Original article

Characterization and selection of dairy lactococci based on statistical analyses of biochemical and technological properties

Claire Server-Busson^{a*}, Marc Danzart^b, Vincent Juillard^c,
Jean-Yves Leveau^a

^a Département de microbiologie industrielle; ^bDépartement de génie industriel alimentaire Ensia,
1, avenue des Olympiades, 91744 Massy cedex, France

^c Unité de recherches laitières, Inra, 78352 Jouy-en-Josas cedex, France

(Received 19 November 1997; accepted 20 March 1998)

Abstract — A total of 18 dairy lactococcal strains isolated from commercial starters, curds and fresh cheeses were screened for 63 biochemical and technological characters and compared to three reference strains. Using hierarchical clustering analyses, all these strains were compared on the basis of eight biochemical criteria or seven technological criteria. The technological characteristics under study were growth kinetics, acidification, diacetyl production and proteolysis during growth in milk, as well as 3 peptidase activities. Clusters obtained from these classifications mainly differed in the proteolytic character of the strains. Principal Component Analyses (PCA) were also carried out on the results of the technological characterization. This statistical method allows to compare simultaneously all the strains and their technological properties. The two statistical methods used made it possible to evaluate the relationships between strains on the one hand and between strains and their technological properties on the other hand. This study allowed to select strains for their abilities to acidify milk or to produce diacetyl which could be of great interest for the fresh cultured dairy industry.
© Inra/Elsevier, Paris.

Lactococcus / selection / technological characterization / hierarchical clustering / principal component analysis

Résumé — Caractérisation et sélection de lactocoques d'origine laitière à partir d'analyses statistiques de leurs propriétés biochimiques et technologiques. Dix-huit souches de lactocoques d'origine laitière, isolées de ferments commerciaux, de caillés et de fromages frais, ont été caractérisées selon 63 caractères biochimiques et technologiques et comparées à trois souches de référence. Grâce à l'analyse par classification hiérarchique ascendante, l'ensemble de ces souches a été comparé sur la base de huit critères biochimiques ou de sept critères technologiques. Les caractéristiques technologiques testées étaient la croissance, l'acidification, la production de diacétyl et la protéolyse

* Correspondence and reprints. E-mail: busson@ensia.inra.fr

lors de cultures en lait, ainsi que trois activités peptidasiques. Les groupes obtenus à la suite de ces classifications diffèrent principalement par le caractère protéolytique des souches. Des analyses en composantes principales (ACP) ont aussi été appliquées aux résultats de la caractérisation technologique. Cette méthode statistique permet de comparer simultanément toutes les souches et leurs propriétés technologiques. Les deux méthodes statistiques utilisées ont permis d'évaluer les relations entre les souches d'une part et entre les souches et les propriétés technologiques d'autre part. Cette étude a permis de sélectionner des souches pour leur capacité acidifiante ou leur aptitude à produire du diacétyle. De telles souches sont potentiellement intéressantes pour leur utilisation dans les produits laitiers frais. © Inra/Elsevier, Paris.

***Lactococcus* / sélection / caractérisation technologique / classification hiérarchique ascendante / analyse en composantes principales**

1. INTRODUCTION

Mesophilic lactic acid bacteria are commonly used as starter cultures by the dairy industry. Starter cultures play several important functions: conversion of lactose into lactic acid, casein degradation, formation of aroma compounds, inhibition of undesired bacteria. The composition of starter used by the dairy industry is most of the time poorly known [3, 5]. Several interactions have been characterized among lactic acid bacteria during growth in milk [16], which might result in a modification of the starter composition. Interactions occurring between strains might affect the technological properties of the starter. Consequently, optimization of the process control first requires a better characterization of the starter cultures.

In this context, strain characterization studies have been developed. They focus on strains from various origins and evaluate specific technological properties in relation to their applications [4, 11, 22, 23]. Technological characterizations taking into account combined properties such as acidifying, flavoring, proteolytic and peptidasic activities were mainly carried out for the yoghurt related strains [1, 2, 27, 28]. Mesophilic lactic acid bacteria and their selection for practical use have received less attention.

In the present study, we have biochemically and technologically characterized 18

dairy lactococcal strains isolated from commercial starters, curds and fresh cheeses. Statistical analyses of our results succeeded in classifying and comparing these strains especially on the basis of relevant technological properties.

2. MATERIALS AND METHODS

2.1. Bacterial strains

A total of 140 lactic acid bacteria were isolated from five commercial cheeses starters, curds and fresh cheeses on MRS [7], on MRS with added vancomycin ($20 \mu\text{g}\cdot\text{mL}^{-1}$) [20], on maltose MRS with added NaCl (4%, w/w) and on M17 media [26]. Three reference strains from the Inra collection (Jouy-en-Josas, France) were also used (table I). All the strains were stored at -20°C in 10% reconstituted sterile litmus milk with added glucose and yeast extract (0.5%, each).

2.2. Biochemical tests

Identification was carried out after preculturing cells in lactose M17 broth for 24 h at 30°C . Cells were harvested by centrifugation (4 000 rpm, 10 min), washed with tryptone/salt and resuspended in the same solution at 0.5 absorbance unit at 600 nm. This bacterial suspension was used to inoculate all the different media at 2%. Cultures were incubated at 30°C . Trials were repeated twice.

To identify the strains, 58 biochemical features were tested. Cell morphology was noted by

Table I. Origin and identification of the 18 isolates and the three reference strains used in this work.
Tableau I. Origine et identification des 18 isolats et des trois souches de référence utilisés dans ce travail.

Strain number	Origin	Identification
076	Inra collection CNRZ 1076	<i>Lc. lactis lactis</i>
075	Inra collection CNRZ 1075	<i>Lc. lactis lactis</i>
L36	Starter A	<i>Lc. lactis lactis</i>
L45	Starter B	<i>Lc. lactis lactis</i>
L68	Starter A	<i>Lc. lactis lactis</i>
L84	Starter B	<i>Lc. lactis lactis</i>
L86	Starter B	<i>Lc. lactis lactis</i>
L58	Starter C	<i>Lc. lactis lactis</i>
LF6	Curd F	<i>Lc. lactis lactis</i>
LS6	Curd F	<i>Lc. lactis lactis</i>
D57	Starter C	<i>Lc. lactis lactis diacetylactis</i>
D58	Starter C	<i>Lc. lactis lactis diacetylactis</i>
D59	Starter C	<i>Lc. lactis lactis diacetylactis</i>
D11	Starter D	<i>Lc. lactis lactis diacetylactis</i>
D18	Starter D	<i>Lc. lactis lactis diacetylactis</i>
D22	Starter E	<i>Lc. lactis lactis diacetylactis</i>
D25	Starter E	<i>Lc. lactis lactis diacetylactis</i>
DF7	Curd G	<i>Lc. lactis lactis diacetylactis</i>
DP7	Fresh cheese G	<i>Lc. lactis lactis diacetylactis</i>
380	Inra collection CNRZ 380	<i>Lc. lactis cremoris</i>

microscopic observation and Gram staining. Catalase activity was tested by dropping H_2O_2 (20%) on colonies grown on M17 agar. Oxidase activity was tested by the method of Kovacs [18] with the oxichrome reagent (AES, Combourg, France). Carbon dioxide production from glucose was determined using inverted Durham tubes in MRS broth (without triammonium citrate) overlaid with a parafin plug according to the method of Gibson and Abd-El-Malek [9]. Cultures were examined after 48 h of growth. Lactic acid configuration and citric acid utilization were determined enzymatically with Boehringer kits (Boehringer, Mannheim, Germany) after incubation for 24 h. Acetoin production from citrate was tested by the Voges-Proskauer test after incubation for 48 h. L-arginine dihydrolase activity was tested by using the Niven medium modified by Hitchener et al. [12]. Carbohydrate fermentation pattern was determined using the API 50 CH system (Biomérieux, Marcy l'Étoile, France).

2.3. Technological tests

Technological characterization of the strains was performed during growth in a reference milk (low heat skim milk powder, Nilac, NIZO, Ede, The Netherlands). Milk was reconstituted at 10% under sterile conditions. Preculture conditions and milk inoculation were identical to those described by Juillard and Richard [13]. The mean inoculation level of the cultures was 1.10^6 cfu·mL⁻¹. Cultures were incubated at 30 °C for 24 h.

Growth in milk was assessed by bacterial enumerations. Cell population was estimated by plating samples on lactose M17 medium with a spiral plater (Spiral System, Cinciratti, USA) after mechanically disrupting the lactococci chains with an Ultra-Turrax (model T25, Janke and Kunkel, Stauffer, Germany) (20 000 rpm, 30 s). The decimal logarithm of the maximum population level, N_m (log₁₀ cfu·mL⁻¹), was calculated. Linear regression analysis was performed on experimental data in the exponential phase

of growth and growth rates, μ_i (h^{-1}), were calculated as described by Hassan et al. [10].

Milk acidification was estimated by pH measurements (pH-meter model 654, Portamess, Knick, Berlin, Germany). The pH difference after 12 h of incubation (dpH) was calculated. Linear regression analyses were performed on pH data in the 6.2 to 4.8 range and the mean acidification rate V_{mar} (pH unit- h^{-1}) was calculated [8].

Proteolytic activity was colorimetrically determined at the stationary phase of growth by measuring the release of free amino groups with trinitro-benzene-sulfonic acid (TNBS; Sigma, Norwalk, USA) [21]. Glycine (Sigma) was used as standard. Proteolytic activity (μmol of Gly- 10^{-10} cfu) was expressed as the amount of free amino groups in the fraction of milk soluble in trichloroacetic acid (0.72 mol-L^{-1}) (Labosi, Élancourt, France).

Flavoring activity was estimated by the amount of diacetyl produced in milk after 24 h of incubation. Diacetyl was measured by static head-space gas chromatography. Samples were prepared as described by Degorce-Dumas et al. [6]. The following operating conditions were used: injector and detector temperatures 150°C , oven temperature 90°C , He flow rate $30 \text{ mL}\cdot\text{min}^{-1}$. Samples were hermetically sealed and heated for 25 min at 90°C . Diacetyl (Sigma), Dia ($\text{mg}\cdot\text{L}^{-1}$), was quantified by the external standard calibration method.

Peptidase activities of cell free extracts were measured by using chromogenic substrates. Lysis was achieved as described by Juillard et al. [17]. The activities of PepN and/or PepC were checked with L-lysyl-p-nitroanilide (Sigma) and that of PepC with alanyl- β -naphthylamide (Sigma). The activity of PepX was measured using alanyl-prolyl-p-nitroanilide as the substrate (Bachem, Budendorf, Switzerland). One unit of PepN and PepX activities was defined as the amount of enzyme producing an absorbance variation of 0.01 per min at 405 nm and 30°C . One unit of PepC activity was defined as the amount of enzyme producing an absorbance variation of 0.01 during 1 hour at 550 nm and 30°C . The protein content of the cell free extract was determined according to the method of Lowry et al. [19] with bovin serum albumin (Sigma) as the standard. Specific activity was defined as the number of activity units per milligram of protein.

2.4. Statistical analysis

Results of the biochemical tests were coded as either '1' (positive or medium response) or '0' (negative or weakly positive response). Statistical analysis of the identification results was carried out by a hierarchical clustering using the Jaccard distance (suitable to qualitative data) with a mean linkage. The same analysis was performed on the results of the technological characterization using normed values and the euclidian distance (suitable to quantitative data). Principal Component Analyses (PCA) were also carried out on the results of the technological characterization.

Confidence intervals of growth and mean acidification rates were calculated as described by Hassan et al. [10]. Some trials were repeated three times in order to confirm experimental results. Mean values and standard deviation were calculated.

The STATITCF software (version 5.0) (Institut des Céréales et des Fourrages, Paris, France) was used to treat data.

3. RESULTS

3.1. Biochemical identification and statistical analysis

Among the 140 isolated lactic acid bacteria, 115 strains belonged to the genus *Lactococcus*. A hierarchical clustering was done on the identification results of these lactococci (data not shown). It allowed to choose 18 strains according to their phenotypes and their origins (table 1). Characteristics of these strains corresponded to those described by Schleifer et al. [24] for the genus *Lactococcus*. Identical characters for all the strains were omitted for the calculation of similarity index among the strains using the Jaccard distance as they were not discriminating. Hierarchical clustering was actually performed with eight biochemical characters which were arginine deamination, citrate utilization, fermentation of ribose, D-xylose, mannitol, amygdaline, maltose and starch yielding a 21×8 matrix. At a similarity

level of 65%, 19 strains were classed into 3 major clusters (A, B, C). The 2 *Lc. lactis cremoris* (1 isolate and 1 reference strains) remained ungrouped (figure 1).

Some strains of the biovar *diacetylactis* belonging to cluster A were grouped with a 100% similarity level, although they originated from different biotopes. The two reference strains 076 and 075 were also considered identical as they only differed from each other in their proteolytic activity. This character was not taken into account in the identification.

Cluster B comprised the two remaining *Lc. lactis lactis diacetylactis* strains which were separated from the others because of their inability to ferment starch.

Some of the eight *Lc. lactis lactis* isolates belonging to cluster C were at 100% phenotypically similar although coming from different origins.

The two *Lc. lactis cremoris* were separated from the other strains because of a poor carbohydrate fermentation patterns with only few positive responses. Especially all the

eight biochemical characters tested gave negative responses for the CY1 isolate.

The reference strains 076, 075, 380 were classified into different clusters from their related isolates. Strains 076, 075 were closer to the *Lc. lactis lactis diacetylactis* isolates than the *Lc. lactis lactis* strains due to their inability to ferment D-xylose. The reference strain 380 differed from the isolate CY1 in being able to hydrolyse arginine and maltose.

3.2. Data analysis of the technological characterization

Hierarchical clustering performed on the technological data shown in table II led to the dendrogram illustrated in figure 2. At a similarity level of 65%, 17 strains were classed into three clusters (A', B', C'). Four strains CY1, 380, 075 and D57 remained ungrouped.

Group A' was composed of five strains which had the same technological properties as the typical Prt^+ *Lc. lactis lactis* 076.

Figure 1. Dendrogram obtained by hierarchical clustering of eight biochemical tests using the Jaccard distance with a mean linkage (STATITCF software version 5.0).

Figure 1. Dendrogramme obtenu par classification hiérarchique ascendante des résultats de huit tests biochimiques en utilisant la distance de Jaccard et la moyenne des distances pondérées (logiciel STATITCF version 5.0).

Table II. Technological characteristics and associated parameters for the 21 lactococci.**Tableau II.** Caractéristiques technologiques et paramètres associés pour les 21 lactocoques.

Strains	Growth					Acidification			Proteolysis	Flavor
	μ_1 (h ⁻¹)		μ_2 (h ⁻¹)		N_m (log cfu·mL ⁻¹)	-Vmar (pH unit·h ⁻¹)	dpH (unit pH)	Gly μmol of Gly·10 ⁻¹⁰ cfu)	Dia (mg·L ⁻¹)	
	value	a	value	a		c				
076	1.52	0.02	1.06	0.07	9.38	0.51	0.02	2.34	9.37	—
L36	1.40	0.04	1.22	0.05	9.34	0.49	0.02	2.35	7.56	—
L45	1.31	0.01	1.15	0.01	9.30	0.47	0.02	2.32	8.62	—
L68	1.39	0.02	0.81	0.01	9.26	0.48	0.02	2.33	9.98	—
LF6	1.35	0.09	1.03	0.03	9.25	0.40	0.02	2.37	8.33	—
LS6	1.39	0.03	1.07	0.01	9.34	0.47	0.02	2.30	8.11	—
380	1.26	0.04	0.87	0.03	9.00	0.34	0.03	2.25	21.50	—
CY1	1.51	0.02	0.81	0.03	9.00	0.35	0.01	2.34	24.30	—
075	1.50	0.04	—	—	8.04	0.03	—	0.30	0.00	—
L84	1.25	0.02	—	—	8.28	0.03	—	0.32	0.00	—
L86	1.07	0.04	—	—	8.20	0.03	—	0.34	0.00	—
L58	1.26	0.02	—	—	8.28	0.03	—	0.32	0.00	—
D57	1.20	0.02	—	—	8.15	0.04	—	0.47	0.00	1.83 (0.43) ^b
D58	1.21	0.02	—	—	8.18	0.03	—	0.39	0.00	0.78
D59	1.12	0.09	—	—	8.18	0.03	—	0.37	0.00	0.73
D11	1.48	0.04	—	—	8.11	0.03	—	0.42	0.00	0.99
D18	1.55	0.07	—	—	7.61	—	0.33	0.00	0.87	—
D22	1.09	(0.09) ^b	—	—	7.76 (0.04) ^b	—	—	0.29 (0.05) ^b	0.00	0.56
D25	1.37	0.03	—	—	7.74	0.03	—	0.35	0.00	1.14
DF7	1.20	0.03	—	—	8.11	0.04	—	0.47	0.00	0.68 (0.08) ^b
DP7	1.48	0.05	—	—	8.11	0.03	—	0.36	0.00	1.17 (0.10) ^b

^a Growth rate confidence limit at 95%; ^b average of three repetitions and mean standard deviation; ^c mean acidification rate confidence limit at 95%.

^a Intervalle de confiance à 95 % du taux de croissance ; ^b moyenne de trois essais et écart type de la moyenne ; ^c intervalle de confiance à 95 % de la vitesse moyenne d'acidification.

Figure 2. Dendrogram obtained by hierarchical clustering of seven normed technological characterization results using the euclidian distance with a mean linkage (STATITCF software version 5.0).

Figure 2. Dendrogramme obtenu par classification hiérarchique ascendante des résultats normés de sept tests technologiques en utilisant la distance euclidienne et la moyenne des distances pondérées (logiciel STATITCF version 5.0).

They rapidly acidified milk and were proteolytic. Their exponential growth phase was biphasic and their maximum population level high (*table II*).

Strains CY1 and 380 were clustered together. Both strains were acidifying and strongly proteolytic. Because of their higher proteolytic activity, these strains were separated from the others. Nevertheless, they resembled strains belonging to cluster A' in their growth kinetics and their acidifying activity. They also displayed two exponential growth phases but with a second exponential growth rate, μ_2 , lower than that of strains of cluster A' (*table II*). Their maximum population was also lower. Consequently, they acidified milk less rapidly. However, after 12 h of growth, the pH decreased from 6.67 down to 4.37, a value which was quite similar to the strains of cluster A'.

Strains belonging to clusters B' and C' and strains 075 and D57 exhibited similar growth, acidifying and proteolytic characteristics. Their exponential growth phase

was monophasic. After 12 h of incubation, they acidified milk down to 6.31 only. This low acidifying activity did not allow to calculate a mean acidification rate. Moreover, measurement of proteolysis by the TNBS method suggested a Prt- phenotype. In fact, these strains were classed into different clusters because of the intensity of their flavoring activity and, to a lesser extent, their maximum population. Strains belonging to cluster B' produced amounts of diacetyl superior to $0.85 \text{ mg} \cdot \text{L}^{-1}$ and reached at a mean $7.7 \cdot 10^7 \text{ cfu} \cdot \text{mL}^{-1}$ at the stationary phase. Strains belonging to cluster C' produced quantities of diacetyl inferior to $0.80 \text{ mg} \cdot \text{L}^{-1}$ and reached a mean population of $1.4 \cdot 10^8 \text{ cfu} \cdot \text{mL}^{-1}$.

Strain D57 differed from the others in producing relatively high amounta of diacetyl (*table II*).

According to this analysis, two major groups can be defined. The first one corresponds to cluster A' and strains 380, CY1 and the second one to clusters B', C' and

strains 075, D57. On the opposite to the second group, the first group was characterized by high acidifying and proteolytic strains.

Comparison of the two dendrograms (figures 1 and 2) shows that the major difference between the two clusterings lies on the proteolytic character. For instance, the *Lc. lactis lactis* isolates which were rather biochemically homogeneous were divided into two different technological groups according to their proteolytic activity. The weakly acidifying and non proteolytic *Lc. lactis lactis* strains were clustered with the *diacetylactis* biovar strains which were

also non proteolytic and produced few amounts of diacetyl. Concerning the *Lc. lactis lactis diacetylactis* isolates, they were mainly clustered according to the intensity of their flavoring activity.

3.3 Principal component analyses

Principal Component Analysis was performed on the results of the technological characterization of the 21 lactococci (tables II and III) and revealed two different groups corresponding to the acidifying and proteolytic bacteria and the weakly acidifying and

Table III. Specific peptidasic activities of the 21 lactococci.

Tableau III. Activités spécifiques peptidasiques des 21 souches de lactocoques.

Strains	Peptidasic activities		
	PepN + PepC (activity unit.mg ⁻¹ protein) ^a	PepC (activity unit.mg ⁻¹ protein) ^b	PepX (activity unit.mg ⁻¹ protein) ^a
076	0.29	8.86	68.2
L36	0.19	7.10	45.1
L45	0.28	6.19	25.9
L68	0.45	13.2	55.4
LF6	0.32	8.70	46.4
LS6	1.14 (0.43) ^c	7.82	25.6 (0.21) ^c
380	2.70	20.7	49.8
CY1	12.9 (2.47) ^c	125 (8.45) ^c	65.9
075	0.33	4.62	147.5
L84	0.34	14.6	31.0
L86	0.78	4.74	66.7
L58	1.42	15.3	46.0
D57	1.32 (0.40) ^c	3.85 (0.80) ^c	74.4
D58	0.60	11.3	76.1
D59	0.21	5.37	20.7
D11	0.63	14.1	84.3
D18	0.43	31.0	139
D22	0.44	6.47	204 (62.6) ^c
D25	0.24	7.53	104
DF7	0.35	7.44	55.4
DP7	0.18	1.86	166

^a 1 unit of activity = amount of enzyme producing an absorbance variation of 0.01 unit.min⁻¹ at 405 nm and 30 °C; ^b 1 unit of activity = amount of enzyme producing an absorbance variation of 0.01 unit in 1 h at 550 nm and 30 °C; ^c average of three repetitions and mean standard deviation.

^a 1 unité d'activité = quantité d'enzyme produisant une variation d'absorbance de 0,01 unité.min⁻¹ à 405 nm et à 30 °C ; ^b 1 unité d'activité = quantité d'enzyme produisant une variation d'absorbance de 0,01 unité en 1 h à 550 nm et à 30 °C ; ^c moyenne de trois essais et écart type de la moyenne.

Figure 3. Principal component analysis for the eight acidifying and proteolytic lactococcal strains for nine technological tests (STATITCF software version 5.0). **a)** Technological parameter position onto the plane (1, 2). **b)** Strain projection onto the plane (1, 2). μ_1 , μ_2 (h^{-1}) first and second exponential growth rates, N_m ($\log \text{cfu} \cdot \text{mL}^{-1}$) log of the maximum population, V_{mar} ($\text{pH unit} \cdot \text{h}^{-1}$) mean acidification rate, dpH (unit pH) pH difference after 12 h, Gly ($\mu\text{mol of Gly} \cdot 10^{-10} \text{cfu}$) proteolysis after 24 h, pN, pC, pX (activity $\text{unit} \cdot \text{mg}^{-1}$ protein) specific activities of peptidases PepN, PepC and PepX, respectively

Figure 3. Analyse en composantes principales pour les huit souches de lactocoques acidifiants et protéolytiques pour neuf tests technologiques (logiciel STATITCF version 5.0). **a)** Position des paramètres technologiques dans le plan (1, 2). **b)** Projection des souches sur le plan (1, 2). μ_1 , μ_2 (h^{-1}) taux de croissance respectifs de la première et de la seconde phase exponentielle de croissance, N_m ($\log \text{ufc} \cdot \text{mL}^{-1}$) log de la population maximale, V_{mar} (unité $\text{pH} \cdot \text{h}^{-1}$) vitesse moyenne d'acidification rapide, dpH (unité pH) différence de pH après 12 h, Gly ($\mu\text{mol de gly} \cdot 10^{-10} \text{ufc}$) protéolyse après 24 h, pN, pC et pX (unité activité $\cdot \text{mg}^{-1}$ protéine) activités spécifiques des peptidases PepN, PepC et PepX, respectivement.

non proteolytic ones. Therefore, two PCA were performed, one on each group.

The first PCA was carried out on the results of the characterization of the eight acidifying and proteolytic strains according to nine technological parameters: μ_1 , μ_2 , N_m , V_{mar} , dpH , Gly, PepN, PepC and PepX. Diacetyl production was not taken into account because these strains were unable to ferment citrate. To discriminate the strains, the first two axes were used since they explain 83% of the information, the first axis deals with 57% and the second one with 26%. N_m , V_{mar} and μ_2 are linked to the axis 1 and are on the opposite to Gly, PepN and PepC.

Both μ_1 and dpH are associated to the axis 2 (figure 3a).

Most strains were rather technologically homogeneous except strains 380 and CY1 (figure 3b). These strains particularly differed in their strong proteolytic activity and, to a lesser extent, in their lower μ_2 , N_m and V_{mar} . Strain CY1 is separated from strain 380 because of its higher peptidasic activities. L68 and LF6 strains are not illustrated very well in this projection.

The second PCA was performed on the technological characterization results of the 13 weakly acidifying and non proteolytic

Figure 4. Principal component analysis for the 13 low acidifying and non proteolytic lactococcal strains for seven technological tests (STATITCF software version 5.0). **a)** Technological parameter position onto the plane (1, 2). **b)** Strain projection onto the plane (1, 2). μ_1 , N_m , dpH , pN , pC , pX , symbols as described in figure 3, Dia ($mg \cdot L^{-1}$) diacetyl production after 24 h.

Figure 4. Analyse en composantes principales pour les 13 souches de lactocoques peu acidifiants et non protéolytiques pour sept critères technologiques (logiciel STATITCF version 5.0). **a)** Position des paramètres technologiques sur le plan (1, 2). **b)** Projection des souches sur le plan (1, 2). μ_1 , N_m , dpH , pN , pC et pX , symboles tels que décrits figure 3, Dia ($mg \cdot L^{-1}$) production de diacétyl après 24 h.

lactococcal strains according to seven parameters: μ_1 , N_m , dpH , Dia , $PepN$, $PepC$ and $PepX$. μ_2 and V_{mar} were omitted from this data analysis because they could not be calculated for these strains.

In this analysis, the first three axes were kept. All together they explain 77% of the information divided in 36% for axis 1, 24% for axis 2 and 17% for axis 3. μ_1 , $PepX$ and N_m are linked to axis 1 (figure 4a). The second axis allows to discriminate the strains according to their production of diacetyl and their acidifying activity. The third axis allows to compare the strains on their $PepC$ activity (figure 5a). $PepN$ parameter is both linked to the axes 1 and 3.

In the plane (1, 2), groups of strains are not easily distinguishable (figure 4b). However, the *Lc. lactis lactis* strains L84, L58, L86 are more technologically homogeneous than the *Lc. lactis lactis diacetylactis* isolates which are scattered in the plane. The 3 *Lc. lactis lactis* isolates were characterized by rather high population levels, low acidification and rather low $PepX$ activity. Strains 075 differed from them in its higher growth rate and $PepX$ activity but also in its weaker maximum population level. The *Lc. lactis lactis diacetylactis* growth rates were various and their maximum population levels were rather low. Their $PepX$ activity was high except for D59 strain. Strain D57 is apart

Figure 5. Principal component analysis for the 13 low acidifying and non proteolytic lactococcal strains (STATITCF software version 5.0). **a)** Technological parameter position onto the plane (2, 3). **b)** Strain projection onto the plane (2, 3). μ_1 , N_m , dpH, pN, pC, pX, Dia, symbols as mentioned in figures 3 and 4.

Figure 5. Analyse en composantes principales pour les 13 souches de lactococques peu acidifiants et non protéolytiques (logiciel STATITCF version 5.0). **a)** Position des paramètres technologiques sur le plan (2, 3). **b)** Projection des souches sur le plan (2, 3). μ_1 , N_m , dpH, pN, pC et pX, Dia, symboles tels que mentionnés figures 3 et 4.

from the others because of its flavoring and acidifying characters.

In the plane (2, 3), strains are also scattered (figure 5b). This illustrates the diversity of their technological behavior, especially their various PepC activity as shown by strain D18.

4. DISCUSSION AND CONCLUSION

Hierarchical clustering allows to illustrate strain similarities or differences thanks to dendrograms. Some strains (DF7, D57 and 076, 075) were classified into different clusters from their related subspecies (figure 1)

because of their inability to ferment starch or D-xylose. Utilization of these carbohydrates is reported to be variable among the strains of *Lc. lactis lactis* and *Lc. lactis lactis diacetylactis* [24]. The reference strain 380 differed from the isolate CY1 in atypical characters for *Lc. lactis cremoris* strains (arginine and maltose fermentations) [24]. These different profiles are likely to be due to the origins of the reference strains. Coming from a laboratory collection, they could have lost some of their original characters along with the successive transfers. Nevertheless, these reference strains belonged to the same technological groups as the industrial isolates. Therefore, they have kept at least some of their original technological

properties. Among the 58 biochemical characters under study, arginine deamination and citrate utilization were the most discriminating criteria. The lack of arginine dihydrolase activity is a common feature which helps to discriminate between the subspecies *lactis* and *cremoris*. Citrate metabolism is also a major taxonomic result for identifying the biovar *diacetylactis* [24].

Comparison of the two dendrograms (figures 1 and 2) shows that the biochemical and the technological clusters mainly differ in the proteolytic activity of the strains.

Furthermore, the two major groups defined after hierarchical clustering of the technological results (figure 2) gives evidence for a relation between the population level attained by the strains, their acidifying character and their proteolytic activity. Proteolytic ability of strains explains the differences in growth in milk. During their growth in milk, Prt⁺ strains can hydrolyze casein. Consequently, they develop high population levels and high acidification parameters in comparison to Prt⁻ strains that stop growing when the source of milk non-protein nitrogen is consumed [14, 15]. Therefore, proteolytic activity is required for milk acidification. Nevertheless, despite the strong proteolytic characters of strains 380 and CY1, their acidifying activities (dpH, V_{mar}) were not significantly higher than that of the strains belonging to cluster A'. This suggests that acidification depends on others parameters than proteolytic activity (e.g., amount of lactate produced per cell, ability to maintain internal pH). From these hierarchical clusterings, three characters can be retained for starter classification: arginine deamination, citrate utilization along with proteolysis.

Multidimensional data analyses allow to select strains since these methods made it possible to compare simultaneously all the strains and all their technological characteristics [2]. Moreover, PCA gives further information to the hierarchical clustering

method. In this study, PCA showed the diversity of the technological behaviors of the aroma-producing bacteria whereas the *Lc. lactis lactis* strains were more homogeneous. This method also highlights particular technological characteristics such as the high proteolytic activities of strains 380, CY1 (figure 3b), the high diacetyl production of strain D57 (figure 4b) or the high specific peptidasic activities of strain D18 (figure 4b and 5b). However, in PCA, distances between the strains can be misunderstood because the graphical representation results from a projection of the strains into a plane. On the contrary, dendrograms illustrate a real distance between strains, once calculated. Thus, the two statistical methods are complementary. The hierarchical clustering helps grouping and classifying strains and PCA choosing and selecting strains for their technological properties.

Strain selection for industrial purposes must consider some specific technological activities such as acidification or flavoring. Some strains growing rapidly in milk, with a rapid acidification and a relatively low proteolysis, like the *Lc. lactis lactis* L36, are interesting for producing an acid curd. Besides these characters, *Lc. lactis lactis* Prt⁻ strains could be selected on their peptidase activities and their potential debittering functions. *Lc. lactis lactis* L58 seems to fit these characteristics, especially its potentially debittering PepN activity [25]. Other strains having good growth and high diacetyl production could be selected as aroma-producing strains. To that respect, *Lc. lactis lactis diacetylactis* D57 seems to be the most suitable strain.

In this study, we achieved to select some interesting strains for their acidifying, flavoring and peptidasic potentials by using statistical methods. In further work, we will characterize these strains in mixed cultures. This implies taking into account other selection criteria such as antibiosis activities and phage sensitivity in order to study compatibilities between strains.

ACKNOWLEDGMENTS

We gratefully thank Dr. M.J. Desmazeaud and Dr. C. Foucaud for their advice during this study.

REFERENCES

- [1] Bouillanne C., Desmazeaud M.J., Étude de quelques caractères de souches de *Streptococcus thermophilus* utilisées en fabrication de yoghourt et proposition d'une méthode de classement. Lait 60 (1980) 458-473.
- [2] Bouillanne C., Desmazeaud M.J., Classement de souches de *Lactobacillus bulgaricus* selon quelques caractères utilisés en fabrication du yoghurt, Association avec *Streptococcus thermophilus*, Sci Aliments 1 (1981) 7-17.
- [3] Canteri G., Les levains lactiques, in: Eck A., Gillis J.C. (eds.), Le Fromage de la science à l'assurance-qualité, Lavoisier Tec and Doc, Paris, 1997.
- [4] 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.
- [5] Cogan T.M., Les levains lactiques mésophiles, Une revue. Lait 60 (1980) 397-425.
- [6] Degorce-Dumas R., Goursaud J., Leveau J.Y., Analyse de composés volatils du yaourt par chromatographie en phase gazeuse-espace de tête (headspace), Ind Agro-Aliment 8 (1986) 805-808.
- [7] De Man J.C., Rogosa M., Sharpe M.E., A medium for the cultivation of Lactobacilli, J. Appl. Bacteriol. 23 (1960) 130-135.
- [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] Gibson T., Abd-el-Malek Y., The formation of carbon dioxide by lactic acid bacteria and *Bacillus licheniformis* and cultural method of detecting the process, J. Dairy Res. 14 (1945) 35-44.
- [10] Hassan A.I., Deschamps N., Richard J., Précision des vitesses de croissance des streptocoques lactiques dans le lait basées sur la méthode de dénombrement microbien par formation de colonies, Étude de référence avec *Lactococcus lactis*, Lait 69 (1989) 433-447.
- [11] Herrero M., Mayo B., Gonzalez B., Suarez J.E., Evaluation of technologically important traits in lactic acid bacteria isolated from spontaneous fermentations, J. Appl. Bacteriol. 81 (1996) 565-570.
- [12] Hitchener B.J., Egan A.F., Rogers P.J., Characteristics of lactic acid bacteria isolated from vacuum-packaged beef, J. Appl. Bacteriol. 52 (1982) 31-37.
- [13] Juillard V., Richard J., Étude de l'interaction entre souches protéolytiques de streptocoques lactiques mésophiles et leurs variants non protéolytiques, au cours de leur croissance dans le lait, Lait 69 (1989) 291-304.
- [14] Juillard V., Richard J., Indirect interaction in milk between proteolytic and isogenic non-proteolytic strains of *Lactococcus lactis*, I. Effect of pre-culturing by a non-proteolytic variant, Lait, 70 (1990) 425-438.
- [15] Juillard V., Richard J., Indirect interaction in milk between proteolytic and isogenic non-proteolytic strains of *Lactococcus lactis*, II. Effect of pre-culturing by a proteolytic strain, Lait, 71 (1991) 55-64.
- [16] Juillard V., Spinnler H.E., Desmazeaud M.J., Boquien C.Y., Phénomènes de coopération et d'inhibition entre les bactéries lactiques utilisées en industrie laitière, Lait 67 (1987) 149-172.
- [17] Juillard V., Le Bars D., Kunji E.R.S., Konings W.N., Gripon J.C., Richard J., Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk, Appl. Environ. Microbiol. 61 (1995) 3024-3030.
- [18] Kovacks N., Identification of *Pseudomonas pyocyanea* by the oxidase reaction, Nature 178 (1956) 703.
- [19] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.
- [20] Mathot A.G., Kihal M., Prevost H., Divies C., Selective enumeration of Leuconostoc on vancomycin agar media, Int. Dairy J. 4 (1994) 459-469.
- [21] Mc Kellar R.C., Development of off-flavors in ultra-high temperature and pasteurized milk as a function of proteolysis, J. Dairy Sci. 64 (1981) 2138-2145.
- [22] 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.
- [23] 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. Alim. Nutr. 12 (1994) 437-442.
- [24] Schleifer K.H., Kraus J., Dvorak C., Kilpper-Bälz R., Collins M.D., Fischer W., Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov., System. Appl. Microbiol. 6 (1985) 183-195.

- [25] Tan P.S.T., Van Kessel T.A.J.M., Van de Veer-donk F.L.M., Zuurendonk P.F., Bruins A.P., Konings W.N., Degradation and debittering of a tryptic digest from β -casein by aminopeptidase N from *Lactococcus lactis* subsp. *cremoris* Wg2, Appl. Environ. Microbiol. 59 (1993) 1430–1436.
- [26] Terzaghi B.E., Sandine W.E., Improved medium for lactic streptococci and their bacteriophages, Appl. Microbiol. 29 (1975) 807–813.
- [27] Zourari A., Desmazeaud M.J., Caractérisation de bactéries lactiques thermophiles isolées de yaourts artisanaux grecs, II. Souches de *Lactobacillus delbrueckii* subsp. *bulgaricus* et cultures mixtes avec *Streptococcus salivarius* subsp. *thermophilus*, Lait 71 (1991) 463–482.
- [28] Zourari A., Roger S., Chabanet C., Desmazeaud M.J., Caractérisation de bactéries lactiques thermophiles isolées de yaourts artisanaux grecs. I. Souches de *Streptococcus salivarius* subsp. *thermophilus*, Lait 71 (1991) 445–461.