Taxonomic Status of Lactic Acid Bacteria in Wine and Key Characteristics to Differentiate Species

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***Oenococcus oeni* is the best malolactic bacterium adapted to low pH and the high SO₂ and ethanol concentrations in wine. *Leuconostoc mesenteroides* and *Leuconostoc paramesenteroides* (now classified as *Weissella paramesenteroides*) have also been isolated from wine. *Pediococcus damnosus* is not often found in wine and is considered a contaminant of high pH wines. *Pediococcus inopinatus, Pediococcus parvulus* and *Pediococcus pentosaceus* have occasionally been isolated from wines. *Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus buchneri, Lactobacillus hilgardii* (previously *Lactobacillus vermiforme*), *Lactobacillus fructivorans* (previously *Lactobacillus trichoides* and *Lactobacillus heterohiochii*) and *Lactobacillus fermentum* have been isolated from most wines. *Lactobacillus hilgardii* and *L. fructivorans* are resistant to high acid and alcohol and have been isolated from spoiled fortified wines. *Lactobacillus vini, Lactobacillus lindneri, Lactobacillus nagelii* and *Lactobacillus kunkeei* have been described more recently. The latter two species are known to cause sluggish or stuck alcoholic fermentations in wine. Although *Lactobacillus collinoides* and *Lactobacillus mali* (previously *Lactobacillus yamanashiensis*) decarboxylate L-malic acid, they are more often found in cider and fruit juices. *Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus diolivorans, Lactobacillus jensenii* and *Lactobacillus paracasei* are seldomly isolated from wines. Some strains of *Lactobacillus casei* may be closer related to *Lactobacillus paracasei* or a distant relative, *Lactobacillus zeae*. *Oenococcus kitaharae*, isolated from compost is genetically closely related to *Oenococcus oeni*, but does not decarboxylate malate, prefers higher growth pH and is phenotypically well distinguished from *O. oeni*. This review summarises the current taxonomic status of malolactic bacteria and lists key phenotypic characteristics that may be used to identify the species.**

Malolactic bacteria (MLB) convert L-malic acid to L(+)-lactic acid and CO_2. The slight increase in pH, accompanied by the fermentation of residual sugars, ensures a microbiologically stable product before bottling (Rankine, 1972; König and Fröhlich, 2009). 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). *Oenococcus oeni* is predominant in wines with a low pH (<3.5) and produces flavor compounds such as acetaldehyde, diacetyl, acetic acid, acetoin, and 2,3-butanediol (Nielsen & Richelieu, 1999; Bartowsky & Henschke, 2004; Swiegers *et al.*, 2005; Unden & Zaunmüller, 2009). Wines from warmer viticultural regions, i.e. South Africa, California and Australia, have a lower acidity (Jackson, 2008). Secondary (malolactic) fermentation (MLF) in these wines could be detrimental and a reduction of the acid level could result in a flat, insipid wine and subsequent growth of spoilage bacteria, e.g. *Pediococcus damnosus* and certain *Lactobacillus* spp. (Bartowsky & Henschke, 2004; Swiegers *et al.*, 2005). It is thus important to control MLF in wine and carefully select starter cultures. Several excellent reviews on MLF, of which most focus on the benefits and disadvantages, and changes in the sensory qualities of wines, have been published (Davis *et al.*, 1985; Wibowo *et al.*, 1985; Henick-Kling, 1988; Kunkee, 1991; Lonvaud-Funel, 1999; Swiegers *et al.*, 2005).

Bacterial numbers increase to 10^3 or 10^4 cfu/ml soon after crushing of the grapes, but decline to almost no detectable levels during alcoholic (yeast) fermentation (Fleet, 2001). *O. oeni, Leuconostoc mesenteroides* subsp. *mesenteroides* and a number of *Lactobacillus* spp. are almost always present throughout primary (alcoholic) and secondary fermentations (Lonvaud-Funel, 1999; Swiegers *et al.*, 2005; Jackson, 2008).

This review focuses on the taxonomic status of MLB within the broader family of LAB and lists key phenotypic and genotypic characteristics that may be used to distinguish MLB from other LAB. In some cases clear species differentiation can only be obtained by comparison of genotypic characteristics.

THE GENUS *OENOCOCCUS*

The name *Oenococcus* pertains to "a little round berry from wine". Only two species have been described, i.e. *O. oeni* and *Oenococcus kitaharae*. Description of the genus is based on characteristics of *Leuconostoc oenos* (Garvie, 1986), later reclassified to *O. oeni* by Dicks *et al.* (1995).

Oenococcus cells are Gram-positive, non-motile, asporogenous and ellipsoidal to spherical in shape, usually arranged in pairs or short chains. Cell morphology may vary, depending on growth conditions and age. Sugars are fermented via a combination of the hexose-monophosphate and phosphoketolase pathways. Glucose-6-phosphate dehydrogenase (G6PDH) and xylulose-5-phosphoketolase are the key metabolic enzymes (Garvie, 1986). Equal amounts of D(−)-lactic acid, CO_2, and ethanol are formed from the metabolism of D-glucose. Pentose phosphate may be converted

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to acetic acid, but depends on the level of dissolved oxygen in the medium (Garvie, 1986). NAD and NADP serve as coenzymes for G6PDH, but only NADP is required (Garvie, 1975). Energy (ATP) is obtained by substrate level phosphorilation. Catalase and cytochromes are not present. Polysaccharides and most alcohols are not metabolized. Strains are usually non-proteolytic, although exoprotease activity has been reported for a strain isolated from red wine produced in Argentina (Manca de Nadra *et al.*, 2004). Nitrate is not reduced and indole is not formed. Growth is optimal at pH 4.8, but some strains may prefer pH 6.0 to 6.8. In general, growth is stimulated in the presence of 10% CO_2 (Garvie, 1986) Colonies on plates usually develop only after a few days and are less than 1 mm in diameter. Best growth is recorded between 20 and 30°C, in a sugar and protein rich medium, supplemented with tomato juice or grape juice (Garvie, 1986).

O. oeni decarboxylates L-malate to L(+)-lactate by using the malolactic enzyme in the presence of NAD and manganese (Radler, 1986). L-malate as sole carbon source does not support growth, suggesting that a malic enzyme and malate dehydrogenase are not present (Schütz & Radler, 1973; Radler, 1986). MLF is regulated by glucose. As little as 2 mM glucose inhibits MLF by 50% (Miranda *et al.*, 1997). Similar results were recorded in the presence of galactose, trehalose, and maltose. Ribose and 2-deoxyglucose, however, had no effect on the conversion of L-malic acid (Miranda *et al.*, 1997). Glucose inhibition is reversed by the addition of fructose or citrate, which leads to an increase in intracellular concentrations of glucose-6-phosphate, 6-phosphogluconate, and glycerol-3-phosphate (Miranda *et al.*, 1997). Phenolic compounds in red wine reduces the rate of sugar consumption by *O. oeni,* but enhances the metabolism of citric acid, which in turn leads to increased levels of acetic acid (Rozés *et al.*, 2003). Genes encoding the malolactic enzyme and malate permease (*mleA, mleP*) have been sequenced (Labarre *et al.*, 1996).

O. oeni requires as many as 13 amino acids for growth (Garvie, 1967a). Glutamic acid, valine, guanine, adenine, xanthine, uracil, riboflavin, folic acid, nicotinic acid, thiamine, biotin and pantothenic acid, or a derivative of pantothenic acid, are required by all strains (Garvie, 1967a; Garvie, 1986). Isoleucine, leucine, methionine, and tryptophan stimulates the growth of certain strains (Garvie, 1967a). Some strains grow in the absence of uracil and Tween 80 (Garvie, 1967a). None of the strains require pyridoxal (Garvie, 1967a), cobalamin, and p-aminobenzoic acid (Garvie, 1986).

O. oeni is distinguished from other lactic acid bacteria based on growth at pH 4.8, resistance to ethanol, requirement for specific growth factors in tomato juice or grape juice, the lack of NAD-dependent G6PDH, and electrophoretic mobility of D(−)-lactate dehydrogenase (D-LDH), G6PDH and alcohol dehydrogenase (ADH) (Garvie, 1986). Antisera prepared from *O. oeni* showed no cross-reactivity with anti-G6PDH and anti-D(-)-LDH of *Leuconostoc lactis* and anti-G6PDH of *Leuconostoc mesenteroides* subsp. *mesenteroides* (Gasser & Hontebeyrie, 1977). Total soluble cell protein patterns recorded for *O. oeni* are unique and different from *Leuconostoc* spp. (Dicks, 1989, 1995; Dicks *et al.*, 1990). Phenotypic characteristics are listed in Table 1.

O. oeni is genotypically not closely related to other LAB and shares 3-15% DNA homology with *Leuconostoc* spp. (Dicks & Holzapfel, 2009). *O. oeni* is also phylogenetically well separated from the *Leuconostoc sensu stricto* group, the *Weissella*

("*Leuconostoc*") *paramesenteroides* group, and all other LAB, as revealed by 16S rRNA (Martinez-Murcia & Collins, 1990, 1991; Collins *et al.*, 1993) and 23S rRNA (Martinez-Murcia *et al.*, 1993) sequencing. This suggests that *O. oeni* originated from a distinct line of descent (Dicks *et al.*, 1995). The phylogenetic relationship of *O. oeni* with other LAB is shown in Fig. 1.

Type strain of *O. oeni*: ATCC 23279, CCUG 30199, CCUG 32250, CIP 106144, DSM 20252, JCM 6125, LMG 9851, NBRC 100497, NCIMB 11648, NRRL B-3472.

GenBank accession number (16S rRNA): AB022924, M35820, X95980.

O. kitaharae does not decarboxylate L-malate, does not grow in the presence of 10% (v/v) ethanol, and does not require the tomato juice growth factor (Endo & Okada, 2006). The species grows optimal at pH 6.0 to 6.8 and at 30°C (Endo & Okada, 2006). Other phenotypic critera that differentiates *O. kitaharae* from *O. oeni* are listed in Table 1. The two species share 96% 16S rRNA sequence homology (Endo & Okada, 2006).

Type strain of *O. kitaharae*: NRIC 0645, JCM 13282, DSM 17330.

GenBank accession number (16S rRNA): AB221475.

Enrichment and isolation

Growth is supported by a complex combination of amino acids, peptides, fermentable carbohydrates, fatty acids, nucleic acids and vitamins. Biotin, nicotine, thiamine, and pantothenic acids, or derivatives thereof, are required by most strains. Growth on plates is greatly enhanced by semi-anaerobic or microaerophilic incubation, i.e. in the presence of 5% CO_2, N_2, and H_2, or in an anaerobic jar with GasPak (Oxoid, Cambridge, UK) or Anaerocult A (Merck, Darmstadt, Germany). Cysteine hydrochloride (0.05-0.1%, w/v) is often added to broth media to produce microaerophilic conditions. Optimal growth occurs between 20 and 30°C, but it may take as long as 10 days (Garvie, 1986).

Several growth media have been described for *O. oeni,* all of which are acidic with an initial pH between 4.8 and 5.5. The acidic tomato broth (ATB) medium described by Garvie (1967a) contains 25% (v/v) tomato juice. A modification of the latter medium, described by Dicks *et al.* (1990) contains 25% (v/v) grape juice (acidic grape medium). Weiler & Radler (1970) described a more complex medium that contains glucose, yeast extract, peptone, Tween-80, diammonium hydrogen citrate and sodium acetate (pH 5.4). Sorbic acid (0.05%, w/v, final concentration) is added to suppress the growth of yeast. Davis *et al.* (1985) preferred to use MRS (De Man *et al.*, 1960) or tomato juice medium (Ingraham *et al.*, 1960), adjusted to pH 5.5 and supplemented with cycloheximide (50 µg/ml) to prevent yeast growth. Enhanced growth was recorded when the latter medium was supplemented with 40 to 80% (v/v) wine (Davis *et al.*, 1985) or apple juice (Kelly *et al.*, 1989). The FT medium of Cavin *et al.* (1988) contains Tween-80, D-fructose and L-malic acid, buffered with $CaCl_2$ and KH_2PO_4. A modification of the FT-medium, which contains low concentrations of fructose and glucose, cellulose MN 300, and bromocresol green, was used to differentiate malolactic strains from non-malolactic strains (Cavin *et al.*, 1989). Sugar fermentation reactions may be studied by using the "sugar basal broth" described by Garvie (1984), modified by adjusting the pH to 5.2, and substituting bromocresol purple with bromocresol green (0.004%, w/v).

Oenococcus kitaharae is less fastidious and can be cultured in MRS containing BHI (brain heart infusion) medium (Endo & Okada, 2006).

THE GENUS *LEUCONOSTOC*

The name *Leuconostoc* pertains to "a colorless nostoc". Currently, the genus consists of eleven species (Table 2). Cells are Gram-positive, catalase negative, non-motile, asporogenous and ellipsoidal or spherical. Cells grown in a glucose-rich or on a solid medium may appear elongated and thus similar to lactobacilli. Cells are either single, arranged in pairs, or form short to medium length chains. Strains of *L. mesenteroides* may produce dextran, visible as an electron-dense coat on the cell surface.

Growth on the surface of solid medium is poor, but is stimulated when incubated in the presence of 19.8% CO_2, 11.4% H_2, and 78% N_2. Growth in broth is stimulated by the addition of 0.05% (w/v) cysteine HCl. Colonies develop usually only after three to five days, are smooth, round grayish white and less than 1 mm in diameter. The species are nonproteolytic and nitrate is not reduced. Indole is not formed and arginine is not hydrolyzed. Optimal growth is recorded at pH 6.5 and between 20 and 30°C. Growth may occur at 5°C, but this is seldomly reported. All species require nicotinic acid, thiamine, biotin and either pantothenic acid or its derivative 4'O-(α-glucopyranosyl)-D-pantothenic acid. Cobalamin and ρ-aminobenzoic acid are not required. Glucose is fermented to D(-)-lactic acid, CO_2 and ethanol, or acetate, via a combination of the hexose-monophosphate and phosphoketolase pathways. Fructose 1,6-diphosphate aldolase is absent. All species contain an active G6PDH. Xylulose 5-P phosphoketolase converts fermentable sugars to ethanol and D(-)-lactic acid. Some strains may produce additional acetate instead of ethanol when grown in the presence of oxygen. L-malate is decarboxylated to L(+)-lactate by some strains and only in the presence of a fermentable carbohydrate. Polysaccharides and

TABLE 1

Phenotypic characteristics of *Oenococcus oeni, Oenococcus kitaharae* and *Leuconostoc mesenteroides*.[a, b]

Characteristic	O. oeni	O. kitaharae	L. mesenteroides subsp. mesenteroides	L. mesenteroides subsp. dextranicum	L. mesenteroides subsp. cremoris
Acid from:					
Arabinose	d	-	+	-	-
Arbutin	ND	ND	d	-	-
Cellobiose	d	d	d	-	-
Cellulose	d	ND	d	d	-
Fructose	+	+	+	+	-
Galactose	d	+	+	d	+
Lactose	-	-	d	+	d
Maltose	-	+	+	+	-
Mannitol	-	-	d	-	-
Mannose	d	+	d	d	-
Melibiose	d	+	d	d	-
Raffinose	-	(+)	d	d	-
Ribose	d	(+)	d	+	-
Salicin	d	d	d	-	-
Sucrose	-	-	+	+	-
Trehalose	+	+	+	+	-
Xylose	d	-	d	d	-
Hydrolysis of esculin	+	ND	+	+	-
Dextran production	-	-	+	+	-
Growth at pH 4.8	+	d	-	-	-
Requirement of TJF	d	-	-	-	-
Growth in 10% ethanol	+	-	-	-	-
NAD-dependent G6PDH[c]	-	ND	+	+	+
Growth at 37 C	d	-	d	+	-
Peptidoglycan type	Lys-Ser$_2$ or Lys-Ala-Ser	ND	Lys-Ser-Ala$_2$	Lys-Ser-Ala$_2$	Lys-Ser-Ala$_2$

[a]+, 90% or more of strains positive; -, 90% or more of strains negative; d, 11–98% of strains positive; (), delayed reaction; ND, no data; D, 90% or more of the lactic acid is D(−). Data from Dicks & Holzapfel (2009).

[b]Arginine hydrolysis: negative for all species. All species produce CO_2, D(−)lactic acid and ethanol or acetic acid from D-glucose.

[c]Glucose-6-phosphate dehydrogenase.

TABLE 2

Phenotypic characteristics differentiating *Leuconostoc* spp.[a,b]

Characteristic	*L. mesenteroides* subsp.			*L. carnosum*	*L. citreum*	*L. fallax*	*L. holzapfelii*	*L. gasicomitatum*	*L. gelidum*	*L. inhae*	*L. kimchii*	*L. lactis*	*L. pseudomesenteroides*
	mesenteroides	*dextranicum*	*cremoris*										
Cell morphology	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Ovoid or Short rods	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid or ovoid	Coccoid to elongated cocci	Coccoid to elongated cocci
Amygdalin	ND	ND	ND	ND	ND	ND	−	−	ND	d	+	ND	ND
Arabinose	+	−	−	−	+	−	+	+	+	d	+	−	d
Arbutin	d	−	−	−	+	−	−	−	+	−	ND	−	d
Cellulose	d	d	−	ND	ND	ND	ND	ND	ND	ND	ND	−	ND
Cellobiose	d	−	−	d	d	−	−	+	+	+	+	−	d
Fructose	+	+	−	+	+	+	+	+	+	+	+	+	+
Galactose	+	d	+	−	−	−	+	D	−	d	+	+	d
Lactose	d	+	d	−	−	−	−	−	−	−	+	+	d
Maltose	+	+	−	−	+	+	+	+	d	+	+	+	+
Mannitol	d	−	−	−	d	(d)	−	−	−	+	+	−	−
Mannose	d	d	−	d	+	+	+	+	+	+	+	d	+
Melibiose	d	d	−	d	−	−	+	+	+	−	−	d	d
Raffinose	d	d	−	−	−	−	+	+	+	−	+	d	d
Ribose	d	+	−	d	−	+	−	+	d	ND	+	−	+
Salicin	d	−	−	d	+	−	−	−	+	d	+	d	d
Sucrose	+	+	−	+	+	+	−	+	+	+	+	+	d
Trehalose	+	+	−	+	+	(d)	+	+	+	+	+	−	+
Xylose	d	D	−	−	−	−	−	−	+	−	−	−	+
Ammonia from arginine	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of esculin	+	+	−	d	+	ND	−	+	+	+	ND	−	d
Dextran production	+	+	−	+	ND	ND	+	+	+	d	+	−	ND
Growth at pH 4.8	−	−	−	ND	ND	ND	+	ND	ND	+	ND	−	ND
Requirement of TJF	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth in 10% ethanol	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	−	ND
NAD-dependent G6PDH present	+	+	+	ND	ND	ND	ND	ND	MD	ND	ND	+	ND
Growth at 37 C	d	+	−	−	d	+	+	−	−	−	+	+	+
Peptidoglycan type	Lys-Ser-Ala2	Lys-Ser-Ala2	Lys-Ser-Ala2	Lys-Ala2	Lys-Ala2	Lys-Ala2	Lys-Ala2	Lys-Ala2	Lys-Ala2	Lys-Ala2	ND	Lys-Ala2	Lys-Ser-Ala2

[a]+, 90% or more of strains positive; −, 90% or more of strains negative; d, 11-98% of strains positive; (+), delayed reaction; ND, no data. D, 90% or more of the lactic acid is D(−). [b]Data obtained from Holzapfel *et al.* (2009a).

alcohols, except mannitol, are not metabolized. Carbohydrate fermentation patterns vary considerably among *Leuconostoc* spp. (Table 2) and are often strain dependent. Certain strains of *Leuconostoc*, when grown under acidic conditions, ferment sucrose (Garvie, 1967b). Nonpolysaccharide-producing strains of *L. mesenteroides* form dextran in media containing tomato juice or orange juice (Pederson & Albury, 1955; Langston & Bouma, 1959). Key phenotypic characteristics that are used to differentiate *Leuconostoc* spp. from other LAB is shown in Table 3.

Leuconostoc spp. are clearly differentiated based on their soluble cell protein profiles and DNA homology (Dicks *et al.*, 1990, 1993). Numerical analysis of 16 and 23S rDNA gene RFLP patterns is also used to differentiate species. Digestion with *Hin*dIII yielded species-specific patterns (Björkroth *et al.*, 2003). The phylogenetic relationship among *Leuconostoc* spp. is shown in Fig. 1. Since *L. mesenteroides* is the main species present in wine, the characteristrics of only the three subspecies are discussed. For the sake of simplicity *Weissella paramesenteroides* (previously *Leuconostoc paramesenteroides*) is included in the same section as *Leuconostoc*. *Leuconostoc durionis, Leuconostoc ficulneum* and *Leuconostoc fructosum* have been reclassified as *Fructobacillus durionis, Fructobacillus ficulneus* and *Fructobacillus fructosus*, respectively (Endo and Okada, 2008) and are not included in this review. A detailed description for each of the *Leuconostoc* sp. and *Weissella paramesenteroides* can be found in Holzapfel *et al.* (2009a) and Björkroth *et al.* (2009), respectively.

Leuconostoc mesenteroides subsp. *mesenteroides*

Some strains convert L-malate to L-lactate. Dextran is produced from the fermentation of sucrose, especially when incubated between 20°C and 25°C. Colonies are not uniform on sucrose agar and depend on the chemical structure of the dominant type of dextran formed. Some strains produce a heme-requiring catalase (Whittenbury, 1964). Growth has been recorded in the presence of 3.0% (w/v) and 6.5% (w/v) NaCl. Milk supplemented with yeast extract and glucose is acidified and curdled, but not by all strains. Glutamic acid and valine are required. Sugar fermentation reactions are listed in Table 2.

Type strain: Strain 12954, ATCC 8293, DSM 20343, CCUG 30066, CIP 102305, HAMBI 2347, JCM 6124, LMG 6893, NBRC 100496, NRRL B-1118, NRRL B-3470, VKM B-1601.

GenBank accession number (16S rRNA): M23035.

Leuconostoc mesenteroides subsp. *cremoris*

Cells may form long chains with resultant flocculent growth in broth. Dextran is not produced from the fermentation of sucrose. Citrate is metabolized to acetoin and diacetyl (Speckman & Collins, 1968). Most strains do not ferment sucrose (Whittenbury, 1966). Compared to the other two subspecies, a limited number of carbohydrates are fermented (Table 2). Growth is optimal between 18°C and 25°C. All known strains have been isolated from dairy products.

Type strain: ATCC 19254, CCUG 21965, CIP 103009, DSM 20346, LMG 6909, NCIMB 12008, NRRL B-3252, VKM B-1420.

GenBank accession number (16S rRNA): M23034.

Leuconostoc mesenteroides subsp. *dextranicum*

Dextran is produced from sucrose, but less actively than by *L. mesenteroides* subsp. *mesenteroides*. Fewer carbohydrates are fermented than by *L. mesenteroides* subsp. *mesenteroides* (Table 2). More amino acids and vitamins are required for growth than are required by *L. mesenteroides* subsp. *mesenteroides*. Growth occurs from 10°C to 37°C (optimum, 20-30°C). Glucose litmus milk is acidified and curdled and gas production may be formed but not by all strains.

Type strain: ATCC 19255, CCUG 21966, CCUG 30065, CIP 102423, DSM 20484, JCM 9700, LMG 6908, NCIMB 12007, NBRC 100495, NCAIM B.01658, NCIMB 12007, NRRL B-3469, VKM B-1225.

GenBank accession number (16S rRNA): AB023246.

Weissella paramesenteroides (previously *Leuconostoc paramesenteroides*)

The cells are spherical, but often lenticular and may occur in pairs and chains. Gram-positive, non-motile, and nonsporeforming. The interpeptide bridge of the peptidoglycan structure is Lys-Ala$_2$; Lys-Ser-Ala$_2$. Ammonia is not produced from arginine. Dextran is not formed from the fermentation of sucrose. Amino acid requirements are complex and vary between strains. Growth may occur at 18–24ºC, but 30ºC is preferred. Pseudocatalase may be formed if cells are grown in medium with low glucose concentration. Growth has been recorded in media with an initial pH below 5.0

Type strain: ATCC 33313, CCUG 30068, CIP 102421, DSM 20288, JCM 9890, LMG 9852, NCIMB 13092, NRIC 1542, NRRL B-1186, NRRL B-3471.

GenBank accession number (16S rRNA): AB023238, M23033, X95982.

Enrichment and isolation

Microaerophilic to anaerobic conditions are recommended, i.e. incubation in the presence of N$_2$, H$_2$ or CO$_2$, or with anaerobic gas-generating kits such as GasPak (Oxoid) or Anaerocult A (Merck). Different growth media may be used. Some of the more general growth media include MRS (De Man *et al.*, 1960), Rogosa SL-medium (Rogosa *et al.*, 1951), glucose-yeast extract (Whittenbury, 1965), acetate medium (Whittenbury, 1965), yeast extract-glucose-citrate (YGC) broth (Garvie, 1967b), and HP Medium (Pearce & Halligan, 1978). Tetrazolium-sucrose (TS) medium (Cavett *et al.*, 1965), thallous-acetate-tetrazolium-sucrose (TTS) medium (Cavett *et al.*, 1965), and HHD-medium (McDonald *et al.*, 1987) have been used in the isolation of leuconostocs from plant material.

THE GENUS *PEDIOCOCCUS*

The name *Pediococcus* pertains to "a coccus growing in one plane". The genus contains eight species, *viz. Pediococcus acidilactici, Pediococcus claussenii, Pediococcus cellicola, Pediococcus damnosus*, *Pediococcus inopinatus*, *Pediococcus parvulus, Pediococcus stilesii, Pediococcus pentosaceus* (subspecies *pentosaceus* and *intermedius*), and the atypical species *Pediococcus dextrinicus* (Holzapfel *et al.*, 2009b). The type species is *P. damnosus*.

Pediococci are Gram-positive, non-motile and spherical, but may be ovoid. The cells divide to form pairs (usually seen during early or mid-exponential growth) or divide in two perpendicular directions to form tetrads. Chain formation has not been recorded. Cells are small (*ca.* 0.5–1.0 mm), asporogenic, and catalase- and oxidase-negative. Some strains of *P. pentosaceus*

TABLE 3

Major phenotypic characteristics differentiating members of the genus *Leuconostoc* from other genera of LAB (Björkroth & Holzapfel, 2003, modified)[a].

Characteristic	*Leuconostoc*	*Weissella*	Homofer-mentative ovoid-shaped LAB (*Enterococcus*, *Lactococcus*, *Streptococcus*)	*Lactobacillus*		*Carnobacterium*	*Pediococcus*
				Homo-fermentative	Hetero-fermentative		
Morphology	Ovoid to short rods (pairs and chains)	Ovoid or rods (pairs and chains)	Ovoid (pairs and chains)	rods	rods	rods	round cocci (tetrads and pairs)
CO_2 from glucose	+	+	–	–	+	– (+)	–
Hydrolysis of arginine	–	+ or –	– or +	+ or –	– or +	+	– or +
Dextran from sucrose	– / +	– / +	– or +	– or +	– or +	–	–
Lactic acid isomer from glucose	D(–)	D(–) or DL	L(+)	D(–), DL, or L(+)	DL	L(+)	DL or L(+)
Peptidoglycan	LysAla/Ser[b]	LysAla/Ser[c]	Lys-Asp	mainly Lys-Asp and m-A2pm	Lys-Asp and others[d]	*m-A2pm*	Lys-Asp

[a]Symbols: +, positive reaction; –, negative reaction; +/–, mostly positive; –/+, mostly negative.

[b]Abbreviations: Lys, lysine; Ala, alanine; Ser, serine; Asp, aspartate; m-A2pm, 2,6-diaminopimelic acid (2,6-diaminoheptanedioic acid).

[c]No Lys-Asp-types, but differentiations of Lys-Ala/Ser types, with the exception of *W. kandleri*.

[d]Related to those of *Leuconostoc* and *Weissella*.

produce pseudocatalase (Simpson and Taguchi, 1995). Nitrate is not reduced. Arginine hydrolysis has only been reported for *P. acidilactici* and *P. pentosaceus*. Glucose is converted to lactic acid via the Embden–Meyerhof pathway. CO_2 and ethanol are thus not produced. Growth has been recorded at pH 5, but not at pH 9 (except for *P. stilesii*). Optimum growth temperature is 25–35°C, but is species dependent. All species, except *P. claussenii*, produce DL-lactic acid from glucose.

Species group in a phylogenetically homogeneous cluster with 16S rRNA sequence similarities greater than 94%. The genus is thus well separated from other LAB (Fig. 1). Identification to species level is best obtained by 16S rDNA gene sequencing (Collins *et al*., 1990; Kurzak *et al*., 1998; Omar *et al*., 2000; Barney *et al*., 2001), species-specific DNA probes (Lonvaud-Funel *et al*., 1993; Mora *et al*., 1997; 1998; Rodrigues *et al*., 1997), ribotyping (Jager & Harlander, 1992; Satokari *et al*., 2000; Barney *et al*., 2001; Santos *et al*., 2005), randomly amplified polymorphic DNA (RAPD) PCR (Kurzak *et al*., 1998; Nigatu *et al*., 1998; Mora *et al*., 2000; Simpson *et al*., 2002; Fujii *et al*., 2005; Tamang *et al*., 2005), and pulsed-field gel electrophoresis (PFGE) (Dellaglio *et al*., 1981; Luchansky *et al*., 1992; Barros *et al*., 2001). Simpson *et al*. (2006) developed a rapid PFGE protocol for species- and strain-level differentiation.

Sugar fermentations vary and genes encoding raffinose, melibiose, and sucrose metabolism by *P. pentosaceus* and some strains of *P. acidilactici* are plasmid-encoded (Gonzalez & Kunka, 1986; Hoover *et al*., 1988). Key phenotypic characteristics that are used to differente pediococci are listed in Table 4. Since only *P. damnosus, P. inopinatus, P. parvulus* and *P. pentosaceus* are most frequently isolated from wine, descriptions of only these species

are given. A detailed description of other species can be found in Holzapfel *et al*. (2009b).

Pediococcus damnosus

Growth is often slow and 2–3 days of incubation at 22°C may be required. Cysteine added to the medium improves growth. Growth on solid media is usually poor if not incubated anaerobically. Optimum growth is recorded at pH 5.5. Cells do not grow at pH 8.5. Growth temperature ranges from 8 to 30°C. No growth occurs at 35°C. Some strains grow in the presence of 4% NaCl, but none in the presence of 5.5% NaCl (Simpson & Taguchi, 1995). Acetoin or diacetyl are readily produced and causes a buttery odor. Exopolysaccharide formation has been reported for some strains (Carr, 1970). *Pediococcus damnosus* produces a 2-substituted (1–3)-beta-D-glucan (Duenas-Chasco *et al*., 1997). The inability to hydrolyze arginine separates *P. damnosus* from *P. acidilactici* and *P. pentosaceus*. Furthermore, *P. damnosus* has low genomic relatedness to *P. acidilactici* and *P. pentosaceus* (Dellaglio *et al*., 1981; Simpson & Taguchi, 1995). In contrast, *P. damnosus* shows a high degree of genomic relatedness to *P. inopinatus* and *P. parvulus* (34–36%) in DNA–DNA hybridization experiments. Such a close relationship is also reflected in the phylogenetic relatedness based on 16S rRNA sequences (Dobson *et al*., 2002).

Type strain: Be.1, ATCC 29358, CCUG 32251, CIP 102264, DSM 20331, JCM 5886, LMG 11484, NCIMB 12010, VKM B-1602.

GenBank accession number (16S rRNA): AJ318414, D87678.

Pediococcus inopinatus

Pediococcus inopinatus is phenotypically similar to *P. parvulus*, but distinguished by not fermenting pentoses and hydrolyzing
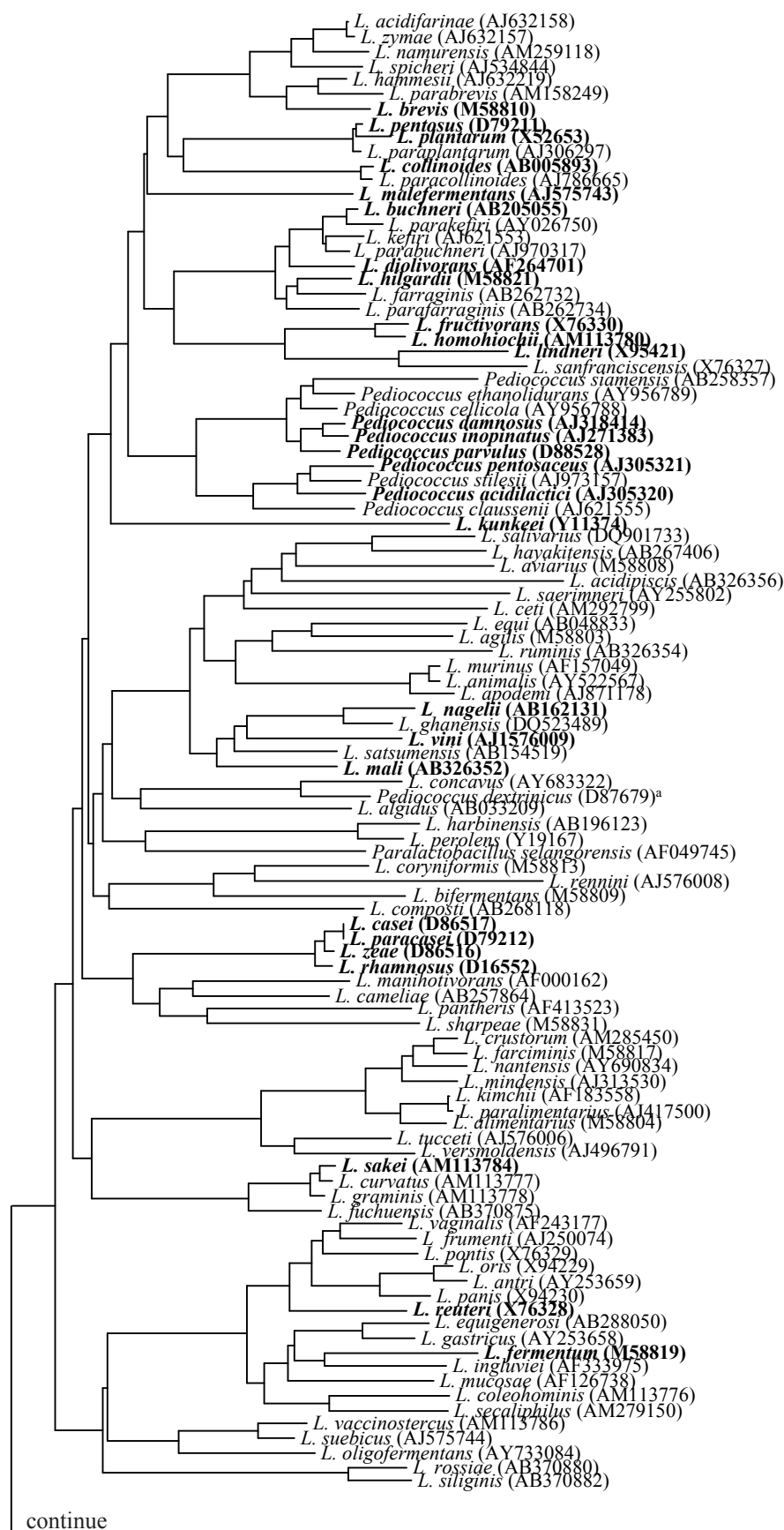
Taxonomic status of malolactic bacteria

L. acidifarinae (AJ632158)
L. zymae (AJ632157)
L. namurensis (AM259118)
L. spicheri (AJ534844)
L. hammesii (AJ632219)
L. parabrevis (AM158249)
L. brevis (M58810)
L. pentosus (D79211)
L. plantarum (X52653)
L. paraplantarum (AJ306297)
L. collinoides (AB005893)
L. paracollinoides (AJ786665)
L. malefermentans (AJ575743)
L. buchneri (AB205055)
L. parakefiri (AY026750)
L. kefiri (AJ621553)
L. parabuchneri (AJ970317)
L. diolivorans (AF264701)
L. hilgardii (M58821)
L. farraginis (AB262732)
L. parafarraginis (AB262734)
L. fructivorans (X76330)
L. homohiochii (AM113780)
L. lindneri (X95421)
L. sanfranciscensis (X76327)
Pediococcus siamensis (AB258357)
Pediococcus ethanolidurans (AY956789)
Pediococcus cellicola (AY956788)
Pediococcus damnosus (AJ318414)
Pediococcus inopinatus (AJ271383)
Pediococcus parvulus (D88528)
Pediococcus pentosaceus (AJ305321)
Pediococcus stilesii (AJ973157)
Pediococcus acidilactici (AJ305320)
Pediococcus claussenii (AJ621555)
L. kunkeei (Y11374)
L. salivarius (DQ901733)
L. hayakitensis (AB267406)
L. aviarius (M58808)
L. acidipiscis (AB326356)
L. saerimneri (AY255802)
L. ceti (AM292799)
L. equi (AB048833)
L. agilis (M58803)
L. ruminis (AB326354)
L. murinus (AF157049)
L. animalis (AY522567)
L. apodemi (AJ871178)
L. nagelii (AB162131)
L. ghanensis (DQ523489)
L. vini (AJ576009)
L. satsumensis (AB154519)
L. mali (AB326352)
L. concavus (AY683322)
Pediococcus dextrinicus (D87679)[a]
L. algidus (AB033209)
L. harbinensis (AB196123)
L. perolens (Y19167)
Paralactobacillus selangorensis (AF049745)
L. coryniformis (M58813)
L. rennini (AJ576008)
L. bifermentans (M58809)
L. composti (AB268118)
L. casei (D86517)
L. paracasei (D79212)
L. zeae (D86516)
L. rhamnosus (D16552)
L. manihotivorans (AF000162)
L. cameliae (AB257864)
L. pantheris (AF413523)
L. sharpeae (M58831)
L. crustorum (AM285450)
L. farciminis (M58817)
L. nantensis (AY690834)
L. mindensis (AJ313530)
L. kimchii (AF183558)
L. paralimentarius (AJ417500)
L. alimentarius (M58804)
L. tucceti (AJ576006)
L. versmoldensis (AJ496791)
L. sakei (AM113784)
L. curvatus (AM113777)
L. graminis (AM113778)
L. fuchuensis (AB370875)
L. vaginalis (AF243177)
L. frumenti (AJ250074)
L. pontis (X76329)
L. oris (X94229)
L. antri (AY253659)
L. panis (X94230)
L. reuteri (X76328)
L. equigenerosi (AB288050)
L. gastricus (AY253658)
L. fermentum (M58819)
L. ingluviei (AF333975)
L. mucosae (AF126738)
L. coleohominis (AM113776)
L. secaliphilus (AM279150)
L. vaccinostercus (AM113786)
L. suebicus (AJ575744)
L. oligofermentans (AY733084)
L. rossiae (AB370880)
L. siliginis (AB370882)

continue

FIGURE 1

Phylogenetic relatedness of malolactic bacteria (printed in bold) with other lactic acid bacteria, as recorded by 16S rRNA sequencing. [a]*Pediococcus dextrinicus* has recently been reclassified as *Lactobacillus dextrinicus* (Haakensen *et al*., 2009). [b]*Fructobacillus durionis, Fructobacillus ficulneus* and *Fructobacillus fructosus* have previously been classified as *Leuconostoc durionis, Leuconostoc ficulneum* and *Leuconostoc fructosum*, respectively (Endo and Okada, 2008). The phylogenetic relatedness of only malolactic bacteria is shown in Fig. 2.
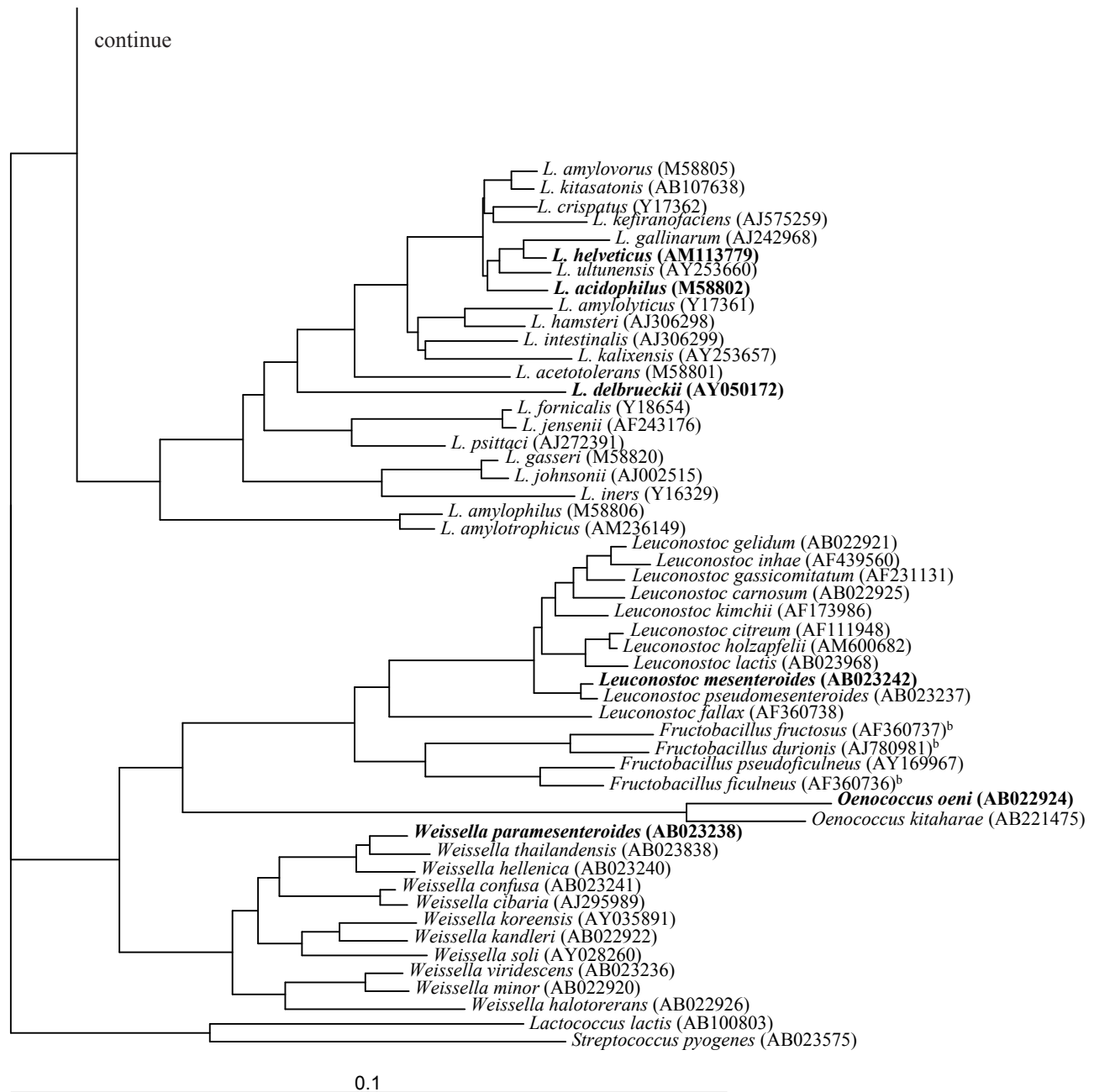
continue

L. amylovorus (M58805)
L. kitasatonis (AB107638)
L. crispatus (Y17362)
L. kefiranofaciens (AJ575259)
L. gallinarum (AJ242968)
L. helveticus (AM113779)
L. ultunensis (AY253660)
L. acidophilus (M58802)
L. amylolyticus (Y17361)
L. hamsteri (AJ306298)
L. intestinalis (AJ306299)
L. kalixensis (AY253657)
L. acetotolerans (M58801)
L. delbrueckii (AY050172)
L. fornicalis (Y18654)
L. jensenii (AF243176)
L. psittaci (AJ272391)
L. gasseri (M58820)
L. johnsonii (AJ002515)
L. iners (Y16329)
L. amylophilus (M58806)
L. amylotrophicus (AM236149)
Leuconostoc gelidum (AB022921)
Leuconostoc inhae (AF439560)
Leuconostoc gassicomitatum (AF231131)
Leuconostoc carnosum (AB022925)
Leuconostoc kimchii (AF173986)
Leuconostoc citreum (AF111948)
Leuconostoc holzapfelii (AM600682)
Leuconostoc lactis (AB023968)
Leuconostoc mesenteroides (AB023242)
Leuconostoc pseudomesenteroides (AB023237)
Leuconostoc fallax (AF360738)
Fructobacillus fructosus (AF360737)[b]
Fructobacillus durionis (AJ780981)[b]
Fructobacillus pseudoficulneus (AY169967)
Fructobacillus ficulneus (AF360736)[b]
Oenococcus oeni (AB022924)
Oenococcus kitaharae (AB221475)
Weissella paramesenteroides (AB023238)
Weissella thailandensis (AB023838)
Weissella hellenica (AB023240)
Weissella confusa (AB023241)
Weissella cibaria (AJ295989)
Weissella koreensis (AY035891)
Weissella kandleri (AB022922)
Weissella soli (AY028260)
Weissella viridescens (AB023236)
Weissella minor (AB022920)
Weissella halotorerans (AB022926)
Lactococcus lactis (AB100803)
Streptococcus pyogenes (AB023575)

0.1

FIGURE 1 (continued)

TABLE 4

Phenotypic characteristics differentiating *Pediococcus* spp.[a,b]

Characteristic	*P. dam-nosus*	*P. acidi-lactici*	*P. celli-cola*	*P. claus-senii*	*P. dextri-nicus*	*P. inopi-natus*	*P. par-vulus*	*P. pento-saceus*	*P. stilesii*
Growth at									
pH 4.5	+	+	+	+	–	+	+	+	+[c]
pH 7.0	–	+	+	+[c]	+	d	d	+	+[c]
pH 8.0	–	+	+	+[c]	+	–	–	+	+[c]
pH 9.0	–	–	–	–	–	–	–	–	+[c]
35 C	–	+	+	+	+	+	+	+	+[c]
40 C	–	+	+	+[c]	+	d	–	d	+[c]
45 C	–	+	–	–	–	–	–	d	+[c]
48 C	–	+	–	–	–	–	–	–	–[c]
Lactic acid configuration	DL	DL	DL	L(+)[c]	L(+)	DL	DL	DL	DL
Acid from									
Arabinose	–	d	d	–	–	–	–	+	–[c]
Galactose	+	+	+	–	d	+	d	+	+[c]
Lactose	–	d	+	–	d	+	–	+	–[c]
Maltose	d	–	+	d	+	+	d	+	+[c]
Melizitose	d	–	–	–	–	–	–	–	–[c]
Ribose	–	+	+	+	–	–	–	+	+[c]
Xylose	–	+	+	–	–	–	–	d	–[c]
Max. NaCl conc. for growth	5	10	?	5	6	8	8	10	8[c]

[a]Symbols: +, 90% or more strains positive; –, 90% or more strains negative; d, 11–89% of strains positive.

[b]Data partially adapted from Simpson & Taguchi (1995), Franz *et al.* (2006), Holzapfel *et al.* (2005), and Zhang *et al.* (2005).

[c]Data have only been obtained with the type strain of *P. claussenii* and *P. stilesii*.

arginine. Cells grow optimally between 30 and 32°C and withstand 6–8% (w/v) NaCl. Some strains form polysaccharides. *Pediococcus inopinatus* is best separated from *P. parvulus* by electrophoresis of L-(+)- and D-(–)-LDH's, DNA–DNA hybridization and comparison of 16S rRNA sequences.

Type strain: ATCC 49902, CCUG 38496, CIP 102406, DSM 20285, JCM 12518, LMG 11409, NCIMB 12564.

GenBank accession number (16S rRNA): AJ271383.

Pediococcus parvulus

Best growth has been recorded in broth supplemented with L-cysteine and Tween-80. Larger colonies develop when cells are streaked onto tomato juice agar and incubated anaerobically at 30°C for 48 h. Some strains require asparagine, but not folic acid. Optimal growth is recorded in medium with an initial pH of 6.5. Growth occurs in the presence of 5.5–8.0% (w/v) NaCl. Pentoses are not fermented. Some strains produce exopolysaccharides and high levels of histamine in wine.

Type strain: ATCC 19371, CCUG 28439, CIP 102262, DSM 20332, JCM 5889, LMG 11486, NBRC 100673, NCIMB 9447, VKM B-1604.

GenBank accession number (16S rRNA): D88528.

Pediococcus pentosaceus

Cells grow equally well aerobically and anaerobically in MRS at 30°C. Litmus milk reactions are variable and may be related to growth requirements. The requirement for folic acid varies between strains. Optimal growth pH is between 6.0 and 6.5, but growth may occur at pH 8.0. Cells grow in the presence of 9–10% (w/v) NaCl. The ability to ferment maltose differentiates *P. pentosaceus* from *P. acidilactici* (although not invariably). Cells may be weakly catalase-positive when grown in medium with low glucose content.

Type strain: ATCC 33316, CCUG 32205, CIP 102260, DSM 20336, JCM 5890, LMG 11488, NCIMB 12012, NCTC 12956.

GenBank accession number (16S rRNA): AJ305321, M58834.

Enrichment and isolation

Pediococcus spp. share several physiological properties with *Lactobacillus*, *Leuconostoc* and *Weissella* spp. Most culture media are thus not selective. SL Medium (Rogosa *et al.*, 1951) and Acetate Agar (Whittenbury, 1965) are semi-selective. *Pediococcus* Selective Medium (PSM), which is a modification of MRS medium (De Man *et al.*, 1961), supplemented with cysteine hydrochloride, novobiocin, vancomycin, and nystatin, may also be used. Growth is usually stimulated under anaerobic conditions.

Several growth media have been developed to detect spoilage strains of *P. damnosus* and *P. claussenii* in beer. MRS agar adjusted to pH 5.5 and MRS broth supplemented with beer (1:1 v/v) proved semi selective, but only when incubated at 22°C in the presence of 90% N_2 + 10% CO_2. Kirin-Ohkochi-Taguchi (KOT) medium, consisting of beer, malt extract, liver concentrate, maltose, L-malic

acid, cytidine, thymidine, actidione and sodium azide, may also be used (Taguchi *et al*., 1990). As far as we know, the medium has not been used to isolate pediococci from wine.

THE GENUS *LACTOBACILLUS*

The name *Lactobacillus* pertains to a small rod isolated from milk. Cells are Gram-positive, catalase negative rods, usually non-motile, long and slender (often bent), or short coryneform-like coccobacilli (Garvie, 1986). Pseudocatalase may be produced by some strains, but only in the presence of heme. Cells occur single, but more than often in chains of varying lengths. Endospores are not produced. Some strains have internal granulations or bipolar cellular inclusions, visible when Gram-stained or stained with methylene blue. Metabolism is fermentative and obligately saccharoclastic. At least half of the end product is lactate. Additional products may include acetate, ethanol, CO_2, formate, or succinate. Lactate is usually not fermented. Growth is facultatively anaerobic, with best surface growth recorded when plates are incubated in the presence of 5 to 10% CO_2. Nitrate reduction is unusual and only detected when the terminal pH is above 6.0 and/or heme is added to the growth medium. Gelatin is not liquefied and casein is not digested. Indole and H_2S are not produced. All species require complex growth media with amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters, and fermentable carbohydrates. Growth temperature ranges from 2 to 53°C, with the optimum between 30 and 40°C. All species are aciduric, with optimal growth between pH 5.5 and 6.2. Species have been isolated from various niches, including grape must, wine, beer, dairy products, grain products, meat and fish, fruit, pickled vegetables, sauerkraut, silage, sourdough, water, soil and sewage. Many of the species are part of the normal flora in the mouth, intestinal tract, and vagina of humans and animals. Pathogenicity is absent or, in rare cases, restricted to individuals with an underlying disease or immune deficiency.

This genus is the largest amongst all LAB. In the 1986 edition of *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986), 44 *Lactobacillus* species were listed. Since then, the number of validly published species escalated to approximately 100, with at least 16 subspecies.

Although many *Lactobacillus* spp. have the ability to convert L-malate to L-lactate and CO_2, only a few survive alcoholic fermentation. The most prominent species are *Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus collinoides, Lactobacillus buchneri, Lactobacillus hilgardii, Lactobacillus fructivorans, Lactobacillus kunkeei, Lactobacillus nagelii, Lactobacillus vini, Lactobacillus mali* and *Lactobacillus fermentum*. At the end of alcoholic fermentation, growth commences and cell numbers may increase to 10^7 cells/mL (König & Fröhlich, 2009).

Lactobacillus spp. less frequently isolated from wine are *Lactobacillus pentosus, Lactobacillus malefermentans, Lactobacillus homohiochii, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus zeae, Lactobacillus rhamnosus, Lactobacillus sakei, Lactobacillus reuteri, Lactobacillus helveticus, Lactobacillus acidophilus* and *Lactobacillus delbrueckii*. *Lactobacillus lindneri* has recently been isolated from Australian wines (Bae *et al*., 2006).

Lactobacillus delbrueckii is separated into four subspecies, e.g. *delbrueckii, bulgaricus, lactis* and *indicus*. Differentiation

between these subspecies is by only a few sugar fermentations. Subspecies *delbrueckii* cannot ferment lactose. Subspecies *bulgaricus* and subspecies *indicus* cannot ferment maltose. Subspecies *bulgaricus* cannot ferment sucrose, whereas trehalose is only fermented by subspecies *lactis*.

Lactobacillus paracasei is separated into subspecies *paracasei* and subspecies *tolerans*. Subspecies *tolerans* is distinguished from subspecies *paracase*i by being unable to ferment amygdalin, cellobiose, esculin, mannitol, melezitose, ribose and sucrose.

Lactobacillus plantarum subsp. *argentoratensis* is difficult to distinguish from *L. plantarum* subsp. *plantarum* and is only differentiated by a single sugar fermentation (subspecies *argentoratensis* cannot ferment melezitose). Further differentiation of these two subspecies from the phenotypically similar species *Lactobacillus paraplantarum* and *Lactobacillus pentosus* is only possible with genotypic methods (Bringel *et al*., 2005).

The two subspecies of *L. sakei* (subsp. *sakei* and subsp. *carnosus*) is phenotypically closely related and only reliably differentiated by numerical analysis of total soluble cell protein patterns and RAPD-PCR.

The phylogenetic relationship of wine *Lactobacillus* spp. with other LAB is shown in Fig. 1. In some cases, species can be differentiated based on simple phenotypic characteristics (Table 5). Differentiation based on sugar fermentation profiles has to be evaluated with care, since many of the genes encoding sugar fermentations are located on plasmids. Identification of species has to be verified by evaluation of genotypic properties. For detailed description of *Lactobacillus* spp. readers are referred to Hammes & Hertel (2009).

Key tests to differentiate malolactic bacteria

The phylogenetic relatedness of malolactic bacteria is shown in Fig. 2. Key characteristics used to differentiate amongst MLB of the genera *Oenococcus, Leuconostoc, Pediococcus* and *Lactobacillus* is summarized in Fig. 3. The type strains of species found in wine are listed below. 16S rDNA gene sequence accession numbers of the species is indicated in Fig. 1.

Lactobacillus acidophilus: ATCC 4356, CCUG 5917, CIP 76.13, DSM 20079, NBRC 13951, JCM 1132, LMG 9433, LMG 13550, NCTC 12980, NRRL B-4495, VKM B-1660.

Lactobacillus brevis: ATCC 14869, CCUG 30670, CIP 102806, DSM 20054, JCM 1059, LMG 6906, LMG 7944, NRRL B-4527.

Lactobacillus buchneri: ATCC 4005, CCUG 21532, CIP 103023, DSM 20057, JCM 1115, LMG 6892, NCAIM B.01145, NRRL B-1837, VKM B-1599.

Lactobacillus casei: ATCC 393, BCRC 10697, CCUG 21451, CECT 475, CIP 103137, DSM 20011, IAM 12473, NBRC 15883, JCM 1134, KCTC 3109, LMG 6904, NCIMB 11970, NCIMB 11970, NRRL B-1922.

Lactobacillus collinoides: ATCC 27612, CCUG 32259, CIP 103008, DSM 20515, JCM 1123, LMG 9194.

Lactobacillus delbrueckii subsp. *delbrueckii:* ATCC 9649, CCUG 34222, CIP 57.8, DSM 20074, NBRC 3202, JCM 1012, LMG 6412, NCIMB 8130, NRRL B-763, VKM B-1596.

TABLE 5

Key characteristics of *Lactobacillus species*[a]

	L. delbrueckii subsp. *delbrueckii*	*L. acido-philus*	*L. helve-ticus*	*L. mali*	*L. nagelii*	*L. casei*	*L. homo-hiochii*
Fermentation type	A[b]	A	A	A	A	B[d]	B
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	mDpm[c]	mDpm	Lys-D-Asp	Lys-D-Asp
G + C content (mol%)	49-51	34-37	37	32.5	ND	45-47	35-38
Lactic acid configuration	D	DL	DL	L	DL	L	DL
Growth at 15 C/45 C	-/+	-/+	-/+	+/d	+/+	+/-	+/-
Gas from glucose	-	-	-	-	-	-	-
Fermentation of:							
Amygdalin	-	+	-	+	+	+	-
Cellobiose	-	+	-	d	+	+	d
Mannitol	+	+	d	+	+	+	d
Melibiose	-	d	-	d	-	-	-
Raffinose	-	d	-	d	-	-	-
Sucrose	+	+	-	+	+	+	-

	L. jensenii	*L. paracasei* subsp. *paracasei*	*L. pentosus*	*L. plantarum* subsp. *plantarum*	*L. rhamnosus*	*L. sakei*	*L. vini*	*L. zeae*
Fermentation type	B	B	B	B	B	B	B	B
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	mDpm	mDpm	Lys-D-Asp	Lys-D-Asp	mDpm	Lys-D-Asp
G + C content (mol%)	33-35	45-47	46-47	44-46	45-47	42-44	39	48-49
Lactic acid isomer(s)	D	L	DL	DL	L	DL/L(D)	DL	L(D)
Growth at 15 C/45 C	-/+	+/d	+/-	+/-	+/+	+/-	-/+	+/+
Gas from glucose	-	-	-	-	-	-	-	-
Carbohydrate fermentation								
Amygdalin	+	+	+	+	+	-	+	+
Cellobiose	+	+	+	+	+	d	+	+
Mannitol	d	+	+	+	+	-	-	+
Melibiose	-	-	+	+	-	+	-	-
Raffinose	-	-	+	+	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+

	L. brevis	*L. buchneri*	*L. collinoides*	*L. fermentum*	*L. fructivorans*	*L. hilgardii*	*L. kunkeei*
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Orn-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp
G + C content (mol%)	44-47	44-46	46	52-54	38-40	39-41	ND.
Growth at 15 C/45 C	+/-	+/-	+/-	-/+	+/-	+/-	+/-
NH3 from arginine	+	+	+	+	+	+	-
Gas from glucose	+	+	+	+	+	+	+
Carbohydrate fermentation							
Arabinose	+	+	+	d	-	-	ND
Cellobiose	-	-	-	d	-	-	-
Esculin	d	d	+	-	-	-	-
Galactose	d	d	+	+	-	d	-
Maltose	+	+	+	+	d	+	-
Mannose	-	-	-	w	-	-	-
Melezitose	-	+	+	-	-	d	-
Melibiose	+	+	+	+	-	-	-
Raffinose	d	d	-	+	-	-	w
Ribose	+	+	+	+	w	+	-
Sucrose	d	d	-	+	d	d	+
Trehalose	-	-	-	d	-	-	-
xylose	d	d	+	d	-	+	-

[a]Data obtained from Hammes & Hertel (2009)
[b]A: Obligately homofermentative
[c]mDpm = meso-diaminopimelic acid
[d]B: Facultatively heterofermentative

TABLE 5 (continued)

Key characteristics of *Lactobacillus species*[a]

	L. lindneri	*L. malefermentans*	*L. reuteri*
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp
G + C content (mol%)	35	41-42	40-42
Growth at 15 C/45 C	+/-	+/-	-/+
NH3 from arginine	-	+	+
Gas from glucose	+	+	+
Carbohydrate fermentation			
Arabinose	-	-	+
Cellobiose	-	-	-
Esculin	-	-	ND
Galactose	-	-	+
Maltose	+	+	+
Mannose	-	-	-
Melezitose	-	-	-
Melibiose	-	-	+
Raffinose	-	-	+
Ribose	-	+	+
Sucrose	-	-	+
Trehalose	-	-	-
Xylose	-	-	-

[a]Obligately heterofermentative lactobacilli

Lactobacillus delbrueckii subsp. *bulgaricus:* ATCC 11842, CCUG 41390, CIP 101027, DSM 20081, NBRC 13953, JCM 1002, LMG 6901, LMG 13551, NCTC 12712, VKM B-1923.

Lactobacillus delbrueckii subsp. *lactis:* ATCC 12315, CCUG 31454, CIP 101028, DSM 20072, JCM 1248, LMG 7942, NRRL B-4525.

Lactobacillus delbrueckii subsp. *indicus:* NCC725, DSM 15996, LMG 22083.

Lactobacillus fermentum: ATCC 14931, CCUG 30138, CIP 102980, DSM 20052, NBRC 15885, JCM 1173, LMG 6902, NCCB 46038, NCIMB 11840, NRRL B-4524.

Lactobacillus fructivorans: ATCC 8288, CCUG 32260, CIP 103042, DSM 20203, NBRC 13954, JCM 1117, LMG 9201, NRRL B-1841.

Lactobacillus helveticus: ATCC 15009, CCUG 30139, CIP 103146, DSM 20075, NBRC 15019, JCM 1120, LMG 6413, LMG 13555, NRRL B-4526.

Lactobacillus hilgardii: ATCC 8290, CCUG 30140, CIP 103007, DSM 20176, NBRC 15886, JCM 1155, LMG 6895, NRRL B-1843.

Lactobacillus homohiochii: ATCC 15434, CCUG 32247, CIP 103141, DSM 20571, NBRC 15887, JCM 1199, JCM 7793, LMG 9478, NRRL B-4559.

Lactobacillus kunkeei: YH-15, ATCC 700308, DSM 12361.

Lactobacillus lindneri: KACC 12445, CIP 102983, DSM 20690, JCM 11027, LMG 14528.

Lactobacillus malefermentans: D2 MF1, ATCC 49373, CCUG 32206, CIP 103367, DSM 5705, NBRC 15905, JCM 12497, LMG 11455, NCIMB 701410.

Lactobacillus mali: ATCC 27053, CCUG 30141, CCUG 32228, CIP 103142, DSM 20444, JCM 1116, LMG 6899, NCIMB 10560, NRRL B-4563, VKM B-1600.

Lactobacillus nagelii: LuE10, ATCC 700692, CCUG 43575, DSM 13675, JCM 12492.

Lactobacillus paracasei subsp. *paracasei:* ATCC 25302, CCUG 32212, CIP 103918, DSM 5622, NBRC 15889, JCM 8130, LMG 13087, NCIMB 700151.

Lactobacillus paracasei subsp. *tolerans:* ATCC 25599, CCUG 34829, CIP 102994, CIP 103024, DSM 20258, NBRC 15906, JCM 1171, LMG 9191, NCIMB 9709.

Lactobacillus pentosus: ATCC 8041, CCUG 33455, CIP 103156, DSM 20314, JCM 1558, LMG 10755, NCAIM B.01727, NCCB 32014, NCIMB 8026, NRRL B-227, NRRL B-473.

Lactobacillus plantarum subsp. *plantarum:* ATCC 14917, CCUG 30503, CIP 103151, DSM 20174, NBRC 15891, JCM 1149, LMG 6907, NCIMB 11974, NRRL B-4496.

Lactobacillus plantarum subsp. *argentoratensis:* DKO 22, CIP 108320, DSM 16365.

Lactobacillus reuteri: ATCC 23272, CCUG 33624, CIP 101887, DSM 20016, NBRC 15892, JCM 1112, LMG 9213, LMG 13557, NRRL B-14171.

Lactobacillus rhamnosus: ATCC 7469, CCUG 21452, CIP A157, DSM 20021, NBRC 3425, JCM 1136, LMG 6400, NCAIM B.01147, NCCB 46033, NCIMB 6375, NCTC 12953, NRRL B-442, VKM B-574.

Lactobacillus sakei subsp. *sakei:* ATCC 15521, CCUG 30501, CIP 103139, DSM 20017, NBRC 15893, JCM 1157, LMG 9468, LMG 13558.
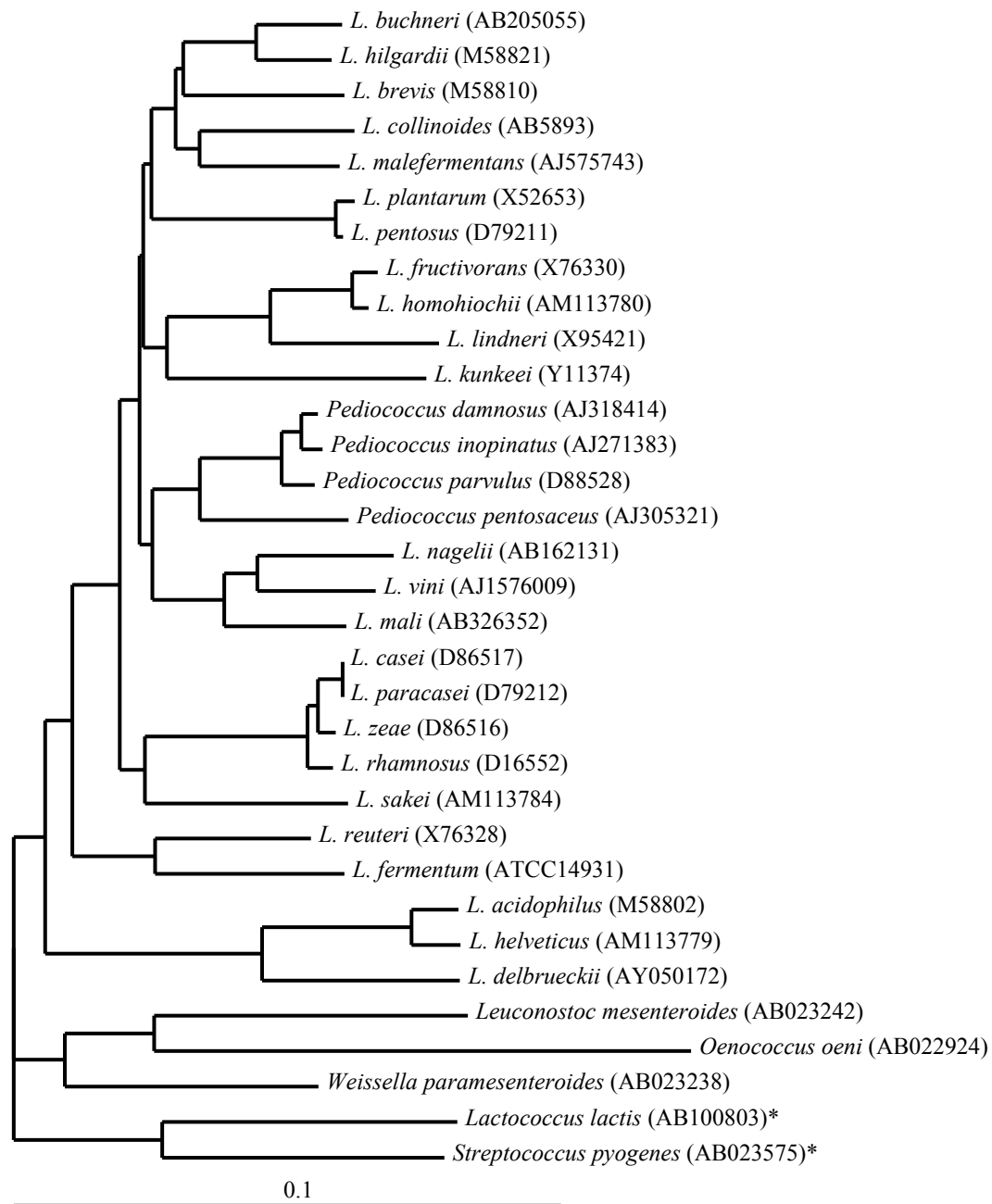
FIGURE 2

Phylogenetic relatedness of malolactic LAB without other LAB, as recorded by 16S rRNA sequencing. *Lactococcus lactis* and *Streptococcus pyogenes* were kept as outside reference strains (as also in Fig. 1).
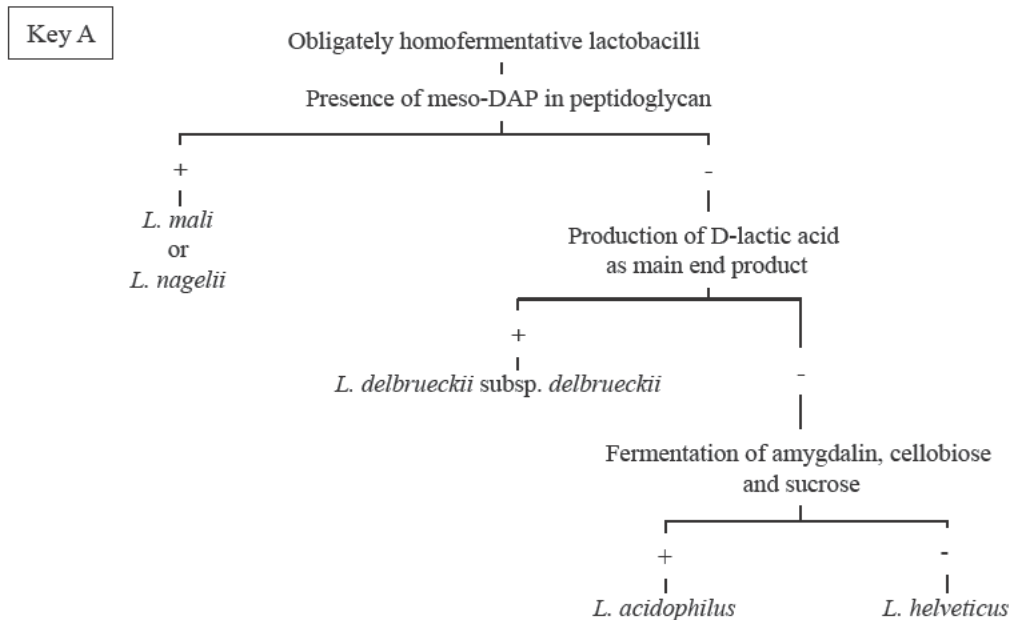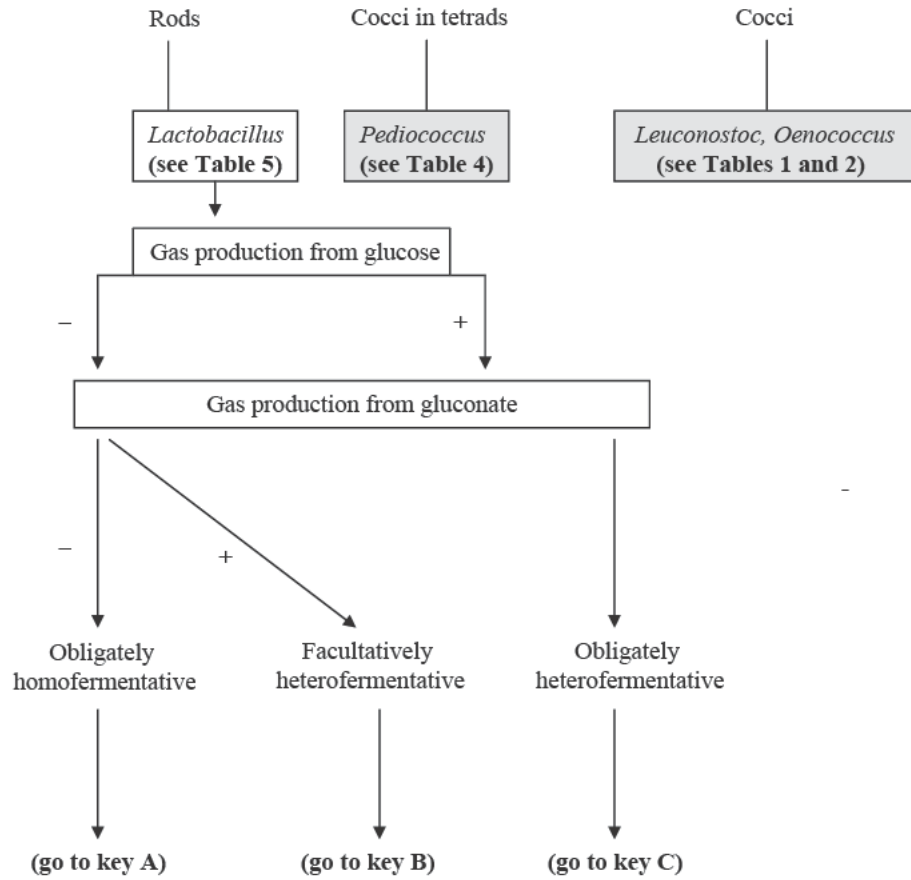
Rods Cocci in tetrads Cocci

| *Lactobacillus* (see Table 5) | *Pediococcus* (see Table 4) | *Leuconostoc, Oenococcus* (see Tables 1 and 2) |

Gas production from glucose

−   +

Gas production from gluconate

−   +

Obligately homofermentative   Facultatively heterofermentative   Obligately heterofermentative

(go to key A)   **(go to key B)**   **(go to key C)**

Key A

Obligately homofermentative lactobacilli

Presence of meso-DAP in peptidoglycan

+   −

L. mali
or
L. nagelii

Production of D-lactic acid
as main end product

+

L. delbrueckii subsp. *delbrueckii*

−

Fermentation of amygdalin, cellobiose
and sucrose

+   −

L. acidophilus   *L. helveticus*

FIGURE 3

Key phenotypic characteristics to differentiate malolactic bacteria.

Key B

Facultatively heterofermentative lactobacilli

Presence of meso-DAP in peptidoglycan

+

Fermentation of gluconate, mannitol, melibiose, raffinose and sorbitol.

+

L. plantarum or *L. pentosus*

(go to key D)

–

L. vini

–

Stereoisomer of lactic acid produced

L

Fermentation of amygdalin, mannitol and melezitose.

+

L. casei,
L. paracasei,
L. rhamnosus or
L. zeae

(go to key E)

–

L. sakei

DL

L. homohiochii

D

L. jensenii

Key C

Obligately heterofermentative lactobacilli

NH₃ from arginine

NH_3 from arginine

+

Ornithine in peptidoglycan

+

L. fermentum

–

Growth at 45°C but not at 15°C

+

L. reuteri

–

Fermentation of melezitose

+

L. buchneri or *L. collinoides*

–

Fermentation of arabinose

+

L. brevis

–

Fermentation of xylose

+

L. hillgardii

–

L. fructivorans or *L. malefermentans*

–

Fermentation of sucrose

+

L. kunkeei

–

L. lindneri

FIGURE 3 (continued)

Key phenotypic characteristics to differentiate malolactic bacteria.

Key D

Species-specific DNA primers for multiplex PCR assay of *L. plantarum* subsp. *plantarum, L. plantarum* subsp. *argentoratensis, L. pentosus,* and *L. paraplantarum*

Primer	Sequence (5' to 3')
paraF	GTCACAGGCATTACGAAAAC
pentF	CAGTGGCGCGGTTGATATC
planF	CCGTTTATGCGGAACACCTA
pREV	TCGGGATTACCAAACATCAC

L. plantarum subsp. *plantarum* produces a 318 bp amplicon, *L. plantarum* subsp. *argentoratensis* a 318 bp amplicon and a 120 bp amplicon, *L. pentosus* a 218 bp amplicon, and *L. paraplantarum* a 107 bp amplicon, as indicated below (from Torriani *et al.*, 2001 and Bringel *et al.*, 2005).

1. *L. plantarum* subsp. *plantarum*
2. *L. plantarum* subsp. *argentoratensis*
3. *L. pentosus*
4. *L. paraplantarum*
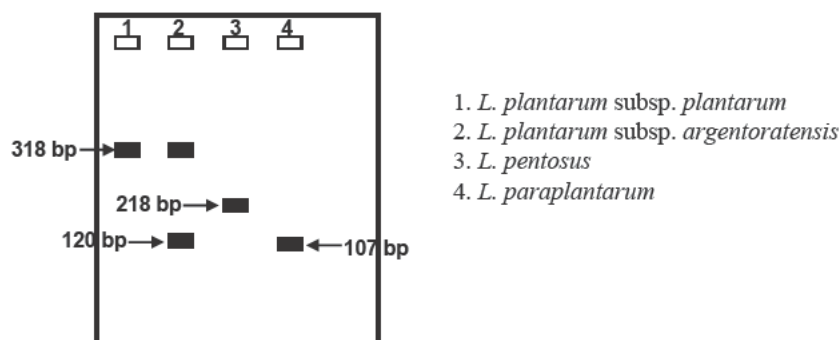
Key E

Species-specific primers to differentiate *L. casei, L. paracasei, L. rhamnosus* and *L. zeae*

Name of primer	Sequence (5' to 3')
W1	TGCACTGAGATTCGACTTAA
W2	CACCGAGATTCAACATGG
W3	TGCATCTTGATTTAATTTTG
D1	TGCATCGTGATTCAACTTAA
Y2	CCCACTGCTGCCTCCCGTAGGAGT

L. casei produces a 295 bp of amplicon with W1-Y2 primer set.

L. paracasei produces a 295 bp of amplicon with W2-Y2 primer set.

L. rhamnosus produces a 295 bp of amplicon with W3-Y2 primer set.

L. zeae produces a 295 bp of amplicon with D1-Y2 primer set.

FIGURE 3 (continued)

Key phenotypic characteristics to differentiate malolactic bacteria.

Lactobacillus sakei subsp. *carnosus:* R 14b/a, CCUG 31331, CIP 105422, JCM 11031, LMG 17302.

Lactobacillus vini: DSM 20605, CECT 5924

Lactobacillus zeae: ATCC 15820, CCUG 35515, DSM 20178, JCM 11302, LMG 17315, NCIMB 9537.

Enrichment and isolation

The most common medium used to isolate *Lactobacillus* spp. is MRS agar (De Man *et al*., 1960) or APT agar (Evans and Niven, 1951). Acetate medium (Rogosa *et al*., 1951) may also be used, but is as MRS and APT, not completely selective. Other LAB such as leuconostocs, pediococci, enterococci and *Weissella* spp., bifidobacteria and yeasts may also grow. Growth of yeast is prevented by the addition of cycloheximide (100 mg/L). Intestinal lactobacilli are commonly isolated on MRS, SL, or LAMVAP agar (Hartemink *et al*., 1997). More anaerobic species may be isolated by supplementing the growth media with L-cysteine (0.05%, w/v) and incubation in an anaerobic jar. In some cases, medium have been supplemented with grape must, tomato juice, fresh yeast extract, malt extract, ethanol, mevalonic acid, beer, fruit juices, cheese whey, or meat extract. It may be necessary to replace glucose with fructose, maltose, sucrose, or arabinose, especially for isolation of heterofermentative lactobacilli. Homohiochii Medium (Kleynmans *et al*., 1989) and NBB medium (Back, 1980) are good examples of specialised growth media. Plates should always be incubated anaerobically, or in the presence of 10% CO_2 and 90% N_2 or H_2. Carr *et al*. (2002) compiled an extensive literature survey on culture media for *Lactobacillus* and other LAB.

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