observations on redox potential, J. Dairy Res. 39 (1972) 161–165.

[17] Kristoffersen T., Mikolajcik E.M., Gould I.A., Cheddar cheese flavor. IV. Directed and accelerated ripening process, J. Dairy Sci. 50 (1967) 292–297.

[18] Law B., Castanon M., Sharpe M., The contribution of starter streptococci to flavour development in Cheddar cheese, J. Dairy Res. 43 (1976) 301–311.

[19] Lievense L.C., Van't Riet K., Noomen A., Measuring and modelling the glucose-fermenting activity of *Lactobacillus plantarum*, Appl. Microbiol. Biotechnol. 32 (1990) 669–673.

[20] Lowrie R.J., Lawrence R.C., Peberdy M.F., Cheddar cheese flavour. V. Influence of bacteriophage and cooking temperature on cheese made under controlled bacteriological conditions, N. Z. J. Dairy Sci. Technol. 9 (1974) 116–121.

[21] Manning D.J., Sulphur compounds in relation to Cheddar cheese flavour, J. Dairy Res. 41 (1974) 81–87.

[22] Okigbo L.M., Oberg C.J., Richardson G.H., Lactic culture activity tests using pH and impedance instrumentation, J. Dairy Sci. 68 (1985) 2521–2526.

[23] Picque D., Perret B., Latrille E., Corrieu G., Caractérisation et classification de bactéries lactiques à partir de la mesure de leur cinétique d'acidification, Lebensm. Wiss. Technol. 25 (1992) 181–186.

[24] SAS, User's guide: statistics version 5, Statistical Analysis System Inst. Cary, N.C., 1985.

[25] Scarinci H.E., Carrasco M.S., Simonetta A.C., Technological properties of strains of lactic acid bacteria for cheese starter: acidifying activity and redox potential control, Microbiol. Aliments Nutr. 12 (1994) 437–442.

[26] Spinnler H.E., Corrieu G., Automatic method to quantify starter activity based on pH measurement, J. Dairy Res. 56 (1989) 755–764.

[27] Urbach G., Contribution of lactic acid bacteria to flavour compound formation in dairy products, Int. Dairy J. 5 (1995) 877–903.

[28] Zanatta P., Basso A., A new approach to the characterization of *Streptococcus salivarius* ssp. *thermophilus* based on acidification rates, Lait 72 (1992) 285–295.