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Identification of lactobacilli from meat and meat products

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Schemes for a rapid and simple identification of lactobacilli from meat and meat products are presented which also include the species only recently published (e.g. L. divergens *and* L. carnis*), and which were verified by the investigation of 229 strains newly isolated from meat and meat products and of nine type strains.*

They are primarily based on differences in the fermentation of sugars and other easily determinable physiological characteristics, and therefore permit a rapid assignment of a new isolate to one of the Lactobacillus *species. However, in most instances this identification has to be confirmed by determining additional characteristics (e.g. the whole range of fermented carbohydrates, types of murein etc.).*

Introduction

Lactobacilli and other lactic acid bacteria are a common component of the microflora of meat and meat products and thus exert an important effect on the quality of these foods.

There are several publications (Rogosa and Sharpe 1959, Cavett 1963, Abo-Elnaga and Kandler 1965, Rogosa 1970, Reuter 1970, Rogosa 1974, Sharpe 1979 and Sharpe 1981) dealing with the identification of lactic acid bacteria, but only a few of them contain an identification key. Moreover, they do not include the new species recently validly described. It was not until 1983 that a valid description of the species *Lactobacillus alimentarius* (Reuter 1983) and *L. farciminis* (Reuter 1983) was given, and the species *L. divergens* (Holzapfel and Gerber 1983) and *L. carnis* (Shaw and Harding 1985) also were officially proposed only recently. It is *L. divergens* and *L. carnis* as well as *L. sake* and *L. curvatus* that are very frequently found on vacuum packaged meat (Schillinger and Lücke 1986). In previous investigations of the 'lactic flora' of meat and meat products (Sharpe 1962, Reuter 1967, Laban et al. 1978, Nordal and Slinde 1980, Hitchener et al. 1982) strains of these species still were described as not identifiable or as so-called 'atypical' streptobacteria, because they could not be grouped into the taxonomic system existing at that time.

For these reasons it seemed necessary to develop an identification scheme for meat lactobacilli, including all species validly described up to 1985.

Materials and Methods

Bacterial strains

229 strains of *Lactobacillus* had been isolated from fresh meat (beef and pork) and different meat products (fermented sausage and bologna-type sausages) on acetate agar (Rogosa et al. 1951) and MRS agar (De Man et al 1960) by Holzapfel, Hechelmann, Lücke and Schillinger at the Federal Centre for Meat

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Research in Kulmbach (Holzapfel et al. 1976, Hechelmann, Lücke and Schillinger, unpublished data). The type strains of *L. alimentarius* (DSM 20249), *L. farciminis* (DSM 20184), *L. halotolerans* (DSM 20190), *L. plantarum* (DSM 20174) and *L. viridescens* (DSM 20410) as well as *L. curvatus* DSM 20010 were obtained from the Deutsche Sammlung von Mikroorganismen (DSM). The type strain of *L. sake* (ATCC 15521) was received from the American Type Culture Collection (ATCC), and the type strains of *L. carnis* (LV 61) and *L. divergens* (LV 60) were obtained from B. J. Shaw (AFRC, Food Research Institute Bristol, Langford BS18 7DY, UK).

Growth conditions

All strains were cultivated at 25°C in MRS broth (Merck), and plates were incubated in anaerobic jars (Gas Pak Anaerobic System, BBL). For maintenance of cultures, strains were freeze-dried in skim-milk. Working cultures were also kept at −25°C in the presence of 50% glycerol.

Cultural and biochemical tests

The *fermentation of carbohydrates* was determined according to Sharpe (1962) using the miniaturized method described by Jayne-Williams (1975, 1976). The test sugars were added to the basal medium (MRS without glucose and meat extract, but with 0·004% chlorophenol red) as filter-sterilized solutions to a final concentration of 0·5% (w/v). The miniplates were incubated at 25°C in the Gas Park Anaerobic System and were examined after 2 and 5 days.

Growth at pH 3·9 was tested in MRS broth adjusted with HCl to pH 3·9.

Salt tolerance was tested in MRS broth supplemented with 7% and 10% NaCl, respectively.

Growth at different temperatures was observed in MRS broth after incubation for 3 days at 15°C or 45°C and for 7 days at 4°C or 8°C.

The *production of dextran from sucrose* was determined on MRS agar in which the glucose had been replaced by 5% sucrose.

Hydrolysis of arginine was tested in MRS broth without glucose and meat extract but containing 0·3% arginine and 0·2% sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent.

Gas (CO_2)-production from glucose was observed in MRS broth containing inverted vials, with citrate omitted.

The *production of H_2S* was tested on modified Lead Acetate agar (Difco), as described by Shay and Egan (1981).

The *production of acetoin* was determined using Voges–Proskauer test (Reuter, 1970). The *hydrogen peroxide production* was determined on manganese dioxide agar (MRS supplemented with 0·75% manganese dioxide and with 0·5% xanthan gum) (Coretti 1958, Whittenbury 1964).

The *configuration of the lactic acid enantiomers* was determined enzymatically (Gutmann and Wahlefeld 1974, Gawehn and Bergmeyer 1974) using D-lactate- and L-lactate dehydrogenase (Boehringer Mannheim GmbH, Mannheim).

The *presence of meso-diaminopimelic acid* (mDpm) in the cell walls was determined according to the chromatographic method described by Abo-Elnaga and Kandler (1965), but using thin-layer chromatography on cellulose plates.

Results and Discussion

1. Distinction of the lactobacilli from closely related taxa

The lactic acid bacteria can be characterized as gram-positive, non-sporing microaerophilic bacteria whose main fermentation product from carbohydrates is lactate (Kandler 1983). Traditionally, they have been subdivided into the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Kandler 1982). In 1984, the enterococci comprising most of the streptococci of the serological group D were excluded from *Streptococcus* by Schleifer and Kilpper-Bälz (1984) and recently the streptococci of the serological group N were transferred to the genus *Lactococcus* (Schleifer et al. 1985).

The lactobacilli generally are rod-shaped and thus can be distinguished

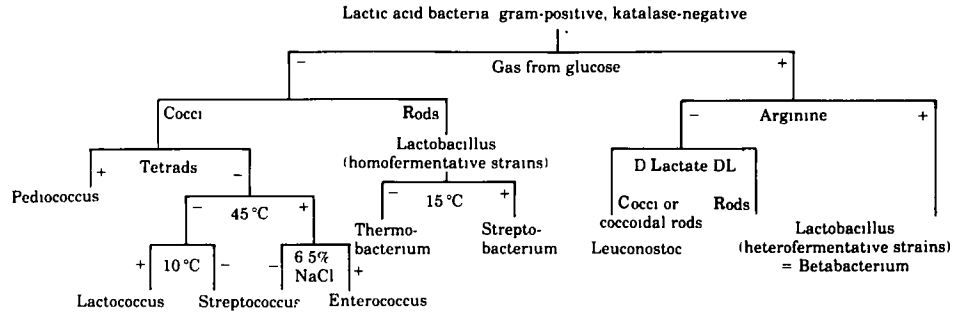

Fig. 1. Differentiation scheme for lactic acid bacteria. 8°C: growth at 8°C; 10°C: growth at 10°C; 15°C: growth at 15°C; 45°C: growth at 45°C; 6·5% NaCl: growth in presence of 6·5% NaCl; arginine: hydrolysis of arginine; lactate: production of lactate: D or L: the isomere recorded makes up 90% or more of the total lactic acid; D(L) or L(D): the isomere given in brackets makes up 15–20% of total lactic acid; DL: 25–75% of total lactic acid are of the L-configuration; ribose: production of acid from ribose; mDpm: presence of meso-diaminopimelic acid in cell wall.

from the other lactic acid bacteria showing a coccal cell morphology. But some species of the heterofermentative lactobacilli are coccoid rods and may be confused with leuconostocs unless additional characteristics are tested. A scheme for differentiation of lactic acid bacteria (into genera and subgenera) is presented in Fig. 1. It is based on easily determinable characteristics allowing a rapid assignment of a new isolate to any of the genera of the lactic acid bacteria.

Gas production from glucose is used as a first step in the differentiation of lactic acid bacteria. For a classification of the homofermentative lactobacilli producing no gas from glucose we use the subdivision of Orla-Jensen (1919) in two main groups on the basis of their growth temperatures. The so-called streptobacteria grow at 15°C whereas thermobacteria do not. The heterofermentative lactobacilli which had been called betabacteria by Orla-Jensen, can be differentiated from leuconostocs by producing NH_3 from arginine (exceptions: *L. viridescens, L. sanfrancisco, L. fructosus*) and by forming DL lactic acid from glucose (exceptions: *L. divergens, L. carnis* which both form the L enantiomer only), whilst leuconostocs do not hydrolyze arginine and form only D-lactic acid (Sharpe 1979).

2. Further differentiation of the lactobacilli

Figures 2 and 3 were developed in order to render possible a further differentiation of the lactobacilli. The schemes are based on a comparison of the typical physiological and biochemical characters of the different species (Rogosa 1974, Kagermeier 1981, Hiu et al. 1984, Shaw and Harding 1985, Schillinger 1985, Kandler and Weiss 1986, as well as own observations). For the differentiation between *L. sake* and *L. curvatus* we used data presented by Holzapfel and Gerber at the 'European Meeting of Meat Research Workers' held in Ghent in 1986. The species most commonly found on meat products (Reuter 1973, Kagermeier 1981, Morishita and Shiromizu 1986, Schillinger and Lücke 1986) are italicized in Figs 2 and 3.

Table 1 presents the lactobacilli we isolated from fresh meat, vacuum packaged meat and different meat products. They have been differentiated by determining the characteristics listed in Table 2 and in some cases those of Table 3 too. As a reference, the type strains of

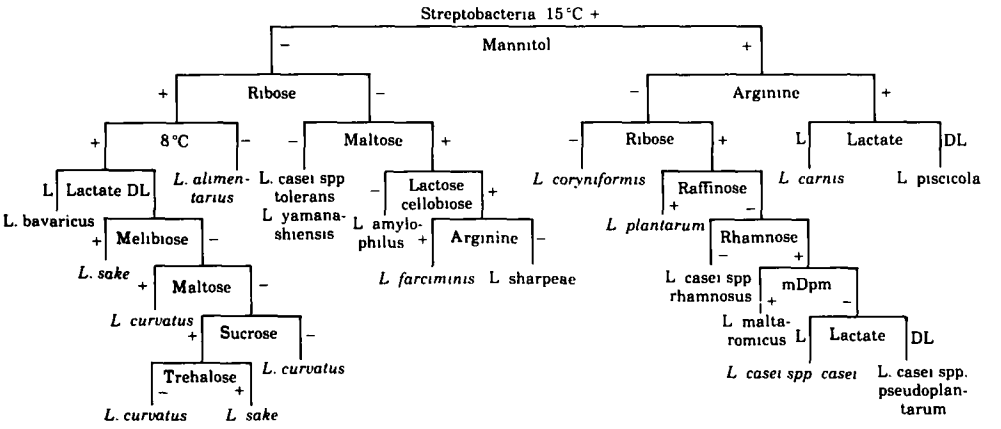

Fig. 2. Scheme for rapid identification of streptobacteria from meat and meat products. 8°C: growth at 8°C; 15°C: growth at 15°C; arginine: hydrolysis of arginine; lactate: production of lactate: D or L: the isomere recorded makes up 90% or more of the total lactic acid; D(L) or L(D): the isomere given in brackets makes up 15–20% of total lactic acid; DL: 25–75% of total lactic acid are of the L-configuration; ribose: production of acid from ribose; mDpm: presence of meso-diaminopimelic acid in cell wall. Names of species most common on meats are italicized.

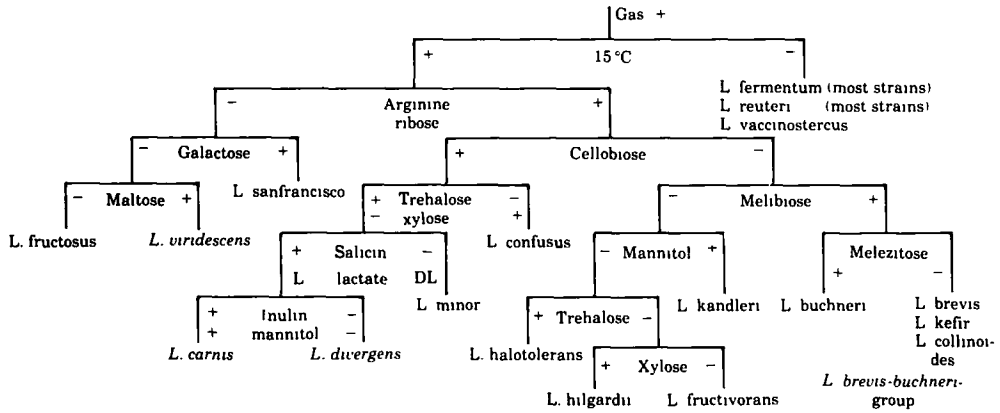

Fig. 3. Scheme for rapid identification of heterofermentative lactobacilli from meat and meat products. 15°C: growth at 15°C; arginine: hydrolysis of arginine; lactate: production of lactate: D or L: the isomere recorded makes up 90% or more of the total lactic acid; D(L) or L(D): the isomere given in brackets makes up 15–20% of total lactic acid; DL: 25–75% of total lactic acid are of the L-configuration; ribose: production of acid from ribose. Names of species most common on meats are italicized.

L. alimentarius, L. carnis, L. divergens, L. farciminis, L. halotolerans, L. plantarum, L. sake, L. viridescens as well as *L. curvatus* DSM 20010 had been included in this study.

In order to verify the new differentiation schemes given in Figs 2 and 3, we re-identified the 229 strains of *Lactobacillus* using only the few features included in these figures. The results of

Table 1. *Lactobacillus* strains investigated in the present study.

Species	Number of strains	Origin	
		Own isolates	Reference strains
L. alimentarius	1	—	marinated meat products
L. brevis	7	fermented sausage, bologna-type sausage	—
L. carnis	4	fermented sausage	vacuum-packaged meat
L. casei spp. casei	4	beef	—
L. coryniformis	1	fermented sausage	—
L. curvatus	51	vacuum-packaged beef and pork, fermented sausage	milk
L. divergens	16	vacuum-packaged beef and pork	vacuum-packaged meat
L. farciminis	3	fermented sausage	sausage
L. halotolerans	3	fermented sausage	fermented sausage
L. hilgardii	4	fermented sausage	—
L. plantarum	7	fermented sausage	pickled cabbage
L. sake	131	vacuum-packaged beef and pork, fermented sausage, bologna-type sausage	sake starter
L. viridescens	6	fermented sausage, bologna-type sausage	
	—	frankfurter-type sausage	cured meat
	238		

this simplified identification procedure agreed very well with the former determinations (97%). Only six out of 229 strains could not be correctly identified by using these identification schemes because of their deviations in important properties. Strain 579 was identified as *L. piscicola* because of its capability to hydrolyze arginine. However, the spectrum of fermentable carbohydrates was very similar to that of *L. plantarum*. Likewise, four heterofermentative strains could not clearly be typed using the system presented in Fig. 3. In this scheme there is a division of the heterofermentative lactobacilli in two groups: the main group comprising those species hydrolyzing arginine and fermenting ribose and the other group containing those which are unable to utilize both compounds. Strains which had previously been identified as *L. divergens* on the basis of their formation of L-lactic acid and the m-diaminopimelic acid content of their cell wall produced ammonia from arginine but no acid from ribose and therefore could not be allocated to one of these groups. Similarly, it was not possible to identify clearly the fourth heterofermentative strain showing the typical physiological properties of *L. viridescens* but differing in fermenting ribose. The physiological and biochemical properties of strain 733 agreed well with those of *L. divergens*, but it was not possible to detect any gas production from glucose. Therefore the differentiation resulted in *L. bavaricus* instead of *L. divergens*.

In contrast with typical heterofermen-

Table 2. Physiological characteristics of the lactobacilli investigated.

Species	Number of strains	Fermentation of																					Gas from glucose	NH_3 from arginine	Growth at				on	in		Slime from sucrose		Formation of	
		arabinose	cellobiose	esculin	galactose	gluconate	glycerol	inulin	lactose	maltose	mannitol	melezitose	melibiose	raffinose	rhamnose	ribose	salicin	sorbitol	sucrose	trehalose	xylose			4°C	8°C	45°C	pH 3·9	acetate agar	7% NaCl	10% NaCl	Voges-Proskauer		H_2S	H_2O_2
L. alimentarius	1	[a]+	+	+	+	+	−	−	−	+	−	−	−	−	−	+	+	−	+	+	−	+	+	−	−	−	−	−	+	+	−	+	−	−
L. brevis	7	57	−	43	+	+	14	−	71	+	−	−	+	−	−	+	29	−	43	−	+	+	+	57	+	86	+	+	+	29	−	−	50	+
L. carnis	4	−	+	+	+	+	50	50	75	+	+	−	−	−	−	+	+	−	50	+	50	−	+	+	+	−	−	−	−	−	−	+	−	50
L. casei	4	50	+	75	+	75	75	−	25	+	+	25	−	−	−	75	+	25	+	+	−	−	−	+	+	25	−	−	+	−	−	50	−	25
L. coryniformis	1	−	+	+	+	+	−	−	+	+	+	−	+	−	−	−	+	−	+	−	−	−	−	−	+	+	+	+	+	+	−	+	N.D.	−
L. curvatus	51	14	67	45	82	37	14	6	20	80	−	8	−	2	2	+	37	4	69	55	16	−	35	94	+	77	71	88	92	77	14	45	77	61
L. divergens	16	−	+	69	6	44	88	−	−	+	−	50	19	6	−	88	+	−	88	+	6	+	+	88	+	12	31	−	75	6	−	88	94	38
L. farciminis	3	−	+	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	+	+	−	−	+	33	+	−	+	+	+	50	−	33	−	−
L. halotolerans	3	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	+	+	−	+	+	+	+	+	+	−	−	66	−
L. hilgardii	4	50	−	−	25	75	−	−	24	+	−	25	−	−	−	+	25	−	−	−	+	+	+	−	75	+	+	+	+	50	−	−	50	−
L. plantarum	7	29	+	+	+	57	−	−	+	+	+	+	+	+	−	+	+	+	86	+	−	−	−	57	+	+	+	+	+	33	−	71	60	−
L. sake	131	43	37	23	97	98	2	−	18	14	−	1	94	−	2	+	47	−	99	94	1	−	88	99	+	87	89	98	99	93	16	57	66	51
L. viridescens	6	−	−	−	−	67	−	−	−	+	−	−	−	−	−	17	−	−	−	−	−	+	−	+	+	+	−	33	+	67	−	−	17	+

[a] Symbols: +: 90% or more strains positive; −: 90% or more strains negative; 57: 57% of the strains are positive; N.D.: not determined.

Table 3. Biochemical characteristics of the *Lactobacillus* species most commonly found on meat and meat products (data from Kandler and Weiss, 1986).

Species	Lactic acid isomer(s)[a]	Murein type[b]	Electrophoretic mobility[c] D-LDH	L-LDH	Mol% G + C
L. alimentarius	L(D)	Lys-D Asp	0·80	1·10	36–37
L. brevis	DL	Lys-D Asp	1·62	1·40	44–47
L. carnis	L	mDpm	N.D.	N.D.	34–36
L. casei spp. casei	L	Lys-D Asp	1·22	0·93	45–47
L. coryniformis	D(L)	Lys-D Asp	0·38	—	45
L. curvatus	DL	Lys-D Asp	1·20	1·60	42–44
L. divergens	L	mDpm	—	1·30	33–35
L. farciminis	L(D)	Lys-D Asp	1·15	1·20	34–36
L. halotolerans	DL	Lys-Ala-Ser	1·75	1·30	45
L. hilgardii	DL	Lys-D Asp	1·31	0·97	39–41
L. plantarum	DL	mDpm	1·44	1·28	44–46
L. sake	DL	Lys-D Asp	1·20	1·60	42–44
L. viridescens	DL	Lys-Ala-Ser	2·03	—	41–44

[a] D or L: the isomere recorded makes up 90% or more of the total lactic acid; DL: 25–75% of total lactic acid are of the L-configuration; D(L) or L(D): the isomere given in brackets makes up 15–20% of total lactic acid.
[b] Abbreviations used by Schleifer and Kandler (1972).
[c] Determined in polyacrylamide disk gel-electrophoresis pH 7·5; L-LDH rabbit iso I served as reference.
N.D.: not determined.

tative lactobacilli producing equimolar amounts of CO_2, lactic acid and ethanol (acetate) from glucose, isolates of *L. divergens* and *L. carnis* produce only small amounts of gas from glucose and therefore often are confused with homofermentative lactobacilli. Holzapfel and Gerber (1983) as well as Shaw and Harding (1985) suggested an atypical heterofermentative metabolism in these two species. They also differ from all other described heterofermentative lactobacilli in their production of virtually pure L- (+) lactic acid from hexoses and pentoses. Because gas formation by *L. carnis* is frequently not detectable, this species is also included in the differentiation scheme for the streptobacteria.

The proposed schemes for a rapid identification of lactobacilli are intended to render possible a first assignment of new isolates from meat and meat products to one of the known *Lactobacillus* species. In most instances the result has to be verified by confirmatory tests.

Moreover, it is important to know that only typical members of a species can be determined by the identification keys suggested. However, many strains of a species differ from the type strain of the same species in some of their physiological properties. For instance, Dellaglio et al. (1984) isolated from silage a large number of *Lactobacillus* strains showing very high DNA–DNA homologies to the type strain of *L. plantarum* but were not fermenting melibiose nor raffinose. In such cases it is indispensable for a clear distinction to test additional properties of the species. Information on the carbohydrate utilization pattern, the type of murein in cell wall and the configuration of the lactic acid produced is very helpful for the differentiation of the isolate. But in many cases a clear identification will not be possible without the determination of the electrophoretic mobility of

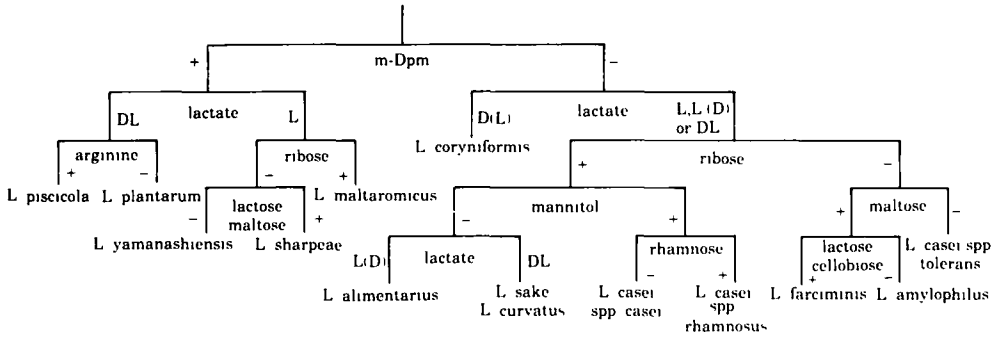

Fig. 4. Identification scheme for 'streptobacteria' based on murein type, lactic acid configuration and some physiological characteristics of the species. Arginine: hydrolysis of arginine; lactate: production of lactate: D or L: the isomere recorded makes up 90% or more of the total lactic acid; D(L) or L(D): the isomere given in brackets makes up 15–20% of total lactic acid; DL: 25–75% of total lactic acid are of the L-configuration; ribose: production of acid from ribose; mDpm: presence of meso-diaminopimelic acid in cell wall.

the lactate dehydrogenases (Gasser, 1970) or the % G + C content of DNA. For some species of the *L. brevis-buchneri* group which are phenotypically very similar only DNA–DNA hybridization will definitely clarify their relationship. However, those determinations need methods like electrophoresis or isolation and hybridization of DNA which are too time-consuming for the routine identification of large numbers of new isolates.

Figure 4 shows an identification scheme for the streptobacteria primarily based on the configuration of lactic acid and presence of mDpm in the cell wall. Due to the greater taxonomic value of these stable characters, this scheme will allow a more reliable identification than Figs 2 and 3, which are primarily based on differences in carbohydrate utilization pattern. Nevertheless, even if strains are identified by using the scheme given in Fig. 4, confirmation of the results by testing additional features remains necessary.

It is the heterofermentative lactobacilli that are more difficult to identify. Many of them, e.g. *L. brevis, L. buchneri, L. hilgardii, L. kefir* and *L. collinoides* are phenotypically very similar, but have to be considered as separate species because of their low DNA–DNA homologies (Schillinger 1985). Their carbohydrate fermentation pattern is similar, and they do not differ in the configuration of lactic acid produced (DL) nor in murein type (Lys-D-Asp) of the cell wall (Kandler and Weiss 1986). In the future, more work has to be done in order to find attributes which will allow not only a clear distinction between those species but are also easy to determine and consequently can be used routinely to screen a large number of *Lactobacillus* isolates.

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