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Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum*, *Lact. fermentum*, *Lact. rhamnosus*, *Lact. sake*, *Lact. parabuchneri*, *Lact. gallinarum*, *Lact. casei*, *Weissella minor* and related taxa isolated from *kocho* and *tef*

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Aim: The study was carried out to assess the agreement of API 50 CH fermentation data of food lactobacilli with their RAPD profiles to determine whether the system could be used alone as a reliable taxonomic tool for this genus.

Methods and Results: API 50 CH, RAPD and DNA:DNA reassociation data for 42 lactobacilli from *tef* and *kocho* were compared with 30 type strains. Discrepancies were observed between the three methods in assigning strains of *Lactobacillus plantarum*, *Lact. fermentum*, *Weissella minor* and *Lact. gallinarum*, and *Lact. fermentum*, *Lact. amylophilus*, *Lact. casei* subsp. *pseudoplantarum* and *Lact. rhamnosus*. DNA reassociation data agreed well with RAPD results.

Conclusions: API 50 CH profiles should be complemented with molecular genetic results for effective identification in *Lactobacillus*.

Significance and Impact of the Study: The study suggested less dependability of metabolic data alone as an identification tool.

INTRODUCTION

Injera from *tef* (*Eragrostis tef*) dough and bread from *kocho* (*Ensete ventricosum*) constitute the main lactic acid bacteria (LAB) fermented dietary sources in Ethiopia. Lactobacilli are amongst the dominant LAB during the natural fermentation processes of these two foods (Gashe 1985, 1987; Nigatu 1998; Nigatu and Gashe 1994a,b, 1998). Previous studies by Gashe (1985, 1987) using metabolic profiles have shown the predominance of *Lactobacillus plantarum*, *Lact. brevis* and *Lact. fermentum* in *tef* dough whereas the first species and *Lact. coryniformis* subsp. *coryniformis* have frequently been isolated from *kocho*.

A set of API 50 metabolic data using fermentable carbohydrates is one of the strongly favoured phenotypic procedures in common use for identification of *Lactobacillus* species. Different studies (Molin *et al*. 1993; Johansson *et al*. 1995b) have shown the reproducibility and reliability of such patterns and hence, their use as a taxonomic tool. Metabolic data are often used complementary to other methods of identification in lactobacilli. According to Kandler and Weiss *et al.* (1986), *Lact. plantarum* and *Lact. fermentum* belong to the *Lactobacillus* supercluster, but in separate groups. *Lactobacillus plantarum* is a facultative heterofermentative species belonging to group II, while *Lact. fermentum* is an obligate heterofermentative species of the group III lactobacilli in the *Bergey's Manual of Systematic Bacteriology*. The first taxon consists of low G + C (44–46 mol%) and the second, high G + C (52–54 mol%) groups (Kandler and Weiss 1986). Phylogenetically, these two species also belong to two distinctly different species groups (Collins *et al.* 1991; Schleifer and Ludwig 1995). Vogel *et al*. (1994) have demonstrated the different allocations of these two species using both whole-cell protein patterns and 16S rRNA sequences.

Randomly amplified polymorphic DNA (RAPD) has become a rapid and reliable tool in *Lactobacillus* taxonomy. Du Plessis and Dicks *et al.* (1995) and Van Reenen and Dicks (1996) have effectively used RAPD–PCR as an accu-

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rate method for differentiation of phenotypically indistinguishable lactobacilli. Johansson *et al*. (1995a) have also used this procedure for typing of *Lact. plantarum* strains.

In a recent taxonomic study using RAPD and DNA-DNA homology data, lactobacilli isolates were accurately assigned to appropriate taxa (Nigatu *et al*. unpublished data). Nevertheless, while using API 50 CH fermentation patterns, it was not possible to find agreement with genotypic affiliations. Therefore, it was difficult to allocate field strains from *tef* and *kocho* to the right species using the API system. This paper reports the appropriate identification of lactobacilli isolates using RAPD profiles and the discrepancy with API 50 CH patterns, and in particular, the difficulty of phenotypically discriminating *Lact. plantarum* strains from *Lact. fermentum*, *Lact. gallinarum* or *Weissella minor*, and *Lact. casei* strains from those of *Lact. oris* and *Lact. rhamnosus*.

MATERIALS AND METHODS

Food samples, sampling, strains and culture conditions

Fermented *kocho* (pH 4·3) samples were purchased from markets in south and south-western Ethiopia. *Tef* flour samples were collected from households in Addis Ababa and fermented in the laboratory according to previously described procedures (Nigatu and Gashe 1994a,b).

Aliquots of 25 g or 25 ml from each food (*kocho* or 48 h-fermented *tef* dough, pH 4·3) were serially diluted using previously described procedures (Gashe 1985, 1987; Nigatu and Gashe 1994a,b) and seeded onto Rogosa Agar (Oxoid). Plates were anaerobically incubated in a BBL GasPak system (BBL GasPak Anaerobic System, Becton Dickinson Microbiological Systems, Cockeysville, Maryland, USA) at 30 °C for 24–72 h.

Gram-positive, catalase-negative, very short to very long rods, occurring singly, in pairs or in chains, grown on Rogosa Agar under anaerobic incubation, were presumptively taken as lactobacilli. Randomly-picked representatives (42) from all morphologically distinct colonies were subcultured in modified *Lactobacillus*-carrying medium, LCM (Efthymiou and Hansen 1962), containing 2% glucose. Isolates were purified using MRS broth (Oxoid,) and further, on Rogosa agar six to eight times, with subsequent transfer into LCM. Pure strains, as judged by microscopic observations for homogeneity of cellular morphologies, were stored in a freezing buffer (Ahrné *et al*. 1989) at −80 °C until used. Thirty type strains of lactobacilli were subcultured on LCM agar, and further in LCM broth, along with the 42 food isolates. The type strains used in the study, their sources and metabolic groups are illustrated in Table 1.

Table 1 Type strains used in the study.

Taxon	Strain	Fermentation*
Lactobacillus acidophilus	DSM 20079^T	OHo
Lact. agilis	DSM 20509^T	FHe
Lact. alimentarius	DSM 20249^T	FHe
Lact. amylophilus	CCUG 30137^T	OHo
Lact. amylovorus	DSM 20531^T	OHo
Lact. brevis	DSM 20054^T	OHe
Lact. casei subsp. *pseudoplantarum*	DSM 20008^T	FHe
Lact. casei subsp. *tolerans*	DSM 20258^T	FHe
Lact. crispatus	DSM 20584^T	OHo
Lact. delbrueckii subsp. *bulgaricus*	DSM 20081^T	OHo
Lact. farciminis	CCUG 30671^T	OHo
Lact. fermentum	DSM 20052^T	OHe
Lact. gallinarum	CCUG 30724^T	OHo
Lact. graminis	CCUG 32238^T	FHe
Lact. homohiochi	CCUG 32247^T	FHe
Lact. jensenii	DSM 20557^T	OHo
Lact. murinus	DSM 20452^T	FHe
Lact. oris	NCFB 2160^T	OHe
Lact. parabuchneri	CCUG 32261^T	OHe
Lact. paracasei subsp. *paracasei*	NCFB 151^T	FHe
Lact. plantarum	ATCC 14917^T	FHe
Lact. reuteri	DSM 20016^T	OHe
Lact. rhamnosus	DSM 20021^T	FHe
Lact. sake	CCUG 30521^T	FHe
Lact. salivarius	DSM 20555^T	OHo
Lact. vaccinostercus	CCUG 30723^T	OHe
Lact. vaginalis	CCUG 31452^T	OHe
Weissella confusa†	CCUG 30113^T	OHe
W. kandleri‡	CCUG 32237^T	OHe
W. minor§	CCUG 30668^T	OHe

*Fermentation, OHe, Obligately Heterofermentative; FHe, Facultatively Heterofermentative; OHo, Obligately homofermentative in metabolism.
†Formerly *Lactobacillus confusus* CCUG30113^T.
‡Formerly *Lactobacillus kandleri* CCUG 32237^T.
§Formerly *Lactobacillus minor* CCUG 30668^T.

Genetic identification

RAPD preparation. Crude cell extracts from overnight cultures were amplified using a PCR procedure and a conventional agarose gel electrophoresis run, after which photography was done according to a previously described procedure (Nigatu *et al*. 1998).

Reproducibility of the RAPD procedure. Cell lysates were prepared in duplicate and in some cases, in triplicate, from pure cultures of the type strains and a few field isolates

which were then amplified independently following the same procedure. Gels were also run, scanned and analysed using the same statistical software (Nigatu *et al*. 1998).

DNA:DNA hybridization. Pure DNA was prepared following the procedure of Ståhl and Molin (1994) as modified by Johansson *et al*. (1995). In preparing crude chromosomal DNA from the isolates and the negative controls, *Lact. reuteri* DSM 20016^T for *Lact. plantarum* and *Lact. casei* subsp. *pseudoplantarum* DSM 20008^T for *Lact. salivarius,* washed pellets of overnight cultures were lysed following the procedure described in the RAPD study. In the pre-hybridization step, chromosomal DNA obtained from the isolates was blotted on positively-charged nylon membrane (Boehringer Mannheim, GmbH, Mannheim, Germany) on a slot-blot apparatus (SlotBlot, Model PR648, Hoefer Scientific Instruments, San Francisco, CA, USA). Hybridization was performed with a vertically-rolling hybridizer (Hybridizer HB-2D, Techne Cambridge Ltd, UK) using a DIG-labelled hybridization procedure described by Johansson *et al*. (1995a). Images were developed on a Kodak X-omatic film in an X-ray developer and cassette (Kodak, Eastman, Rochester, NY, USA) as recommended by the manufacturer. Results obtained on the X-ray film were then visually compared with the intensity of the pure homologous DNA for the type strain (data not shown).

Phenotypic identification

API 50 CH fermentation assays. Overnight cultures of lactobacilli isolates and type strains grown in 10 ml MRS broth at 30 °C were washed twice with sterile physiological saline (0·9% sodium chloride) and pellets were suspended in API 50 CHL medium (API systems, BioMéreux). Using sterile Pasteur pipettes, homogenized suspensions of the cells in the medium, with subsequent vortex mixing, were transferred into each of the 50 wells on the API 50 CH strips. This was done for all isolates and type strains. All wells were overlaid with sterile paraffin oil (Merck) to effect anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 30 °C. Changes in colour from violet were monitored after 1, 2 and 7 days. Results for each of the 49 strips were graded from 0 to 5, where 5 denoted a complete change to yellow and 0, no change at all. The first strip served as a control well. Aesculin hydrolysis (revealed by a change to a darker colour or black) was represented by a positive sign (+) while a negative sign (–) represented no change.

Numerical analyses

Image preparation, comparison and analyses. Gel images of RAPD bands produced on the photo negative were scanned with a flatbed scanner (UMAX UC630 Max Color) at a resolution of 200 dots per inch and analysed using the UPGMA (unweighted pair group method using arithmetic averages) algorithm in the statistical software GelCompar version 4·0 (Applied Maths, Kortrijk, Belgium) following earlier procedures (Nigatu *et al*. 1998).

API 50 CH fermentation profile analysis. Grades of fermentation results 3, 4 and 5 were interpreted as positive (+) whereas 0, 1 and 2 were negative (–). Thus, while computing, they corresponded to 'one' for positive and 'zero' for negative results for all the type and field strains. Percentages of positive results for the 67 *Lactobacillus* strains, based on 49 carbohydrates fermented by each strain, were calculated and test vigor values generated; values below 20% were taken as low (Table 2). The fermentation patterns were evaluated for relationships (similarities and differences) between the type and field strains by cluster analysis using GelCompar version 4·0 program and the dendrogram produced (Fig. 1). Strains which aggregated to form a cluster were taken as metabolically closely related organisms and in total, seven major clusters were defined at a relationship of 70% (Table 2). For all statistical comparisons, the Ward's arithmetic algorithm was employed along with the UPGMA clustering method. In cases of difficulty in identifying the most closely related type strain to the isolate(s), where multiple type strains aggregate in a cluster, further comparison of each type strain independently with the isolates ensured the determination of the degree of relatedness.

RESULTS

RAPD analyses

RAPD electrophoretic band profiles of the type strains and the food isolates gave clearly distinct and typical patterns. Those isolates possessing bands of corresponding molecular weight to a type strain clustered together and hence, affiliated to known *Lactobacillus* and *Weissella species*.

RAPD clusters were therefore produced where the isolates belonging to the same species grouped with their genetically closely related type strains. Figure 1 shows the band pattern relationships between some representative type strains and isolates. Table 3 shows the clustering pattern of the isolates with their closely related type strains.

Table 2 Percentage frequencies of positive characteristics found in clusters 1 (*Lactobacillus graminis*), 2a, 2b (*Lact. fermentum*), 3a, 3b (*Lact. plantarum*), 4 (*Lact. sake*), 5 (*Lact. vaccinostercus*/*Lact. oris*), 6a (*Lact. casei* subsp. *pseudoplantarum*), 6b (*Lact. rhamnosus*) and 7 (*Lact. parabuchneri*) based on API 50 CH fermentation grouping

	Clusters* (percentage positive strains)									
Carbohydrates	1 ($n=1$)	2a ($n=8$)	2b ($n=1$)	3a ($n=7$)	3b ($n=3$)	4 ($n=4$)	5 ($n=3$)	6a ($n=1$)	6b ($n=5$)	7 ($n=8$)
Glycerol	0	50	0	0	0	0	33	0	0	0
D-Arabinose	0	0	0	0	0	0	100	0	40	0
L-Arabinose	0	0	100	100	100	50	100	0	0	100
Ribose	0	87	100	100	100	100	0	100	100	100
D-Xylose	0	0	0	0	67	25	100	0	0	0
Adonitol	0	0	0	0	0	0	0	0	80	0
D-Mannose	100	100	100	100	100	100	100	100	100	0
L-Sorbose	0	0	0	0	0	0	0	100	0	0
Rhamnose	0	50	0	0	67	25	0	0	20	0
Dulcitol	0	0	0	43	0	0	0	0	20	0
Inositol	0	0	0	43	0	0	0	0	60	0
Mannitol	0	100	100	71	100	0	0	100	100	75
Sorbitol	0	0	0	43	100	0	0	100	100	0
α-Methyl-D-mannoside	0	0	100	0	67	0	33	0	0	0
α-Methyl-D-glucoside	0	0	0	29	0	0	100	100	100	0
Lactose	0	100	100	100	100	100	67	100	80	67
Melibiose	0	100	100	100	100	75	100	0	0	100
Saccharose	0	87	100	100	3	25	100	100	100	100
Inulin	0	0	0	43	0	0	67	100	80	0
Melezitose	0	0	0	100	100	0	33	100	100	87
D-Raffinose	0	100	100	100	100	0	100	0	0	87
Amidon	0	0	100	28	0	25	100	0	0	87
Glycogen	0	0	100	0	0	0	33	0	0	0
β-Gentiobiose	100	100	100	100	100	100	100	100	80	12
D-Turanose	0	50	100	43	33	0	100	100	100	0
D-Lyxose	0	0	0	0	0	0	33	100	0	0
D-Tagatose	0	25	0	100	67	100	100	100	100	0
L-Fucose	0	0	0	0	0	0	67	0	0	0
D-Arabitol	0	62	100	0	0	0	0	0	0	0
L-Arabitol	0	0	0	0	0	0	0	100	0	0
Gluconate	0	100	100	57	100	25	100	100	80	12
5-keto-gluconate	0	0	0	86	33	0	0	0	0	87

*Cluster numbering corresponds to the API 50 CH grouping.
n = The number of strains included in the cluster. Strain 481b (straggler in API 50 CH clustering) fermented L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, N-acetyl glucosamine, amygdaline, salicine, cellobiose, maltose, lactose, melibiose, trehalose, β-gentiobiose, D-turanose, gluconate and 5-ketogluconate.

DNA:DNA hybridization

To ensure the validity of the RAPD results obtained, seven of the isolates assigned to the *Lact. plantarum* cluster, two from the *W. minor* cluster, one each from the *Lact. gallinarum*, *Lact. homohiochi*, *Lact. amylophilus* and *Lact. paracasei* clusters, and two from straggler (unidentified) strains, were picked and their pure chromosomal DNAs hybridized with DNA probes prepared from *Lact. plantarum* ATCC 14917^T and/or *Lact. salivarius* CCUG 31453^T. DNA homology results for all the seven tested isolates drawn from the *Lact. plantarum* RAPD cluster were positive in the slot-blot data. Thus, both genetic identification procedures were in harmony, as shown in Table 3, but disagreed with the phenotypic data except for one strain (strain

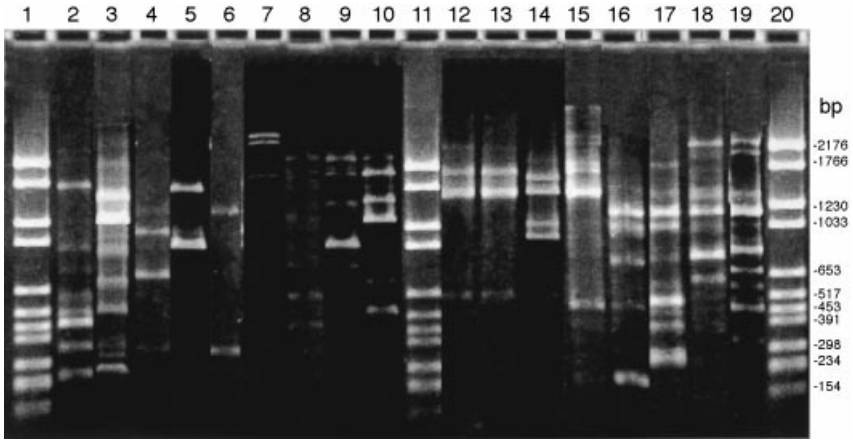

Fig. 1 RAPD electrophoresis band patterns of type strains and food isolates of *Lactobacillus* and *Weissella* species. Lanes 1, 11 and 20: DNA molecular weight marker VI (154–2176 bp); lanes 2 and 15: unidentified *Lactobacillus* species (481b and 94a); lane 3: *Lact. parabuchneri* CCUG 32261^T; lane 4: *Lact. homohiochi* CCUG 32247^T; lane 5: *W. confusa* CCUG 30113^T; lane 6: *W. minor* (486b); lane 7: *Lact. salivarius* DSM 20555^T; lane 8: *Lact. casei* subsp. *pseudoplantarum* (74a); lane 9: *Lact. casei* subsp. *pseudoplantarum* DSM 20008^T; lane 10: *Lact. rhamnosus* DSM 20021^T; lanes 12 and 13: *Lact. gallinarum* (218a and 227a); lane 14: *Lact. gallinarum* CCUG 30724^T; lanes 16, 17 and 18: *Lact. plantarum* (99a, 488a and 490b); lane 19: *Lact. plantarum* ATCC 14917^T

488b). For the 10 *Lact. plantarum* strains, however, the RAPD and API 50 CH results were not in agreement.

API 50 CH fermentation

Correlation of the 42 field strains with their metabolically-related type strains, based on their carbohydrate utilization patterns using API 50 CH, gave the results shown in Table 2. All of the isolates were able to ferment galactose, D-glucose, D-fructose and maltose but not erythritol, L-xylose, β-methyl xyloside, xylitol, D-fucose and 5-ketogluconate. With the exception of five strains, all isolates fermented lactose while only two strains degraded glycogen. All of the tested isolates except the eight strains in cluster 7 (*Lact. parabuchneri*) assimilated D-mannose, N-acetyl-glucosamine, amygdaline, arbutin, aesculin, salicin, cellobiose and trehalose. Ten of the 41 strains readily fermented starch, while the overall percentage of starch hydrolysers after 20 days exceeded 39%. Fifteen of the 16 starch hydrolysing strains were isolated from *kocho* while only one strain from seven isolates belonged to *tef* dough.

From this phenotypic affiliation, it was observed that a large proportion of the isolates (10 strains, or more than 23·9%) had metabolic traits similar to *Lact. plantarum* followed by *Lact. fermentum* (nine strains) and *Lact. parabuchneri* (eight strains); all these three together shared over 64% of the total number of isolates. Metabolic patterns of the three species appear quite common and essential in these two traditional, plant-derived, lactic acid fermented foods. Carbohydrate fermentation results of the food isolates showed their wider arrays of enzymatic activities, as shown in Table 2.

Figure 2 and Table 3 depict the metabolic and genetic clustering patterns of the type strains with food isolates, respectively. Except for a *Lact. plantarum* isolate (strain 488b), which was clearly identified by all of the three procedures, and the rest of the *Lact. plantarum* strains, which were genetically but not phenotypically identified, there was no agreement between the three procedures, as clearly seen particularly in the non-*Lact. plantarum* strains. Thus, the RAPD clusters did not correspond with the API 50 CH grouping pattern of isolates. Therefore, there were no relationships established between the two identification procedures.

DISCUSSION

Previous studies have shown that the genus *Lactobacillus* is a heterogenous group with unstable taxonomy (Schleifer and Ludwig 1995). Genetic taxonomy based on phylogenetic data, such as 16S rRNA, has shown the relatedness of the currently recognized and reorganized *Lactobacillus* species. As a consequence, some species previously included in the genus are now affiliated to a separate genus, *Weissella*, such as *W. confusa*, *W. viridescens*, *W. halotolerans*, *W. hilgardii*, *W. kandleri*, *W. minor* and *W. hellenica* (formerly *Lact. confusus*, *Lact. viridescens*, *Lact. halotolerans*, *Lact. hilgardii*, *Lact. kandleri*, *Lact. minor* and *Lact. hellenica*,

Table 3 Phenotypic and genetic affiliation and API 50 CH fermentation vigors of isolates and type strains*

Isolate (strain)	Source	Phenotypic affiliation	DNA:DNA hybridization†	RAPD identity	Test vigor (%)
		Lact. graminis§			31
477b	*Tef* dough	*Lact. graminis*	ND	Straggler††	27
		Lact. fermentum§	ND	*Lact. plantarum*	43
433a	*Kocho*	*Lact. fermentum*	*Lact. plantarum*	*Lact. plantarum*	47
489a	*Kocho*	*Lact. fermentum*	*Lact. plantarum*	*Lact. plantarum*	47
497a	*Kocho*	*Lact. fermentum*	*Lact. plantarum*	*Lact. plantarum*	49
480b	*Tef* dough	*Lact. fermentum*	*Lact. plantarum*¶	*Lact. plantarum*	45
490b	*Tef* dough	*Lact. fermentum*	*Lact. plantarum*¶	*Lact. plantarum*	45
521a	*Kocho*	*Lact. fermentum*	*Lact. plantarum*	*Lact. plantarum*	45
99a	*Kocho*	*Lact. fermentum*	ND	*Lact. plantarum*	39
276a	*Kocho*	*Lact. fermentum*	ND	*Lact. plantarum*	45
267a‡	*Kocho*	*Lact. fermentum*	ND	*Lact. plantarum*	53
		Lact. plantarum§			59
508a	*Kocho*	*Lact. plantarum*	ND	Straggler††	49
539a	*Kocho*	*Lact. plantarum*	ND	*Lact. gallinarum*	47
79a	*Kocho*	*Lact. plantarum*	Not *Lact. plantarum*	*W. minor*	43
227a	*Kocho*	*Lact. plantarum*	Not *Lact. plantarum*	*Lact. gallinarum*	59
218a‡	*Kocho*	*Lact. plantarum*	ND	*Lact. gallinarum*	63
238a‡	*Kocho*	*Lact. plantarum*	Not *Lact. plantarum*	W. minor	57
488b	*Tef* dough	*Lact. plantarum*	*Lact. plantarum*	*Lact. plantarum*	53
449a	*Kocho*	*Lact. plantarum*	ND	*W. minor*	49
484b	*Tef* dough	*Lact. plantarum*	*Lact. plantarum***	*Lact. homohiochi*	51
486b	*Tef* dough	*Lact. plantarum*	*Lact. plantarum***	*W. minor*	53
		Lact. sake§			41
317a	*Kocho*	*Lact. sake*	ND	*Lact. fermentum*	39
338a‡	*Kocho*	*Lact. sake*	*Lact. salivarius***	*Lact. amylophilus*	39
487a	*Kocho*	*Lact. sake*	Not *Lact. plantarum*	*W. minor*	41
376a	*Kocho*	*Lact. sake*	ND	*W. minor*	33
		Lact. vaccinostercus§			51
		Lact. oris§			47
74a‡	*Kocho*	*Lact. oris*	ND	*Lact. casei* subsp. *pseudoplantarum*	55
55a‡	*Kocho*	*Lact. oris*	ND	*Lact. sake*	53
167a‡	*Kocho*	*Lact. oris*	Not *Lact. salivarius*	*Lact. paracasei* subsp. *paracasei*	61
		Lact. casei subsp. *pseudoplantarum*§			59
115a‡	*Kocho*	*Lact. casei* subsp. *pseudoplantarum*	ND	*W. kandleri*	57
		Lact. rhamnosus§			57
140a‡	*Kocho*	*Lact. rhamnosus*	Not *Lact. plantarum*	Straggler††	55
2a‡	*Kocho*	*Lact. rhamnosus*		Straggler††	57
137a	*Kocho*	*Lact. rhamnosus*	*Lact. casei* subsp. *tolerans*		53
250a	*Kocho*	*Lact. rhamnosus*	ND	*Lact. casei* subsp. *pseudoplantarum*	49
122a	*Kocho*	*Lact. rhamnosus*		Straggler††	49
481b	*Tef* dough	Straggler		Straggler††	33
		Lact. parabuchneri§			25
560a	*Kocho*	*Lact. parabuchneri*		Straggler††	27
308a	*Kocho*	*Lact. parabuchneri*		Straggler††	27
289a	*Kocho*	*Lact. parabuchneri*		Straggler††	25
350a	*Kocho*	*Lact. parabuchneri*	*Lact. salivarius*	Straggler††	25
94a	*Kocho*	*Lact. parabuchneri*		Straggler††	25
405a	*Kocho*	*Lact. parabuchneri*		Straggler††	27

Table 3 *continued*

Isolate (strain)	Source	Phenotypic affiliation	DNA:DNA hybridization†	RAPD identity	Test vigor (%)
108a	*Kocho*	*Lact. parabuchneri*		Straggler††	20
380a	*Kocho*	Straggler		Straggler††	23

[a]Non-clustering type strains in the RAPD results: *Lact. farciminis* CCUG 30671^T, *Lact. alimentarius* DSM 20249^T, *Lact. crispatus* DSM 20584^T, *Lact. agilis* DSM 20509^T, *Lact. delbrueckii* subsp. *bulgaricus* DSM 20081^T, *Lact. amylovorus* DSM 20531^T, *Lact. murinus* DSM 20452^T, *Lact. jensenii* DSM 20557^T, *Lact. acidophilus* DSM20079^T, *Lact. vaginalis* CCUG 31452^T, *Lact. brevis* DSM 20054^T, *Lact. fermentum* DSM 20052^T, *Lact. reuteri* DSM 20016^T, *Lact. gallinarum* CCUG 30724^T, *Lact. homohiochi* CCUG 32247^T, *Lact. casei* subsp. *tolerans* DSM 20258^T, *Lact. parabuchneri* CCUG 32261^T, *Lact. rhamnosus* DSM 20021^T, *Lact. sake* CCUG 30521^T, *W. confusa* CCUG 30113^T, *W. kandleri* CCUG 32237^T and *W. minor* CCUG 30668^T. The last nine type strains however, clustered with one or the other of the food isolates in the RAPD study.

†Results of slot-blot DNA:DNA hybridization with *Lact. plantarum* ATCC14917^T and/or *Lact. salivarius* CCUG 31453^T.

‡Starch fermenting strains.

§Type strains mentioned in Table 1.

¶Not *Lact. salivarius*.

**Had a very similar band pattern, otherwise there might have been cross-reactions.

††These are unidentified strains using their RAPD band profiles as they separately clustered from the known *Lactobacillus* or *Weissella* species.

respectively) (Vandamme *et al*. 1996; Stiles and Holzapfel 1997). Although phenotypic techniques such as the API system are still taken as powerful tools capable of discriminating between the species of *Lactobacillus*, the use of genetic methods for *Lactobacillus* taxonomy has become the backbone of reliable identification (Vandamme *et al*. 1996). Previous studies by Du Plessis and Dicks *et al.* (1995) have clearly demonstrated differentiation of *Lact. acidophilus*, *Lact. crispatus*, *Lact. amylovorus*, *Lact. gallinarum*, *Lact. gasseri* and *Lact. johnsonii* using a RAPD–PCR procedure. Van Reenen and Dicks (1996) have also used a similar method and distinguished *Lact. plantarum* and *Lact. pentosus*, which had similar API 50 CH fermentation profiles and could therefore not be discriminated using phenotypic traits.

RAPD–PCR is apparently becoming a rapid, accurate and relatively cheaper genetic tool for LAB identification (Du Plessis and Dicks 1995; Van Reenen and Dicks 1996; Nigatu *et al*. 1998). In the current study, all food isolates were apparently discriminated based on the electrophoretic mobility of their RAPD band profiles and were thus separated and assigned into closely related species. However, the API 50 CH clustering, despite its claimed sensitivity due to the involvement of a large number of fermentable carbohydrates, was unable to distinguish the genetically separated isolates. The results therefore exhibited discrepancy with the RAPD data, as a consequence of which the isolates could not be accurately assigned to the appropriate species with the metabolic data. These findings are supported by the DNA reassociation data, at least for *Lact. plantarum* strains, which were entirely in agreement with the RAPD results. The disagreement between the RAPD and API 50 CH data therefore showed the reduced accuracy of the phenotypic procedure to clearly identify *Lactobacillus* isolates.

As shown in Table 3, all food isolates genetically belonging to *Lact. plantarum*, except for one strain, were phenotypically affiliated with *Lact. fermentum*. Both species phylogenetically belong to the *Lact. casei–Pediococcus* group of Vandamme *et al*. (1996) and Stiles and Holzapfel (1997). However, Collins *et al*. (1991) and Vogel *et al*. (1994) have clearly shown that these two species are genetically unrelated and hence belong to different lines. Metabolically, *Lact. plantarum* is a facultative heterofermenter while *Lact. fermentum* is obligately heterofermentative. The RAPD results and the DNA-DNA hybridization data are in agreement with previous genetic taxonomic studies. These two species have unrelated patterns and hence, clustered in separate groups (Table 3).

In a similar fashion, isolates genetically identified as *Lact. gallinarum* were phenotypically related to *Lact. plantarum* (Fig. 2 and Table 3). *Lactobacillus gallinarum* is metabolically an obligately homofermentative species and phylogenetically belongs to the *Lact. delbrueckii* group (Collins *et al*. 1991; Vandamme *et al*. 1996~; Stiles and Holzapfel 1997). In contrast, isolates genetically related to *W. minor* were metabolically allocated to *Lact. plantarum* (Fig. 2), and a field strain belonging to *Lact. fermentum* was metabolically associated with *Lact. sake* (Table 3). *Weissella*

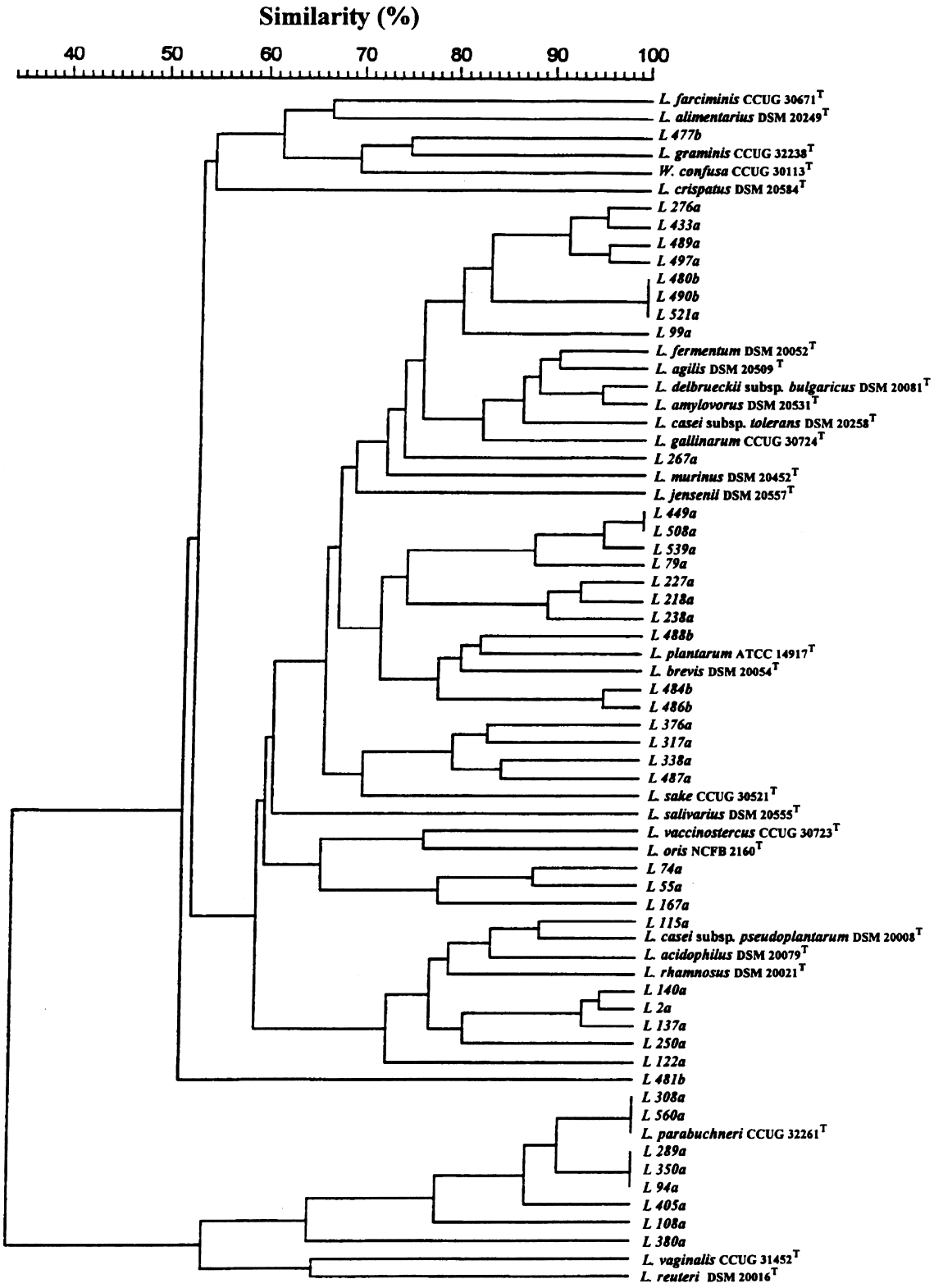

Fig. 2 Simplified dendrogram showing clustering and relationships of type strains and food isolates used in the study based on API 50 CH fermentation of 49 carbohydrates. Associations were produced using UPGMA in the GelCompar program version 4·0

minor is an obligately heterofermentative species. *Lactobacillus sake* and *Lact. plantarum* belong to the same phylogenetic and metabolic group. Genetically heterogeneous *Lactobacillus* isolates such as *Lact. casei* subsp. *tolerans*, *Lact. casei* subsp. *pseudoplantarum* and *Lact. salivarius* have also been phenotypically misidentified as *Lact. rhamnosus* or *Lact. oris*. An isolate identified as *W. kandleri* by RAPD was phenotypically affiliated with *Lact. casei* subsp. *pseudoplantarum*. *Lactobacillus oris* is an obligate heterofermenter while both *Lact. casei* and *Lact. rhamnosus* are facultative heterofermenters. On the other hand, *Lact. salivarius* is an obligately heterofermentative species. Phylogenetically, all these three species belong to the *Lact. casei–Pediococcus* group (Collins *et al*. 1991). Whole-cell protein pattern analyses by Vogel *et al*. (1994) have also shown the close relatedness of *Lact. casei* and *Lact. rhamnosus*. The genetic identity of *Lact. casei*-related taxa, including *Lact. rhamnosus*, was, however, controversial until very recently (Dellaglio *et al*. 1991). Mori *et al*. (1997) have resolved the major controversy by elucidating the 16S rRNA sequence signatures for *Lact. casei*-related organisms and also *Lact. rhamnosus*, revealing their distinct species status. The present RAPD study results for these two species are consistent with the findings of Mori *et al*. (1997).

Fourteen isolates found to be stragglers (unidentified) in the RAPD study (Table 3) were likewise phenotypically assigned to the different species. Amongst these, one strain (strain 350a) was phenotypically closely related to *Lact. parabuchneri* (Fig. 1) but its DNA hybridized with that of *Lact. salivarius*. Another interesting observation was the cross-reaction of some species such as *Lact. homohiochi* and *Weissella minor* with *Lact. plantarum*, and *Lact. amylophilus* with *Lact. salivarius*. The two food isolates phenotypically identified as *Lact. plantarum* (strains 484b and 486b) also had similar DNA homologies but have developed similar or very closely related band patterns with *Lact. homohiochi* and *W. minor*, respectively (Fig. 2). Likewise, a strain phenotypically affiliated to *Lact. salivarius* (strain 338a) had DNA homology with *Lact. sake* but with RAPD band profiles very close to that of *Lact. amylovorus*, indicating disagreement of the RAPD affiliations with the rest of the data. As a matter of fact some isolates might share many bands with some other species although their genetic relatedness being close to other species. Since the RAPD band profiles result from arbitrarily primed short patterns, they might not be exhaustive enough to arrive at a conclusion, or they may not be suitable for some species. Nevertheless, the DNA-DNA hybridization results, though affected by cross-reactions, are more dependable compared with other procedures, as previously reported (Kandler and Weiss 1986; Dellaglio *et al*. 1991; Schleifer and Ludwig 1995).

The basic disagreement between the phenotypic and genetic data in the present study lies in the use of two sets of data as single tools for lactobacilli identification, whereas the RAPD and DNA-DNA hybridization results were supportive to one another, particularly in *Lact. plantarum*, in agreement with previous studies (Johansson *et al*. 1995). One major reason for such a discrepancy might be the loss and gain of plasmids, leading to inconsistency in metabolic traits of a strain, as most carbohydrate fermentation capacities are plasmid-encoded (Ahrné *et al*. 1989). The wide heterogeneity of strains in most of the mentioned *Lactobacillus* species may also have contributed to the disagreement, as previously demonstrated for *Lact. plantarum* (Johansson *et al*. 1995a) and *Lact. casei*-related taxa (Collins *et al*. 1989; Dykes and von Holy 1994; Van Reenen and Dicks 1996; Mori *et al*. 1997). From the comparative evaluation of the phenotypic and genetic techniques, a clear mismatch between the two methods in *Lactobacillus* identification for food isolates from *tef* and *kocho* was observed. Due to the portrait of misidentification and the absence of correspondence with genetic affiliation, the API 50 CH fermentation profiles may not be a useful technique in lactobacilli taxonomy. It might, however, serve to characterize isolates to show their metabolic profiles, provided that the fermentation patterns are consistent. In a recent study on the patterns of API 50 fermentation performances across the genus *Lactobacillus*, inconsistency was similarly observed (Nigatu, unpublished data). Based on the findings of the present study, less dependence on such a phenotypic procedure as a single tool for identification of *Lactobacillus* isolates from such complex habitats is suggested. The applicability of RAPD for this genus as a discriminatory tool is, however, very encouraging.

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REFERENCES

Ahrné, S., Molin, G. and Ståhl, S. (1989) Plasmids in *Lactobacillus* strains isolated from meat and meat products. *Systematic and Applied Microbiology* **11**, 320–325.

Collins, M.D., Phillips, B.A. and Zanoni, P. (1989) Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tol-*

erans, and *Lactobacillus rhamnosus* sp. nov., comb. nov. *International Journal of Systematic Bacteriology* **39**, 105–108.

Collins, M.D., Rodrigruez, U., As, C. et al. (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S RNA. *FEMS Microbiology Letters* **77**, 5–12.

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). Request for an opinion. *International Journal of Systematic Bacteriology* **41**, 340–342.

Du Plessis, E.M. and Dicks, L.M.T. (1995) Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*. *Current Microbiology* **31**, 114–118.

Dykes, G.A. and von Holy, A. (1994) Strain typing in the genus *Lactobacillus*. *Letters in Applied Microbiology* **19**, 63–66.

Efthymiou, C. and Hansen, P.A. (1962) An antigenic analysis of *Lactobacillus acidophilus*. *Journal of Infectious Diseases* **110**, 258–267.

Gashe, B.A. (1985) Involvement of lactic acid bacteria in the fermentation of TEF (*Eragrostis tef*), an Ethiopian fermented food. *Journal of Food Science* **50**, 800–801.

Gashe, B.A. (1987) Kocho fermentation. *Journal of Applied Bacteriology* **62**, 473–474.

Johansson, M.-L., Quednau, M., Molin, G. and Ahrné, S. (1995a) Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* strains. *Letters in Applied Microbiology* **21**, 155–159.

Johansson, M.-L., Sanni, A., Lönner, C. and Molin, G. (1995b) Phenotypically based taxonomy using API 50 CH of lactobacilli from Nigerian Ogi, and the occurrence of starch fermenting strains. *International Journal of Food Microbiology* **25**, 159–168.

Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus* Beijerinck 1901,212^{AL}. In *Bergey's Manual of Systematic Bacteriology*, **Vol. 2**, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1209–1234. Baltimore: Williams & Wilkins.

Molin, G., Jeppsson, B., Johansson, M-L., Nobaek, S., Ståhl, S. and Bengmark, S. (1993) Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. *Journal of Applied Bacteriology* **74**, 314–323.

Mori, K., Yamazaki, K., Ishiyama, T. et al. (1997) Comparative sequence analyses of the genes coding for 16S rRNA of *Lactobacillus casei*-related taxa. *International Journal of Systematic Bacteriology* **47**, 54–57.

Nigatu, A. (1998) Systematics of *Lactobacillus* and *Pediococcus* Isolates from Fermented Tef (*Eragrostis tef*) *and* Kocho (*Ensete ventricosum*) *and* Microbiological Safety of the Baked Products. PhD Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Nigatu, A., Ahrné, S. and Molin, G. (1998) Randomly amplified polymorphic DNA for discrimination of *Pediococcus pentosaceus* and *Ped. acidilactici* and rapid grouping of *Pediococcus* isolates. *Letters in Applied Microbiology* **26**, 412–416.

Nigatu, A. and Gashe, B.A. (1994a) Survival and growth of selected pathogens in fermented kocho (*Ensete ventricosum*). *East African Medical Journal* **71**, 514–518.

Nigatu, A. and Gashe, B.A. (1994b) Inhibition of spoilage and food-borne pathogens by lactic acid bacteria isolated from fermenting tef (*Eragrostis tef*) dough. *Ethiopian Medical Journal* **32**, 223–229.

Schleifer, K.H. and Ludwig, W. (1995) Phylogeny of the genus *Lactobacillus* and related genera. *Systematic and Applied Microbiology* **18**, 461–467.

Ståhl, M. and Molin, G. (1994) Classification of *Lactobacillus neuteri* by restriction endonuclease analysis of chromosomal DNA. *International Journal of Systematic Bacteriology* **44**, 9–14.

Stiles, M.E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology* **36**, 1–29.

Vandamme, P., Pot, B., Gillis, M. et al. (1996) Polyphasic taxonomy a consensus approach to bacterial systematics. *Microbiological Reviews* **60**, 407–438.

Van Reenen, C.A. and Dicks, L.M.T. (1996) Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. *Current Microbiology* **32**, 183–187.

Vogel, R., Böcker, G., Stolz, P. et al. (1994) Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *International Journal of Systematic Bacteriology* **44**, 223–229.