Identification of *Lactobacillus* spp. Isolated from Different Phases During the Production of a South African Fortified Wine

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Fortified wines contain a high level of unfermented sugars and are prone to spoilage by alcohol-tolerant lactic acid bacteria. A total of 62 strains were isolated from various production stages of one of the more popular fortified wines produced in South Africa. The strains were identified by using numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analyses. The species most frequently isolated were *Lactobacillus vermiforme* (24 strains) and *Lactobacillus casei* subsp. *casei* (32 strains). Twenty-four of the strains of *L. vermiforme*, three strains of *Lactobacillus buchneri*, one strain of *Lactobacillus plantarum* and two strains of *L. casei* subsp. *casei* were isolated from spoiled fortified wine which contained 22% (vol/vol) ethanol. The majority of strains of *L. casei* subsp. *casei* (25 of the 32) and two strains of *Lactobacillus zeae* were isolated from wine before submerged fermentation. Five strains of *L. casei* subsp. *casei* were isolated from wine undergoing submerged fermentation, with an alcohol content of 11.92% (vol/vol). No strain was isolated from unbottled wine which underwent the complete fermentation process and with an alcohol content of 17.20% (vol/vol). Three distinct phenotypic groups of *L. vermiforme* were identified at $r \geq 0.70$, separate from *Lactobacillus brevis, L. buchneri* and *Lactobacillus hilgardii*. Three phenotypic clusters have been identified for *L. casei* subsp. *casei*. This is the first report of the presence of *L. vermiforme, L. zeae, L. casei* subsp. *casei* and *L. plantarum* in fortified wines.

During the primary fermentation of wine, grape must is fermented by *Saccharomyces cerevisiae* to mainly ethanol (Goswell, 1986). In a secondary fermentation L-malic acid is converted to L(+)-lactic acid and CO_2 by *Oenococcus oeni* (previously *Leuconostoc oenos*), and members of the genera *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Davis *et al.*, 1985; Wibowo *et al.*, 1985; Dicks *et al.*, 1995). Wines produced in cold regions, i.e. Germany, France and the Eastern United States, have a high acid content and may benefit from deacidification by malolactic fermentation (MLF). However, wines from warmer viticultural regions, i.e. South Africa, California and Australia, have a lower acidity and a further increase in pH could result in a flat, insipid wine with undesirable sensory characteristics (Davis *et al.*, 1985; Wibowo *et al.*, 1985) and subsequent growth of spoilage bacteria such as *Pediococcus* and *Lactobacillus* spp. (Rankine and Bridson, 1971).

Little is known about the bacterial population in fortified wines. Malolactic bacteria are generally adapted to alcohol levels of up to 14% (vol/vol), low pH conditions of 3.2 to 3.8, and SO_2 levels as high as 30 to 50 mg/L (Wibowo *et al.*, 1985). The alcohol levels in fortified wines are, however, usually higher than 15% (vol/vol) and prevent the growth of most malolactic bacteria. However, *Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus brevis* and *Lactobacillus buchneri* can tolerate ethanol levels as high as 20%, vol/vol (Fornachon *et al.*, 1949; Farrow *et al.*, 1986) and should thus be able to survive the conditions in most fortified wines, depending on the method of production.

Most fortified wines are produced by adding distilled alcohol after alcoholic fermentation (Goswell, 1986). Some of the wines have undergone complete fermentation prior to fortification (flor sherry), whereas others have had their fermentation halted by fortification, i.e. sweet dessert wines (Goswell, 1986). The high level of sugars that remain in these wines may become a source of energy for microbial growth and spoilage (Goswell, 1986). *L. hilgardii, L. fructivorans* (including previously identified strains of *Lactobacillus trichodes,* (Fornachon *et al.*, 1949), *Lactobacillus collinoides* and *Lactobacillus mali* have been isolated from Douro fortified wines (Couto and Hogg, 1994).

To date microorganisms responsible for spoilage in South African fortified wines have not received much attention. The aim of this study was to identify the *Lactobacillus* spp. isolated from a South African fortified wine. The phenotypic relatedness of the strains was determined by using numerical analysis of total soluble cell protein patterns and the genetic relatedness by 16S rRNA sequencing.

MATERIALS AND METHODS

Isolation of bacteria and reference strains used

Bacteria were isolated from a popular sweetened fortified wine produced in South Africa. Samples were taken from three different stages during production and from a spoiled bottled product. The first sample was taken from dry white wine before the onset of submerged-culture flor sherry fermentation. The second sample was taken from fortified wine during submerged fermentation with an alcohol content of 11.92% (vol/vol). The third sample was from fortified wine after completion of the fermentation process and with an alcohol content of 17.20% (vol/vol), before the addition of sweet wine. The fourth sample was taken from a bottle of sweetened fortified wine with an alcohol content of 22%

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(vol/vol) and which underwent microbial spoilage. The spoilage was visible as a haze and a sediment in the bottle.

Seven-hundred-and-fifty mL from each of the four samples were centrifuged (8 500 x *g*, 10 min), the pellet resuspended in 1 mL saline solution (0.80%, w/vol, NaCl) and then serially diluted in 10 mL saline. Aliquots from these dilutions were spread-plated onto MRS agar (Biolab). All plates were incubated at 30°C for five days, after which pure cultures were obtained following several streaks on MRS agar.

The reference strains included in this study (listed in Table 1) were obtained from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland).

Preliminary identification

All isolates were Gram stained and tested for the production of catalase by using 5% (vol/vol) hydrogen peroxide. Catalase-negative, Gram-positive rods or cocci were selected and screened for the production of CO_2 from glucose and gluconate, according to the methods described by Dicks and Van Vuuren (1987). All isolates were stored at -80°C in glycerol (40%, vol/vol).

Numerical analysis of total soluble cell protein patterns

The strains were cultured in 50 mL MRS broth for 18 h at 30°C. The methods used for the preparation of whole-cell protein extracts, SDS-PAGE, and preparation of the gels for numerical analysis, were as described by Pot *et al.* (1994b). The software package GEL COMPAR (version 4.0) of Applied Maths (Kortrijk, Belgium) was used to analyse the protein fingerprints (Vauterin and Vauterin, 1992). This program recorded the normalised electrophoretic protein patterns of the densitometric traces. Similarity between all pairs of protein patterns was expressed using the Pearson product moment correlation coefficient (*r*), and cluster analysis was performed by the unweighted average pair-group (UPGMA) method.

16S rRNA sequencing

16S rRNA sequencing was performed on representative strains selected from the protein profile clusters. The method described by Collins *et al.* (1991) was used. PCR was used to amplify a 16S rRNA gene using conserved primers close to the 3' and 5' ends of this gene. The PCR products were purified by using a Prep-A-gene kit (Bio-Rad, Hercules, Ca., USA) according to the manufacturer's instructions and were sequenced by using a *Taq* Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Inc. Foster City, USA) and a model 373A automatic sequencer (Applied Biosystems, Inc.). The closest known relatives of the new isolates were determined by performing sequence data base searches and the sequences of closely related strains were retrieved from GenBank or Ribosomal Database Project libraries. Sequences were aligned by using the program PILEUP (Devereux *et al.*, 1984) and the alignment was corrected manually.

RESULTS

A total of sixty-two Gram-positive and catalase negative rods were isolated from the wines (Tables 2 and 3). Twenty-seven strains were isolated from wine before the onset of submerged fermentation and five strains from wine which was at the time undergoing submerged fermentation. No strains were isolated from wine after the complete fermentation process and with an alcohol content of 17.20% (vol/vol). Thirty strains were isolated from bottled fortified wine which contained 22% (vol/vol) alcohol.

TABLE 1

Reference strains included in this study.

Species	Strain	Source	Comments
Lactobacillus brevis	ATCC 14869^T	Human faeces	Type strain
L. brevis	ATCC 8291	Beer	Previously *Lactobacillus pasteurianas*
Lactobacillus buchneri	ATCC 4005^T	Tomato pulp	Type strain
L. buchneri	ATCC 12935	Oral cavity	
L. buchneri	ATCC 11305	Beer	
Lactobacillus hilgardii	ATCC 8290^T	Wine	Type strain
Lactobacillus sp.	ATCC 11540	Ginger beer	Previously *Betabacterium vermiforme*
Lactobacillus sp.	ATCC 13133	Unknown	Previously *B. vermiforme*
Lactobacillus sakei subsp. *sakei*	DSM 20017^T	Sake	Type strain
L. sakei subsp. *sakei*	NCFB 2714	Sake	Same as NCIMB 13090
Lactobacillus plantarum	ATCC 14917^T	Pickled cabbage	Type strain
L. plantarum	ATCC 8014	Various sources	Previously *Lactobacillus arabinosus*
Lactobacillus casei subsp. *casei*	ATCC 393^T	Cheese	Type strain. Proposed to be reclassified as *Lactobacillus zeae* (Dicks *et al.*, 1996)
Lactobacillus paracasei subsp. *paracasei*	ATCC 25180	Unknown	Previously *L. casei* subsp. *alactosus*. Proposed to be reclassified as *L. casei* subsp. *casei* (Dicks *et al.*, 1996)

TABLE 2

Classification of obligately heterofermentative strains based on numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analysis.

Strain[a]	PAGE[b]	Identification based on 16S rRNA sequencing	Classification
ATCC 14869^T	I		*L. brevis*
ATCC 8291	I		"
ATCC 12935	I		*L. buchneri*
ATCC 4005^T	I		"
ATCC 11305	I		"
ATCC 8290^T	I		*L. hilgardii*
85224a	II	*L. buchneri*	*L. buchneri*
85759a	II		"
85224b	II	*L. buchneri*	"
ATCC 11540	III		*L. vermiforme*
ATCC 13133	III		"
OBS-LEES	III	*L. vermiforme*	"
85752 (1)	III		"
T392	III		"
85757 (2)	III	*L. vermiforme*	"
87591	III	*L. vermiforme*	"
85018 (1)	III		"
A	III		"
LB100 (5)	III	*L. vermiforme*	"
91476	IV		"
85760 (1)	IV	*L. vermiforme*	"
85755 (2)	IV		"
89833	IV		"
85759b	IV	*L. vermiforme*	"
93337	V		"
85758	V	*L. vermiforme*	"
8445601	V		"
93992	V		"
84456	V		"
85758	V	*L. vermiforme*	"
85760 (2)	V		"
93992	V		"
87602	V	*L. vermiforme*	"
92698	V		"
92734	V		"

[a]All strains were isolated from bottled fortified wine which underwent spoilage.

[b]Grouping of strains based on numerical analysis of total soluble cell protein patterns (Fig. 1).

Twenty-seven strains produced CO_2 from glucose and were classified as obligately heterofermentative (group III; Kandler and Weiss, 1986). All of these strains were isolated from bottled fortified wine which underwent spoilage. The phenotypic relatedness of these strains, as determined by numerical analysis of total soluble cell protein patterns, is shown in Fig. 1. Five clusters were delineated at $r = 0.70$, with reference strains of *L. brevis, L. buchneri* and *L. hilgardii* in one cluster at $r \geq 0.72$. Three strains formed cluster II at $r \geq 0.85$. Cluster III consisted of eight strains which clustered at $r \geq 0.80$. The fourth cluster comprised five strains which clustered at $r \geq 0.75$. Eleven strains formed cluster V at $r \geq 0.79$. Based on 16S rRNA sequence analyses, the strains in cluster II are members of *L. buchneri*, whereas the strains in clusters III to V belong to the species *Lactobacillus vermiforme* (Table 2).

Thirty-five strains produced CO_2 from gluconate, but not from glucose and were classified as facultatively heterofermentative. Twenty-seven of these strains were isolated from wine before the onset of submerged fermentation, five strains were isolated from wine which at that stage underwent submerged fermentation and three strains from a bottle of spoiled fortified wine (Table 3). The phenotypic relatedness of these strains, based on their protein banding patterns, is shown in Fig. 2. Four clusters were delineated at $r = 0.84$. Cluster I contained the type strain of *Lactobacillus plantarum* (ATCC 14917^T), *L. plantarum* ATCC 8014 and strain LB100 (2) at

TABLE 3

Classification of facultatively heterofermentative strains based on numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analysis.

Strain[a]	PAGE[b]	Identification based on 16S rRNA sequencing	Classification
ATCC 14917^T	I		*L. plantarum*
ATCC 8014	I		"
LB100 (2)	I	*L. plantarum*	"
ATCC 393	IIa		*L. zeae* (*L. casei* subsp. *casei*)
A27	IIa		"
A29	IIa		"
ATCC 25180	IIb		*L. casei* subsp. *casei* (*L. paracasei* subsp. *paracasei*)
A15	IIb		*L. casei* subsp. *casei*
A17	IIb		"
A1	IIb		"
A3	IIb		"
A2	IIb		"
A9	IIb	*L. casei* subsp. *casei*	"
A14	IIb		"
A16	IIb		"
A4	IIb	*L. casei* subsp. *casei*	"
A6	IIb		"
T394	IIb		"
A21	IIb	*L. casei* subsp. *casei*	"
A22	IIb		"
A23	IIb		"
A24	IIb		"
A18	IIb	*L. casei* subsp. *casei*	"
A25	IIb		"
B2	III		"
B3	III		"
B1	III		"
B4	III		"
A28	III		"
B5	III		"
A11	III		"
A12	III		"
T395 (1)	III		"
A31	III	*L. casei* subsp. *casei*	"
A32	III	*L. casei* subsp. *casei*	"
A26	III	*L. casei* subsp. *casei*	"
A19	IV		"
A20	IV	*L. casei* subsp. *casei*	"
A5	IV		"

[a]Numbers starting with an "A" refer to strains isolated from wine before the onset of submerged fermentation; a "B" refers to strains isolated from wine undergoing submerged fermentation. Strains LB100 (2), T394 and T395 (1) were isolated from bottled fortified wine which underwent spoilage.

[b]Grouping of strains based on numerical analysis of total soluble cell protein patterns (Fig. 2).

$r \geq 0.91$. Nineteen wine strains grouped in cluster II at $r \geq 0.88$; two strains (A27 and A29) grouped with the type strain of *Lactobacillus casei* subsp. *casei* (ATCC 393^T) at $r \geq 0.90$ in subgroup 1, separate from 17 wine strains and *Lactobacillus paracasei* subsp. *paracasei* ATCC 25180, which grouped at $r \geq 0.90$ in subgroup 2. The 12 strains in cluster III grouped at $r \geq 0.85$ and linked with the strains in clusters I and II at $r \geq 0.80$. The three strains in cluster IV formed a phenotypic group at $r \geq 0.84$, but were less closely related to the strains in clusters I to III. Based on 16S rRNA performed on strains selected from the clusters, strain LB100 (2) in cluster I is a member of *L. plantarum*. The strains in clusters II to IV belonged to the same 16S rRNA homology group as *L. casei* subsp. *casei* (Table 3).

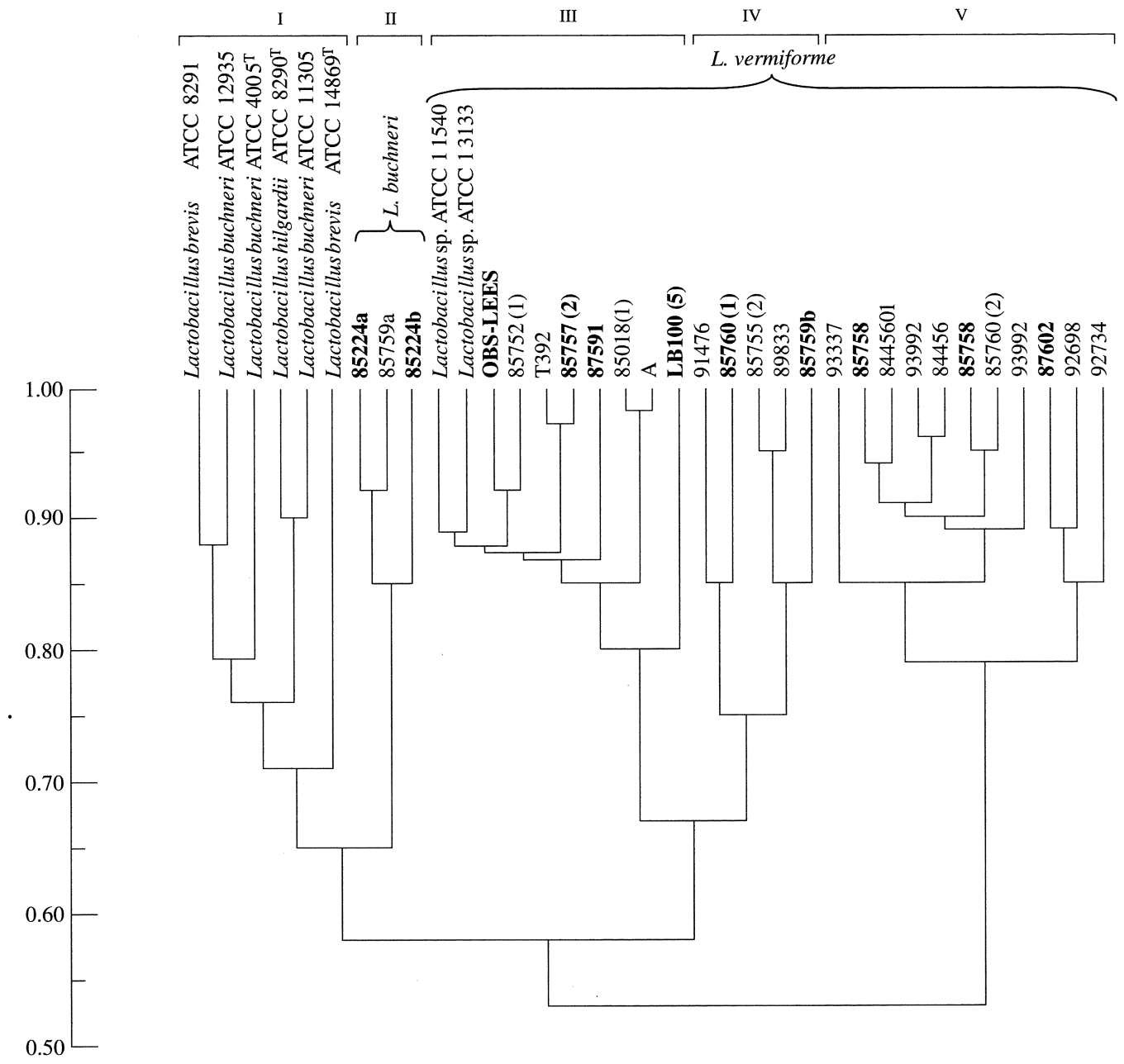

FIGURE 1

Dendrogram showing the clustering based on numerical analysis of total soluble cell protein patterns, of obligately heterofermentative stains of lactobacilli isolated from fortified wine. All strains were isolated from bottled fortified wine which has been spoiled, except strain A, which was isolated from wine before the onset of submerged fermentation. Grouping was by the unweighted average pair-group method. Strains indicated in bold numbers were selected for 16S rRNA sequencing.

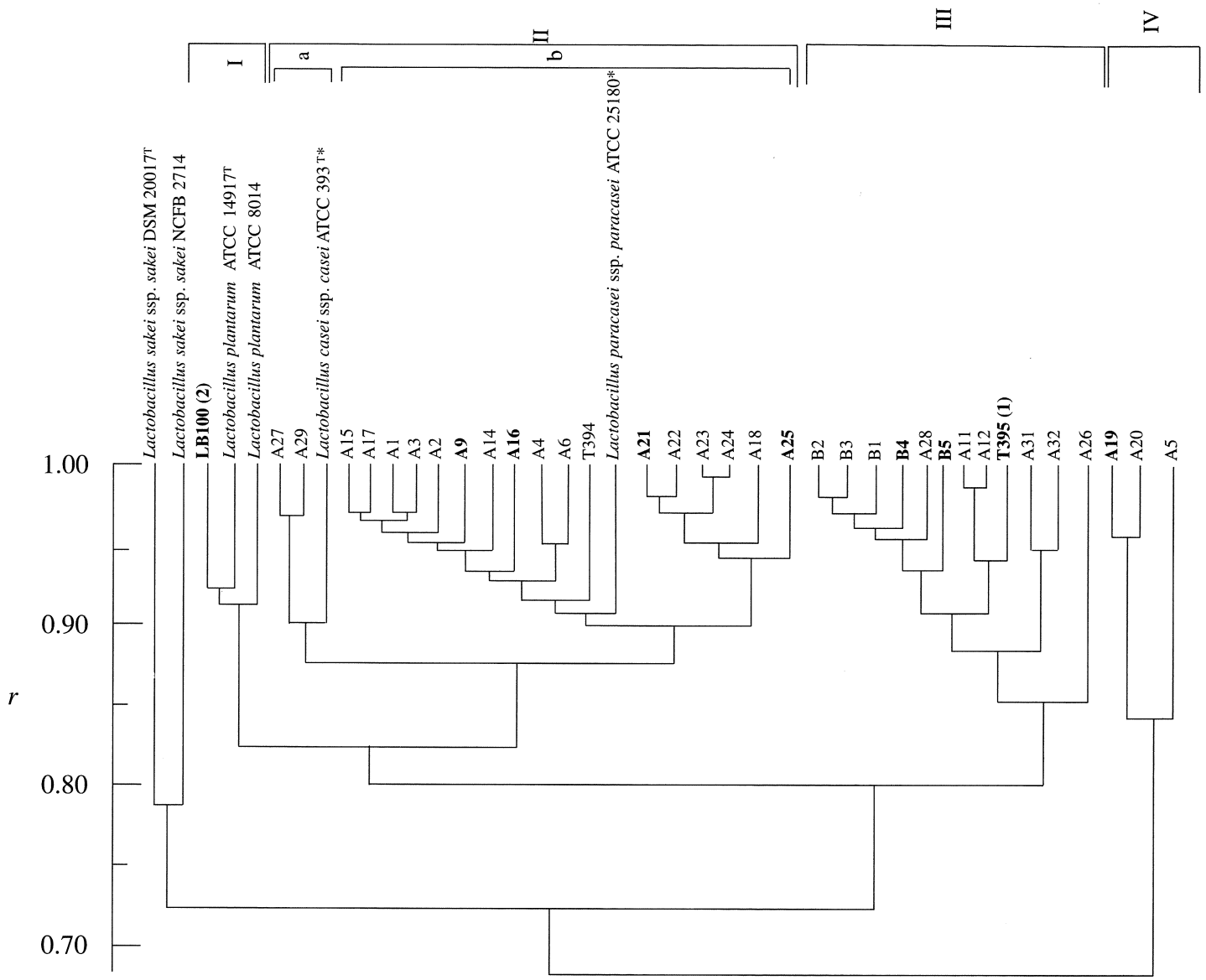

FIGURE 2

Dendrogram showing the clustering based on numerical analysis of total soluble cell protein patterns, of facultatively heterofermentative stains of lactobacilli isolated from fortified wine. Numbers starting with an "A" refer to strains isolated from wine before the onset of submerged fermentation; a "B" refers to strains isolated from wine which was at the time undergoing submerged fermentation. Strains LB100 (2), T394 and T395 (1) were isolated from bottled fortified wine which underwent spoilage. Grouping was by the unweighted average pair-group method. Strains indicated in bold numbers were selected for 16S rRNA sequencing.

*Dicks *et al.* (1996) proposed the reclassification of strain ATCC 393 as *Lactobacillus zeae* and the rejection of the name *Lactobacillus paracasei*, with the effect that all strains classified as *L. paracasei* subsp. *paracasei* be reclasified as *L. casei* subsp. *casei*.

DISCUSSION

Numerical analysis of total soluble cell protein patterns grouped the five reference strains of *L. brevis, L. buchneri* and *L. hilgardii* into one cluster at $r \geq 0.72$ (Fig. 1), suggesting that the three species are phenotypically not that distinct. This is in correlation with our previous findings, i.e. strains of *L. buchneri, L. brevis* and *L. hilgardii* cannot be differentiated by using simple physiological tests (Dicks, 1985). Sharpe (1981) proposed the reclassification of *L. buchneri* as a subspecies of *L. brevis*, based on the many phenotypic similarities between the two species.

Previous results obtained by numerical analysis of total soluble cell protein patterns (Dicks and Van Vuuren, 1987) have clearly indicated that *L. brevis* is a phenotypically heterogeneous species and related to the species *L. buchneri*. Furthermore, three DNA homology groups have been described for *L. brevis* (Vescovo *et al.*, 1979). In the present study strains 85224a, 85759a and 85224b (cluster II) grouped with the type strain of *L. buchneri* into the same 16S rRNA cluster (Table 2), despite their low phenotypic relatedness ($r \geq 0.65$) with *L. buchneri* (Fig. 1). Results obtained in this study and discrepancies noted from previous studies (Dicks and Van Vuuren, 1987; Vescovo *et al.*, 1979) question the taxonomic status of the species *L. buchneri* and *L. brevis*. It may well be that they belong to one genetic group. This necessitates a taxonomic re-investigation of strains currently designated as *L. buchneri* and *L. brevis*. The isolation of *L. buchneri* from fortified wine is not surprising, since the species is known for its ability to tolerate high alcohol levels (Farrow *et al.*, 1986).

The strains in clusters III, IV and V formed tight groups within each cluster, suggesting that they belong to three phenotypically well-defined groups. Furthermore, the overall protein patterns of these strains were different from those obtained for the strains in clusters I and II, as evident by the low correlation values recorded (Fig. 1). Results obtained by 16S rRNA sequence analyses have clearly shown that the strains in clusters III to V are members of *L. vermiforme* (Table 2), well separated from *L. hilgardii* and any other *Lactobacillus* sp.

DNA hybridisation studies performed by Farrow *et al.* (1986) on three strains, designated as *L. vermiforme* NCDO 961, NCDO 962 and NCDO 1965, indicated that they shared a high DNA homology (72 to 90%) with the type strain of *L. hilgardii* (NCDO 264^T). Based on these results, the species name *L. vermiforme* was rejected (Kandler and Weiss, 1986). However, more recent taxonomic studies on two strains (ATCC 11540 and ATCC 13133), which resembled the original description of *Betabacterium vermiforme* (later reclassified as *L. vermiforme*), could not be designated to any of the presently known *Lactobacillus* spp. and were classified as unknown *Lactobacillus* spp. (ATCC Culture Collection Catalogue, 1999). Strain ATCC 11540 was isolated from a ginger-beer plant (Mayer, 1938). The origin of strain ATCC 13133 is not known. Both strains (ATCC 11540 and ATCC 13133) grouped with strains isolated from bottled fortified wine (cluster III, Fig. 1), suggesting that they belong to the same phenotypic group. The strains in clusters III – V (Fig. 1) are also genetically related, as shown by 16S rRNA sequencing (Table 2). It might thus very well be that the strains we have isolated from fortified wine resemble the authentic strains of *B. vermiforme*. If so, the name *L. vermiforme* will have to be revived.

Strain LB100 (2), which formed a tight phenotypic cluster with the type strain of *L. plantarum* (ATCC 14917^T) and *L. plantarum* ATCC 8014 (cluster I, Fig. 2), is also genetically closely related to *L. plantarum*, as determined by 16S rRNA sequencing (Table 3). Strain LB100 (2) is thus classified as *L. plantarum*.

The remaining strains of the facultatively heterofermentative lactobacilli grouped into three well-separated protein profile clusters (Fig. 2), indicating that they belong to at least three phenotypically diverse groups.

L. casei subsp. *casei* (ATCC 393^T) grouped with two wine strains (A27 and A29) in one subgroup, separate from the other strains of *L. casei* subsp. *casei* in cluster II (Fig. 2). Similar results were recorded in our previous studies (Dellaglio *et al.*, 1991; Dicks *et al.*, 1996), which at the time led to a proposal to reclassify *L. casei* subsp. *casei* ATCC 393 (and *Lactobacillus rhamnosus* ATCC 15820) as *Lactobacillus zeae* nom. rev. (Dicks *et al.*, 1996). The proposed reclassification of strain ATCC 393 as *L. zeae,* followed by the designation of strain ATCC 334 as the neotype of *L. casei* subsp. *casei,* was supported by results obtained from DNA-DNA hybridisation studies (Dicks *et al.*, 1996). High levels of DNA homology (above 80%) were recorded between strains ATCC 393 and ATCC 15820, whereas both of these strains shared only a moderate DNA homology (8 to 46%) with strains of *L. casei* subsp. *casei* and its subspecies, including *L. casei* subsp. *alactosus* (Dellaglio *et al.*, 1973). Strains belonging to *L. casei* subsp. *alactosus* have been reclassified as *L. paracasei* subsp. *paracasei* based on DNA hybridisation studies (Collins *et al.*, 1989). However, we have argued that strains originally classified as *L. casei* subsp. *alactosus* be reclassified as *L. casei* subsp. *casei*, based on total soluble cell protein patterns and DNA-DNA hybridisation studies (Dicks *et al.*, 1996). Thus, based on the data previously presented (Dellaglio *et al.*, 1991; Dicks *et al.*, 1996) and the results obtained in the present study, the strains in subgroup a of cluster II should be classified as *L. zeae* and the strains in subgroup b as *L. casei* subsp. *casei* (Table 3). This classification is supported by results obtained from 16S rRNA sequencing (Table 3).

Concluded from the 16S rRNA sequencing data, the strains in clusters III and IV belong to the species *L. casei* subsp. *casei* (Table 3). The protein profiles of the strains from these two clusters differed from the protein profiles recorded for strains in cluster II (Fig. 2), indicating that they are phenotypically not closely related to *L. casei* subsp. *casei*. The strains in clusters III and IV may thus represent additional subspecies of *L. casei*. It is interesting to note that all five strains isolated from wine during submerged fermentation (strains B2, B3, B1, B4 and B5) grouped in cluster III (Fig. 2).

The taxonomic status of *L. casei* and its subspecies is uncertain. The species has been subjected to considerable nomenclatural changes (Collins *et al.*, 1989; Pot *et al.*, 1994a). This is not surprising, since the *L. casei – Pediococcus* phylogenetic group is the largest and most heterogeneous of all lactic acid bacteria (Collins *et al.*, 1991). An in-depth taxonomic study is needed on all members of *L. casei*, which should also include strains from various niches.

The conclusion of the present study is that the strains most frequently isolated from the wines were *L. vermiforme* and *L. casei* subsp. *casei*. The absence of homofermentative or facultatively heterofermentative species from the bottled fortified wine is perhaps not surprising, since members of these two groups are less tolerant

to alcohol than species from the obligately heterofermentative group (group III, Kandler and Weiss, 1986). It is furthermore interesting to note that only a few strains (5 out of 62) were isolated from wine during submerged fermentation. The reason for this is unknown. Strains of *L. buchneri* and *L. plantarum* were less predominant. *L. plantarum* has been isolated from table wines (Sharpe, 1981) and grape must (Costello *et al.*, 1983). The species seldom proliferates during the grape-must phase of winemaking and is usually suppressed during alcoholic fermentation, but some strains of *L. plantarum* may multiply (Ribéreau-Gayon *et al.*, 1975).

No strains of *L. brevis, L. hilgardii* and *L. fructivorans* were isolated, despite their ability to tolerate alcohol levels as high as 20% (Fornachon *et al.*, 1949; Farrow *et al.*, 1986). Many reports exist regarding the isolation of *L. hilgardii* from spoiled fortified wines. *L. hilgardii* has, for example, been isolated from Portuguese Douro fortified wine (Couto and Hogg, 1994). Strains of *L. hilgardii* have also been isolated from fortified wines with an ethanol content of 10 to 20% (vol/vol) and a pH of 3 to 4 (Hecker and Völker, 1990).

Strains of *L. casei* have been isolated from fresh grape must (Costello *et al.*, 1983). Prior to the addition of sweet fortified wine, the alcohol concentration of the submerged-culture flor fortified wine is adjusted to approximately 17% (vol/vol) by the addition of distilled alcohol. The isolated strains of *L. casei* probably survived the alcoholic fermentation, but were inhibited during the submerged-culture sherry-production process. The apparent absence of isolates from the final fortified wine sample was probably due to the final alcohol fortification of 17.20% (vol/vol), which seems to be too high for the bacteria to survive. The reasons as to why several strains were isolated from bottled wines with an alcohol content of 22% (vol/vol) and not from wines with a 17.20% (vol/vol) alcohol level remain uncertain. It is tempting to speculate that the lower oxygen levels in the bottle contributed to the survival of the bacteria. It is also possible that viable but non-culturable strains may exist, as shown to be the case for some wines during storage (Millet and Lonvaud-Funel, 2000).

This is the first report on *L. casei, L. zeae* and *L. plantarum* isolated from South African fortified wine. The few strains of each of the latter species isolated suggest that they do not play a major role in the spoilage of fortified wines.

CONCLUSIONS

The majority of strains tolerant to high alcohol levels (22%, vol/vol) belonged to the species *L. vermiforme*, suggesting that they are the major spoilage organisms in bottled fortified wine. Only a few strains of *L. casei* subsp. *casei* and *L. zeae*, prominent before submerged fermentation, were detected in the fortified product, which leads to the speculation that the lactic acid bacteria undergo a major population shift towards the end of the fermentation. This is the first report on the presence of *L. vermiforme*, *L. zeae, L. casei* subsp. *casei* and *L. plantarum* in fortified wines. Only one growth medium (MRS) was used in the isolation of the wine strains. Another medium might reveal the presence of more species. Further studies need to be done on these spoilage organisms to determine their impact on the organoleptic quality and texture of the wine.

LITERATURE CITED

Collins, M.D., Phillips, B.A. & Zanoni, P., 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei, Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. Int. J. Syst. Bacteriol. 39, 105-108.

Collins, M.D., Rodrigues, U.M., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. & Wallbanks, S., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. 77, 5-12.

Costello, P.J., Morrison, G.J. Lee, T.H. & Fleet, G.H., 1983. Numbers and species of lactic acid bacteria in wines during vinification. Food Technol. Aust. 35, 14-18.

Couto, J.A. & Hogg, T.A., 1994. Diversity of ethanol-tolerant lactobacilli isolated from Douro fortified wine: clustering and identification by numerical analysis of electrophoretic protein profiles. J. Appl. Bacteriol. 76, 487-491.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H., 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 36, 290-301.

Dellaglio, F., Bottazzi, V. & Trovatelli, L.D., 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. J. Gen. Microbiol. 74, 289-297.

Dellaglio, F., Dicks, L.M.T., Du Toit, M. & Torriani, S., 1991. Designation of ATCC 334 in place of ATCC 393 (NCDO 161) as the neotype strain of *Lactobacillus casei* subsp. *casei* and rejection of the name *Lactobacillus paracasei* (Collins *et al.*, 1989). Int. J. Syst. Bacteriol. 41, 340-342.

Devereux, J., Haeberli, P. & Smithies, O., 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387-395.

Dicks, L.M.T., 1985. Taxonomy and physiology of heterofermentative *Lactobacillus* species, including isolates from South African red wines. Thesis, University of Stellenbosch, Private Bag X1, 7602 Matieland, South Africa.

Dicks, L.M.T. & Van Vuuren, H.J.J., 1987. Relatedness of heterofermentative *Lactobacillus* species revealed by numerical analysis of total soluble cell protein patterns. Int. J. Syst. Bacteriol. 37, 437-440.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395-397.

Dicks, L.M.T., Du Plessis, E..M., Dellaglio, F. & Lauer, E., 1996. Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. Int. J. Syst. Bacteriol. 46, 337-340.

Farrow, J.A.E., Phillips, B.A. & Collins, M.D., 1986. Nucleic acid studies on some heterofermentative lactobacilli: Description of *Lactobacillus malefermentans* sp. nov. and *Lactobacillus parabuchneri* sp. nov. FEMS Microbiol. Lett. 55, 163-168.

Fornachon, J.C.M., Douglas, H.C. & Vaughn, R.H., 1949. *Lactobacillus trichodes* nov. spec., a bacterium causing spoilage in appetizer and dessert wines. Hilgardia 19, 129-132.

Goswell, R.W., 1986. Microbiology of fortified wines. In: Robinson, R.K. (ed.), Developments in Food Microbiology, vol 2. Elsevier Applied Science Publishers, London. pp. 1-20.

Hecker, M. & Völker, U., 1990. General stress proteins in *Bacillus subtilis*. FEMS Microbiol. Ecol. 74, 197-214.

Kandler, O. & Weiss, N., 1986. Section 14, Regular, non-sporing Gram-positive rods. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. (eds). Bergey's Manual of Systematic Bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore. pp. 1208-1234.

Mayer, H.D., 1938. Das "Tibi" Konsortium nebst einem Beitrage zur Kenntnis der Bakterien-Dissoziation. Thesis, University of Delft, The Netherlands.

Millet, V. & Lonvaud0Funel, A., 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett. Appl. Microbiol. 30, 136-141.

Pot, B., Ludwig, W., Kersters, K. & Schleifer, K-H., 1994a. Taxonomy of lactic acid bacteria. Microbiology, genetics and applications. In: De Vuyst, L. & Vandamme, E.J. (eds). Bacteriocins of Lactic Acid Bacteria. Chapman and Hall, London. pp. 13-90.

Pot, B., Vandamme, P. & Kersters, K., 1994b. Analysis of electrophoretic whole-organism protein fingerprints. In: Goodfellow, M. & O'Donnell, A.G. (eds). Chemical Methods in Prokaryotic Systematics. John Wiley and Sons Ltd., Chichester. pp. 493-521.

Rankine, B.C. & Bridson, D.A., 1971. Bacterial spoilage in dry red wine and its relationship to malo-lactic fermentation. Austr. Wine Brew. Spir. Rev. 90, 44, 46, 48, 50.

Ribéreau-Gayon, J., Peynaud, E., Ribéreau-Gayon, P. & Sudraud, P., 1975. Caractértes des Vins. Maturation du Raisin. Levures et Bactéries. Traité d'Oenologie. Sciences et Techniques de Vin, Tome 2. Paris: Dunod.

Sharpe, M. E., 1981. The genus *Lactobacillus*. In: Starr, M., Stolp, H., Trüper, H.G., Balows, A. & Schlegel, H.G. (eds). The Prokaryotes, vol. 2. Springer-Verlag, Berlin. pp. 1653-1679.

Vauterin, L. & Vauterin, P., 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. 1, 37-41.

Vescovo, M., Dellaglio, F., Botazzi, V. & Sarra, P.G., 1979. Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenera *Betabacterium* Orla-Jensen. Microbiologica 2, 317-330.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.