Selection of dairy *Leuconostoc* isolates for important technological properties

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Summary. Twenty-four dairy *Leuconostoc* strains isolated from French commercial starters and four reference strains were screened for 58 morphological and biochemical characters. Hierarchical clustering analysis was performed on the results of dextran production and carbohydrate fermentation tests. Most strains fitted into three clusters at a similarity level of 65% and were identified as belonging to the species *lactis* or *mesenteroides*. Fourteen strains were then selected and further characterized for their technological properties. They were examined for growth, acidification kinetics and proteolytic capacity when growing in milk, and peptidase activities and diacetyl production after growth in MRS broth. Principal component analysis was carried out to group strains and compare their technological properties. This allowed selection of strains of dairy interest. Special attention was given to the flavouring capacity of *Leuconostoc lactis* isolates that could be useful in designing commercial starters.

Leuconostoc spp. are commonly used as aroma producers in mesophilic starters for the production of fresh or soft cheeses and fermented milks (Vedamuthu, 1994). They metabolize citrate and produce volatile compounds, diacetyl being the major flavour compound in cultured dairy products (Cogan & Jordan, 1994). These microorganisms are always cultured in association with an acid-producing *Lactococcus lactis* strain to encourage their flavouring properties to develop. During growth in milk, interactions between strains occur that lead to considerable variations in the composition of the starter (Bellengier *et al.* 1997*b*). As the technological properties of the starter might be affected, optimization of process control first requires improved characterization of the strains.

Leuconostoc strains of dairy origin have seldom been characterized. Recently, growth and acidification capacities have been determined in milk (Demirci & Hemme, 1994; Bellengier *et al.* 1997*a*). Biochemical properties such as citrate metabolism, esterase activities and carbohydrate fermentation have been evaluated (Bellengier *et al.* 1994; Herrero *et al.* 1996; Levata-Jovanovic & Sandine, 1996; Vafopoulou-Mastrojiannaki *et al.* 1996). Bacteriocin production and plasmid content have been described (Johansen & Kibenich, 1992; Herrero *et al.* 1996). Combined studies including both growth and acidification capacities together with biochemical features, and in particular flavouring capacities, have never been reported.

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Thus, in the present study, we first screened 24 *Leuconostoc* strains isolated from French commercial starters for biochemical characters. We then investigated relevant technological traits of 14 strains of potential industrial interest. Finally, comparison of strains on the basis of statistical analysis of these results contributed to selection for their flavouring capacities.

MATERIALS AND METHODS

Bacterial strains

Dairy *Leuconostoc* strains were isolated from four commercial starters on MRS agar medium (De Man *et al*. 1960), on MRS agar medium with additional vancomycin (20 mg/l) (Mathot *et al*. 1994) or containing maltose and NaCl (40 g/l) instead of glucose. Four reference strains were obtained from the CNRZ collection (INRA, F-78350 Jouy-en-Josas, France) and from the Pasteur Institute collection (F-75724 Paris, France). Strains were stored at −20 °C in reconstituted (100 g/l) sterile litmus milk supplemented with 5 g glucose/l and 5 g yeast extract/l.

Morphological and biochemical tests for strain identification

Bacterial cultures were routinely propagated in MRS broth. Working media were inoculated with exponentially grown cells washed in tryptone–salt broth (Server-Busson *et al*. 1998) and incubation was at 30 °C.

Strains were identified as described by Server-Busson *et al*. (1998). Fifty-eight phenotypic features were tested including cell morphology, Gram staining, catalase and oxidase activities (Kovacks, 1956), CO_2 production from glucose (Gibson & Abdel-Malek, 1945) and L-arginine dihydrolase activity (Hitchener *et al*. 1982). Lactic acid configuration and citrate utilization were determined enzymically (Boehringer Mannheim, D-68305 Mannheim 31, Germany); acetoin production was detected by the Voges-Proskauer method (Barritt, 1936); dextran production was tested by growing strains on a sucrose (100 g/l) agar medium (Mayeux *et al*. 1962); carbohydrate fermentation was tested by the API 50 CH test system according to the manufacturer's instructions (Biomérieux, F-69280 Marcy l'Etoile, France). Lactose fermentation was also estimated on MRS agar medium overlaid with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 mg/ml in dimethylformamide; Sambrook *et al*. 1989).

Technological tests

The growth medium was reconstituted (100 g/l) milk (low-heat skim milk powder; Nilac, NIZO, NL-6710 BA Ede, The Netherlands) in sterile water. It was inoculated with $\sim 5 \times 10^6$ cfu/ml in the exponential phase in the same medium containing glucose and yeast extract (5 g/l of each). Incubation was at 30 °C.

Technological characterization was carried out as described by Server-Busson *et al*. (1998). Growth was assessed by bacterial enumeration on MRS agar medium (Bellengier *et al*. 1997*a*) and characterized by the maximum population level, N_m (log (cfu/ml)), and the growth rate, μ (/h). Acidification was estimated by calculating the pH difference (ΔpH) after 12 h growth. Proteinase activity was assessed qualitatively by plating bacterial cultures on to Fast–Slow Differentiating Agar medium (Huggins & Sandine, 1984). Milk proteolysis was measured using 2,4,6-trinitrobenzenesulphonic acid (McKellar, 1981) and expressed as the number of free amino groups (μmol Gly/10^{10} cfu) in the fraction of milk soluble in 0·72 M-trichloroacetic acid.

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Cells were grown in MRS broth to the stationary phase and peptidase activities (PepN, PepC and PepX) were measured in cell-free extracts using chromogenic substrates (Juillard *et al*. 1995). Specific activity was defined as units/mg protein. Protein content of the cell-free extracts was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Flavouring activity was expressed as the amount of diacetyl in mg/l produced by non-proliferating cells after 24 h at 30 °C. Bacterial cultures were centrifuged (8000 ***g***, 4 °C, 15 min) at the end of the exponential growth phase in MRS broth containing 5 g lactose/l and washed twice with 150 mM-sodium acetate buffer, pH 5·3. The cell pellet was resuspended in the same buffer containing 8·2 mM-sodium citrate and 0·1 mM-lactose to a final population of 1×10^9 cfu/ml (Cogan *et al*. 1981). Diacetyl was measured by the static headspace gas chromatography technique on a Perkin-Elmer Sigma 300 FID chromatograph (Perkin-Elmer Sigma, Norwalk, CT 06859, USA) as described by Server-Busson *et al*. (1998).

Statistical analysis

All identification tests were repeated twice. Carbohydrate fermentation profiles obtained from the API 50 CH tests were analysed by APILAB plus software (Biomérieux). Results of the biochemical tests were coded as '1' (positive or moderate response) or '0' (negative or weakly positive response). Statistical analysis of these results was carried out by hierarchical clustering using the Jaccard distance with a mean linkage (Sneath, 1957). Identical characters for all the strains were omitted for the calculation of similarity using the Jaccard distance since they were not discriminating. Stat-ITCF software (Institut des Céréales et des Fourrages, F-75016 Paris, France) was used.

Mean values with SD for technological characteristics were calculated on the basis of three independent experiments. Results of the technological tests were analysed by principal component analysis using Stat-ITCF software.

RESULTS

Identification and characterization of the strains

Twenty-four *Leuconostoc* strains were first isolated from commercial starters (Table 1). Their characteristics corresponded to those described by Garvie (1986). All strains metabolized citrate, although acetoin production was not detected by the Voges-Proskauer test. Three carbohydrate substrates (e.g. glucose, galactose and *N*-acetylglucosamine) were fermented by all the strains whereas 25 substrates always gave negative results. No strains except the reference strains 523 and 468 produced dextran from sucrose. Hierarchical clustering was performed on the results of 20 discriminating biochemical traits that gave positive results with at least one strain (Table 2). The dendrogram shown in Fig. 1 illustrates the results of this statistical analysis.

At a similarity level of 65%, 25 strains were classified into three clusters (A, B and C; Fig. 1). Two reference strains (468 and 009) and one isolate (A54) remained unclustered.

Cluster A was rather heterogeneous and consisted of ten strains partly identified as *Leuconostoc* spp. and two reference strains, 422 and 523. The APILAB plus software designated these isolates as *Leuconostoc mesenteroides* strains belonging to either subsp. *mesenteroides* or subsp. *dextranicum*. As they all failed to metabolize

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Table 1. *Origins and identification of the* Leuconostoc *strains investigated in this study*

Strain	Origin	Identification
A51	Starter A	*Leuconostoc lactis*
A52	Starter A	*Ln. lactis*
A53	Starter A	*Ln. lactis*
A54	Starter A	*Ln. lactis*
A55	Starter A	*Ln. lactis*
A56	Starter A	*Ln. lactis*
A57	Starter A	*Ln. lactis*
A58	Starter A	*Ln. lactis*
A59	Starter A	*Ln. lactis*
A03	Starter A	*Ln. lactis*
I58	Starter A	*Ln. lactis*
I59	Starter A	*Ln. lactis*
B21	Starter B	*Leuconostoc* sp.§
B22	Starter B	*Leuconostoc* sp.§
B23	Starter B	*Leuconostoc* sp.§
B24	Starter B	*Leuconostoc* sp.§
B25	Starter B	*Leuconostoc* sp.§
B26	Starter B	*Leuconostoc* sp.§
B28	Starter B	*Leuconostoc* sp.§
I21	Starter B	*Leuconostoc* sp.§
I51	Starter A	*Leuconostoc* sp.§
I52	Starter A	*Leuconostoc* sp.§
MR1	Starter D	*Ln. mesenteroides* subsp. *cremoris*
MZ1	Starter C	*Ln. mesenteroides* subsp. *cremoris*
422	PIC 102422 †	*Ln. lactis*
523	NCDO 523 †	*Ln. mesenteroides* subsp. *mesenteroides*
468	CNRZ 1468 ‡	*Ln. mesenteroides* subsp. *dextranicum*
009	PIC 103009 †	*Ln. mesenteroides* subsp. *cremoris*

† From the collection of the Pasteur Institute (F-75724 Paris, France).
‡ From the CNRZ collection (INRA, F-78350 Jouy-en-Josas, France).
§ *Ln. mesenteroides* or *Weissella paramesenteroides*, formerly *Ln. paramesenteroides* (Collins *et al*. 1993).

L-arabinose, they were more likely to be strains of *Ln. mesenteroides* subsp. *dextranicum* (Garvie, 1986). However, on the basis of their inability to produce dextran, they may also be *Weissella paramesenteroides* strains. Most strains belonging to this cluster metabolized fructose (except B22 and B23), sucrose (except B26, I51 and I52), maltose, mannose and trehalose.

Cluster B comprised 11 isolates identified as *Ln. lactis*. At a similarity level of 65%, *Ln. lactis* isolate A54 was not classified with these strains because it had a distinctive carbohydrate fermentation pattern, being unable to ferment ribose, D-xylose or α-methyl-D-glucoside.

Cluster C contained two *Ln. mesenteroides* subsp. *cremoris* isolates which were 100% phenotypically similar although their origins were different. These strains displayed very distinctive carbohydrate fermentation profiles with only lactose, glucose, galactose and *N*-acetylglucosamine being fermented.

The reference strains 422, 468 and 009 were classified into different clusters from their related isolates. *Ln. lactis* 422 differed from the isolates identified as *Ln. lactis* and clustered in group B mainly owing to its ability to ferment sucrose, β-gentiobiose, turanose, cellobiose and trehalose. The last two features were atypical for *Ln. lactis* strains. *Ln. mesenteroides* subsp. *cremoris* 009 differed from the isolates MR1 and MZ1 in fermenting sucrose but not lactose. *Ln. mesenteroides* subsp. *dextranicum* 468 remained unclassified when clustering at a 65% similarity level

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Table 2. *Differentiating traits of the* Leuconostoc *strains used for the hierarchical clustering analysis*

Strain	Dextran	Ribose	D-xylose	Fructose	Mannose	L-arabinose	α-Methyl-D-glucoside	Aesculin	Salicine	Cellobiose	Lactose	Melibiose	Sucrose	Trehalose	D-raffinose	Starch	β-Gentiobiose	Turanose	Gluconate	2-Oxo-gluconate
A51, A57, A58, A59	–	+/–	+/–	+	+	–	+	+/–	–	–	+	+	–	–	–	–	–	–	–	–
A52	–	+	+	+	+	–	+	–/+	–	–	+	+	–	–	–	–	–	–	–	–
A53	–	+/–	+/–	+	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	+/–
A54	–	–	–	+	+	–	–	–	–	–	+	+	–	–	+	–	–	–	–	–
A55	–	+/–	+/–	+	+	–	+	–/+	–	+/–	+	+	–	–	+	–	–	–	–	–
A56	–	+/–	+/–	+	+	–	+	–/+	–	–	+	+	–	+	+	–	–	+	–	–
A03	–	+	+	+	+	–	+/–	–	–	–	+	+	–	–	+	–	–	–	–	–
I58	–	+/–	+/–	+	+	–	+	+	–	–	+	+	–	–	+	–	–	–	–	–
I59	–	+/–	+/–	+	+	–	+	+/–	–	–/+	+	+	–	–	+	–	–	–	–	–
B21, B28, 422	–	+	+	+/–	+	–	+	+	–	+/–	+	+	+	+	+	–	+/–	+	–	–
B22, B23	–	+	+	–	+	–	+	+	–	+	+	+	+	+	+	–	+/–	+	–	–
B24	–	+	+	+	+	–	+	+	+/–	+	+	+	+	+	+	–	+/–	+	–	–
B25	–	–	+	+	+	–	+	+	–/+	+	+	+	+/–	+	–	–	+/–	+	–	–
B26	–	+	–	+	+	–	+	+/–	+	+	+	+	–	+	–	+/–	+	+	–	–
I21	–	+	+	+/–	+	–	+	+	–/+	+/–	+	+	+	+	+	+/–	+/–	+	–/+	–
I51	–	+/–	+/–	+	+	–	+/–	+	–	+/–	+	+	–	+	+	–	+/–	–	–	–/+
I52	–	+/–	+/–	+	+	–	+/–	–	–	+	+	+	–	+	+	–	+/–	–	–	+/–
MR1, MZ1	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
523	+	+/–	+	+	+	+/–	+	+	+/–	+	+	+	+	+	+	–	+	+	–/+	–/+
468	+	+/–	+	+	+	–	+	–	–	–	–	–	+	+	–	–	–	–	+/–	–/+
009	–	–	–	–	–	–	–	–	–	–	–	–	+/–	–	–	–	–	–	–	–

+, Positive response; –, negative response; +/–, medium positive response; –/+, weakly positive response.

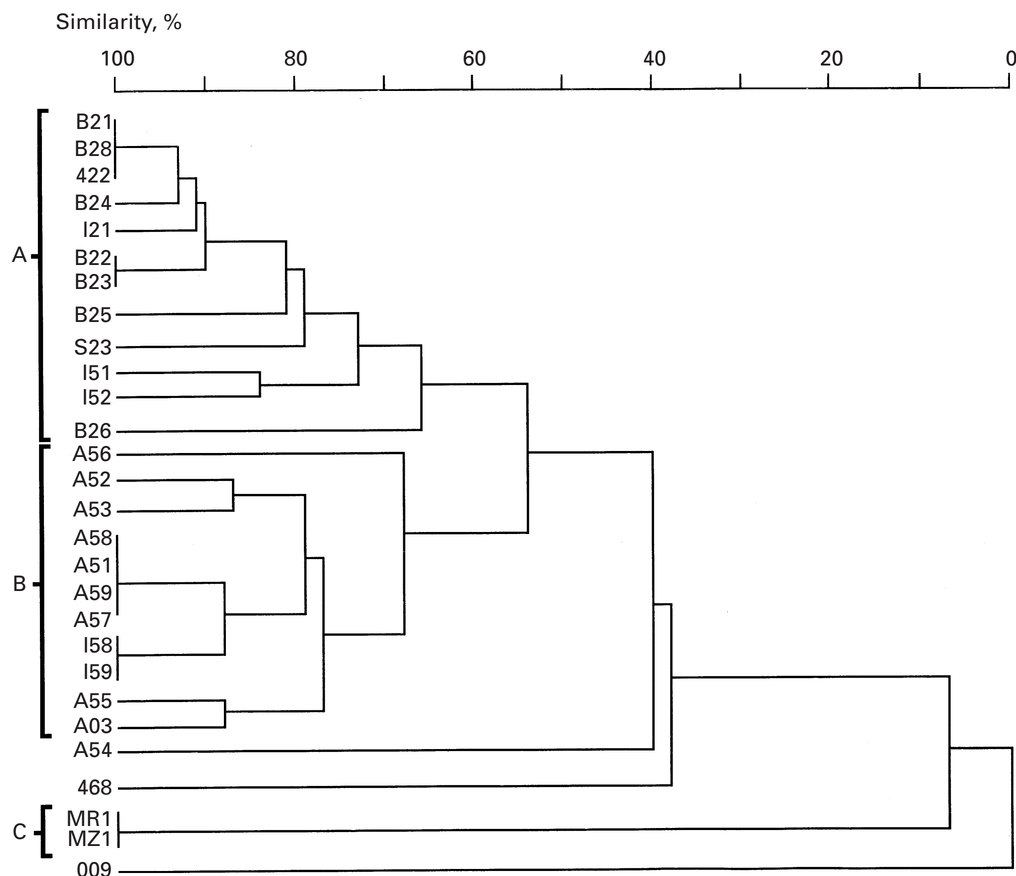

Fig. 1. Dendrogram obtained by hierarchical clustering of 20 biochemical tests on *Leuconostoc* isolates using the Jaccard distance with a mean linkage (Stat-ITCF software). See Table 1 for identification of strains. Clusters A, B and C were defined at a 65% similarity level. Cluster A contained ten strains partly identified as *Leuconostoc* sp. and two reference strains, cluster B strains were identified as *Ln. lactis* and cluster C contained two *Ln. mesenteroides* subsp. *cremoris* strains and one reference strain. For detailed discussion, see text.

mainly because of its inability to ferment lactose and its ability to produce dextran. These different profiles may be related to the laboratory origins of the reference strains.

On the basis of this clustering, 14 strains were then chosen for their phenotypes and were further characterized.

Principal component analysis of the technological characterization results

The results of the technological characterization of the 14 *Leuconostoc* strains are shown in Table 3. Principal component analysis was carried out on the results of seven technological characteristics: μ, N_m, ΔpH, diacetyl production, and the activities of PepN (PepN+PepC), PepC and PepX. As proteolytic activity was not significant for all strains (i.e. not discriminating), it was omitted from the analysis.

To discriminate among the strains, the first three axes were used. The first axis dealt with 50% of the information, the second with 20% and the third with 16%. Axis 1 made it possible to differentiate strains according to their growth and

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Table 3. *Technological characteristics and associated characteristics for 14* Leuconostoc *strains*

Strains	Growth		Acidification, ΔpH	Proteolysis, μmol Gly/10^{10} cfu	Flavour, Dia	Peptidase activities, units/mg protein†		
	μ	N_m				PepN+PepC (a) Lys-pNA‡	PepC (b) Ala-βNA‡	PepX (a) Ala-Pro-pNA‡
A51	0·97	8·24	0·39	0·08	1·15	2·26	24·49	0·66
A52	0·97	8·26	0·41	0·04	2·26	1·44	25·94	2·99±0·45§
A53	0·92	8·18	0·37	−0·06	< 0·50	1·85	25·64	1·42
A55	1·19	8·19	0·38	0·03	< 0·50	1·85	27·13±4·53§	1·37
A56	0·95	8·33	0·36	0·03	< 0·50	3·75	24·48	0·92
A59	1·03±0·05§	8·29±0·09§	0·41±0·05§	0·02±0·01§	3·58±0·68§	1·52	23·85	1·69
A03	1·12	8·07	0·35	0·08	0·69	2·56	26·58	2·03
B21	0·56	7·04	0·07	ND	0·67	1·35	19·91	1·14
B22	0·60±0·12§	7·44±0·28§	0·15±0·02§	ND	1·60	1·67	22·06	2·74
B24	0·72	7·53	0·12	ND	0·71±0·08§	0·99	13·69	1·45
B25	0·91	8·24	0·38	0·03	1·47±0·39§	3·04±0·13§	17·97±3·56§	7·48±0·32§
B26	0·95	8·21	0·37	−0·03	0·76	1·16	21·42	0·67
MZ1	0·87	7·53	0·40	0·02	< 0·50	1·14	19·80	1·19
422	0·75	7·49	0·13	ND	2·10	1·30	18·59	1·36

μ, growth rate, /h; N_m, maximum population, log (cfu/ml); ΔpH, change in pH units/12 h; Dia, diacetyl production, mg/l; ND, not determined.

† A unit of activity is the amount of enzyme producing a change in (a) A_{405} of 0·01/min or (b) A_{550} of 0·01/h at 30 °C.

‡ Substrates were Lys-pNa, lysyl-p-nitroanilide; Ala-βNA, alanyl-β-naphthylamide; Ala-Pro-pNA, alanyl-prolyl-p-nitroanilide.

§ Means±SD for three independent experiments.

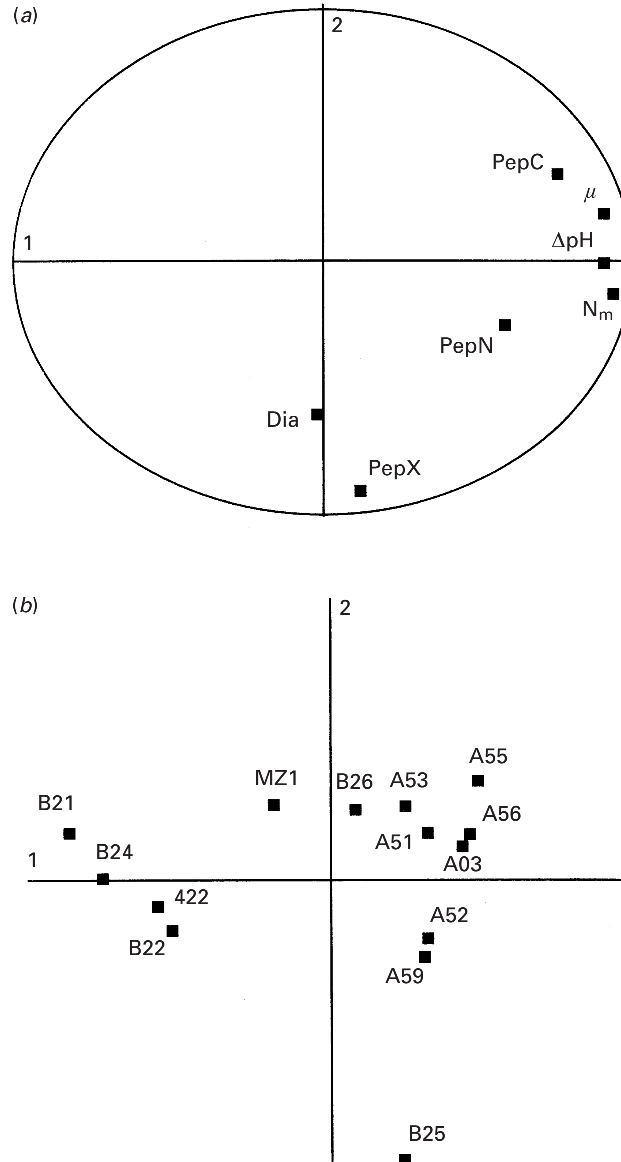

Fig. 2. Principal component analysis for 14 *Leuconostoc* strains for seven technological tests (Stat-ITCF software): plane (1, 2). (*a*) Technological characteristic position on plane (1, 2); (*b*) strain projection on plane (1, 2). μ, Growth rate; N_m, maximum population; ΔpH, change in pH; Dia, diacetyl production; PepN, PepC, PepX, enzyme activities. See Table 1 for identification of strains and Table 3 for units of measurements.

acidifying capacities in milk and their PepC activity (Fig. 2*a*). Axis 2 enabled us to compare strains according to their PepX activity and diacetyl production and axis 3 according to their PepN activity and, again, their diacetyl production (Fig. 3*a*). It should be noted that the flavouring character was linked to plane (2, 3). Plane (2, 3) was not taken into account because it dealt with only 36% of the information compared with 66% for plane (1, 3).

In plane (1, 2), two groups of strains were easily distinguishable (Fig. 2*b*). Strains B21, B22, B24 and 422 grew poorly in milk. Their mean growth rate was 0·66/h and

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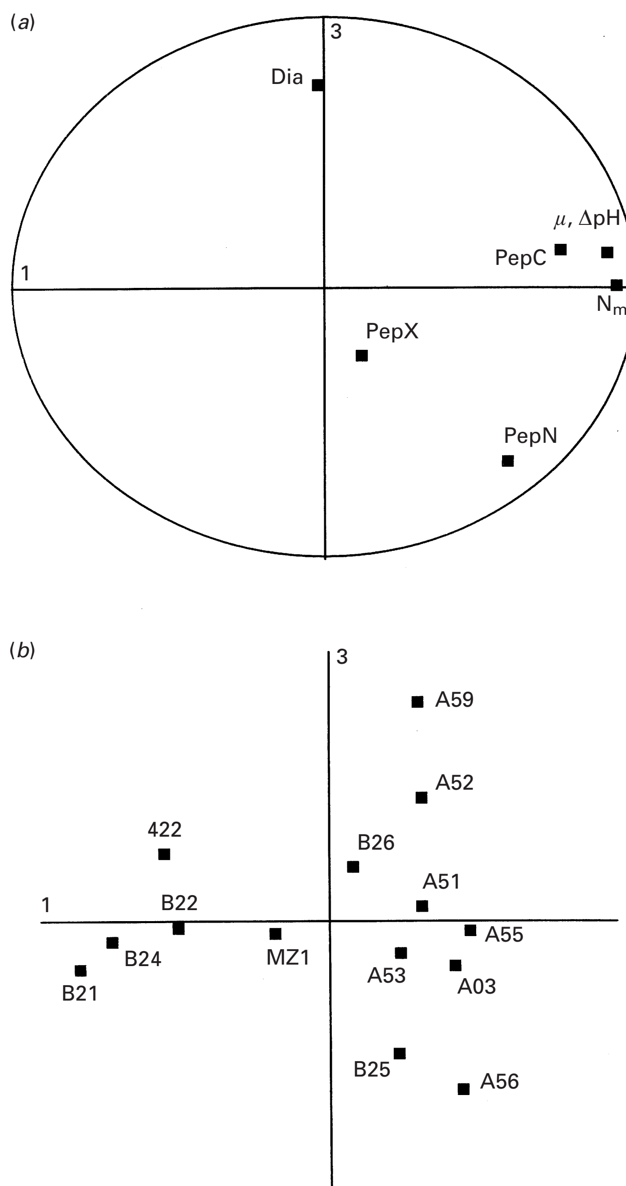

Fig. 3. Principal component analysis for the 14 *Leuconostoc* strains for seven technological tests (Stat-ITCF software): plane (1, 3). (*a*) Technological characteristic position on plane (1, 3); (*b*) strain projection on plane (1, 3). μ, Growth rate; N_m, maximum population; ΔpH, change in pH; Dia, diacetyl production; PepN, PepC, PepX, enzyme activities. See Table 1 for identification of strains and Table 3 for units of measurements.

their mean maximum population level only $2{\cdot}4 \times 10^7$ cfu/ml. Acidification of milk was $< 0{\cdot}2$ pH units/12 h (Table 3). These strains were also characterized by weak PepC activity, except for strain B22. Their flavouring activity was either weak (strains B21 and B24) or moderate (strains 422 and B22). In contrast, the seven *Ln. lactis* isolates and strains B25 and B26 were characterized by relatively good growth with a mean growth rate of 1/h and a maximum population level of $1{\cdot}7 \times 10^8$ cfu/ml (Table 3). Milk was slightly acidified (ΔpH up to 0·4 units/12 h) and did not

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coagulate after 24 h. PepC activity of most strains was high, except for strain B25, which was separated from the others mainly because of its higher PepX activity. Diacetyl production varied with the strain.

In plane (1, 3) (Fig. 3*b*), strains were clustered similarly to Fig. 2*b* according to their growth and acidifying characteristics (axis 1). The seven *Ln. lactis* and strains B25 and B26 were scattered along axis 3. This illustrates the diversity of both their flavouring capacity and their PepN activity. Strains A59 and A52 were characterized by relatively high diacetyl production and mean PepN activity. In contrast, other strains were characterized by either a weak (B24 and MZ1) or moderate (422, B21 and B22) PepN activity (Table 3). Diacetyl production was rather low for all strains.

Discussion

Most *Leuconostoc* isolates belonged to the species *lactis*, corresponding with the composition of starters commonly used in the dairy industry (Garvie, 1986; Thunell, 1995). Like the isolates in the present study, *Ln. lactis* strains are characterized by good growth and acidification in milk (Devoyod & Poullain, 1988; Thunell, 1995). Ten strains were partly identified as either *Ln. mesenteroides* or *Weissella paramesenteroides* (formerly *Ln. paramesenteroides*; Collins *et al.* 1993). Biochemical differentiation between these two bacteria relies mainly on dextran production and fermentation of carbohydrates such as L-arabinose (Devoyod & Poullain, 1988; Millière *et al.* 1989; Collins *et al.* 1993). However, as dextran production is an unstable character, some non-dextran-forming strains of *Ln. mesenteroides* have already been isolated (Devoyod & Poullain, 1988), and this contributes to the difficulty of classifying them.

Most *Leuconostoc* isolates were characterized by poor and slow growth in milk, especially strains B21, B22 and B24, as they lacked proteinase activity and milk is a poor growth medium (Bellengier *et al.* 1997*a*). All *Leuconostoc* isolates had PepN and PepC aminopeptidase activities, together with PepX dipeptidyl aminopeptidase activity. Their levels were different from those of *Lc. lactis* strains: PepN and PepC activities being higher and PepX activity lower (Server-Busson *et al.* 1998). However, differences in peptidase activities cannot explain the poor growth of *Leuconostoc* strains in milk found in the present study and reported previously (Bellengier *et al.* 1997*a*). Thus, the role of peptidases in the growth of *Leuconostoc* in milk remains questionable. A potential role in the assimilation of casein degradation products in mixed cultures with *Lc. lactis* should be considered.

The results of both hierarchical clustering analysis and principal component analysis clearly showed that technological properties of *Leuconostoc* isolates were related to strains rather than to species, as reported previously (Bellengier *et al.* 1994; Levata-Jovanovic & Sandine, 1996). Technological characteristics (e.g. maximum population levels and acidifying properties) of *Ln. lactis* isolates and strains B25 and B26 were similar to those of proteinase-negative strains of *Lc. lactis* (Server-Busson *et al.* 1998). This could lead to using *Leuconostoc* strains rather than citrate-fermenting *Lc. lactis* strains as aroma producers in starters. In buttermilk cultures, *Leuconostoc* are indeed preferred to *Lc. lactis* subsp. *lactis* biovar *diacetilactis* strains because they reduce acetaldehyde into ethanol and this gives a balanced flavour (Vedamuthu, 1994). On the basis of these criteria, *Ln. lactis* A59 and A52, which grew well in milk and produced relatively high amounts of diacetyl, would be most valuable for use as starters for cultured dairy products that rely on aroma compounds for maximum quality.

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In conclusion, comparison of *Leuconostoc* strains on the basis of their biochemical and technological properties using statistical methods made it possible to select strains of interest for their flavouring capacity. In the field of dairy technology, special attention should now be paid to the expression of their flavouring ability in mixed cultures with an acidifying *Lc. lactis*.

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