Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products

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Review article

Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products

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Abstract – During the last fifteen years, the *Lactobacillus* genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks. Quality assurance programmes associated with research, development, production and validation of the health or technological benefits of these bacteria require their relevant isolation, counting and identification. This review presents the different selective media to isolate lactobacilli, and the numerous different available tools to characterise lactobacilli at genus, species or strain level using either culture-dependent methods: phenotypical, molecular or global methods, or using new culture-independent advanced molecular methods. Enzymes used for PFGE, hybridisation probes and PCR-based method primers are listed in seven tables. In conclusion, the main advantages and disadvantages associated with these techniques are presented.

Lactobacillus **/ media / PFGE restriction enzyme / probe / primer / cheese / dairy product**

Résumé – Isolement, caractérisation et identification des lactobacilles des produits laitiers. Durant les quinze dernières années, le genre *Lactobacillus* a subi de nombreux remaniements et compte actuellement plus de 80 espèces. Les lactobacilles sont présents dans le lait cru, les produits laitiers tels que les fromages, les yaourts et les laits fermentés. Pour s'inscrire dans une démarche qualité visant au développement de lactobacilles à effet santé ou d'intérêt technologique, l'isolement, le comptage et la caractérisation parfaite de ces bactéries sont nécessaires. Cette analyse bibliographique présente les différents milieux sélectifs pour isoler les lactobacilles, ainsi que les outils disponibles à ce jour pour caractériser les lactobacilles au niveau du genre, de l'espèce ou de la souche, aussi bien par des méthodes culture dépendantes : phénotypique, moléculaire, globale, que par les nouvelles méthodes se réalisant à partir d'échantillons bruts. Les enzymes utilisées en PFGE, les sondes d'hybridation et les oligonucléotides utilisés pour les différentes techniques de PCR sont répertoriés en sept tableaux. En conclusion, les avantages et inconvénients de ces techniques sont présentés.

Lactobacillus **/ milieu sélectif / enzyme PFGE / sonde moléculaire / amorce PCR / fromage / produit laitier**

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1. INTRODUCTION: BIODIVERSITY OF LACTOBACILLI IN DAIRY PRODUCTS

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. Their common traits are: Gram-positive, usually catalase-negative, growth under microaerophilic to strictly anaerobic conditions and lactic acid production. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microflora. *Lactobacillus* is one of the most important genera of LAB. In raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer.

1.1. Cheeses and milks

In cheese, lactobacilli (*Lb. helveticus* and *Lb. delbrueckii* ssp. and *lactis*) are present in industrial starter cultures for processing hard cheese (e.g. Emmental, Comté, Italian Grana and Argentinean hard cheeses) [21, 43, 92]. Pasta filata cheeses (Mozarella) and Italian hard cheeses (Canestrato Pugliese and Parmigiano Reggiano) are also processed with *Lb. delbrueckii* ssp. *bulgaricus* [42, 45, 83]. Lactobacilli starters are normally present at levels of 10^9 bacteria/g, contribute to the lactic fermentation, and are involved at the beginning of ripening [70]. For example, in Emmental cheeses, lactobacilli ferment galactose excreted by *Streptococcus thermophilus*, achieve acidification processes, and contribute to primary proteolysis [31, 35, 59, 74]. Their numbers decrease rapidly during ripening, at a rate depending to some degree on the sensitivity of the starters to salt [110], on the water activity, and on the autolysis power of the strains [185]. Some lactobacilli are also present in the natural microflora of the dairy products (non-starter lactic acid bacteria: NSLAB) and originate from animals, farms and cheese dairies: *Lb. casei* ssp. *casei/Lb. paracasei* ssp. *paracasei, Lb.rhamnosus, Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. buchneri, Lb. curvatus, Lb. acidophilus* and *Lb. pentosus* [43, 58, 83, 110, 120, 133]. NSLAB, which are initially present in small numbers (10^2 to 10^3 NSLAB/g after pressing in Cheddar cheese), increase to high numbers in cheese varieties that require long ripening times (10^7 to 10^8 bacteria/g within about 3 months in Cheddar cheese) [10, 21, 34, 58, 69, 73, 86, 110, 145].

Coppola et al. [44] studied the microbiological characteristics of raw milk, natural whey starter and cheese during the first months of ripening of Parmigiano Reggiano: thermophilic lactobacilli – *Lb. helveticus, Lb. delbrueckii* ssp. *lactis. Lb. delbrueckii* ssp. *bulgaricus* – disappeared within 30 d. Rod-shaped mesophilic facultatively heterofermentative lactobacilli – *Lb. casei, Lb. paracasei* ssp. *paracasei, Lb. paracasei* ssp. *tolerans* and *Lb. rhamnosus* – progressively increased in number until the fifth month of ripening. The results showed that the thermophilic lactobacilli and *Lb. rhamnosus* were derived from natural whey starter, whereas the other components of non-starter lactobacilli were derived from raw milk. Similarly, Comté cheese contains mesophilic lactobacilli strains. They originate from the raw milk, and this source was probably more important than the factory environment [17, 58]. Differences have been observed between raw milk cheeses and pasteurised or microfiltered milk cheeses [58, 70, 164]. Eliskases-Lechner et al. [70] found that Bergkäse (Austrian regional cheese) cheeses made from pasteurised milk contained less than one-thousandth the number of facultatively heterofermentative lactobacilli (FHL) present in raw milk cheeses. Differences in citrate metabolism, which occur in raw milk cheeses, can be attributed to the presence of FHL in these cheeses. Very little effort is currently devoted to controlling the numbers

and types of NSLAB present, although these organisms are thought to have a significant influence on cheese flavour development and to participate directly in the production of some major aroma compounds such as acetic acid, formic acid and gas [31, 56, 59, 173]. However, NSLAB may also cause defects. For example, in Emmental cheeses, *Lb. plantarum* may disturb the metabolism of propionic acid bacteria, resulting in lower quality cheeses due to opening and changes in flavour development [34].

It has repeatedly been claimed that the use of selected strains as adjunct cultures improves and accelerates flavour development. The use of *Lactobacillus* adjunct cultures to Cheddar cheese or to cow's milk cheeses ripened for short periods of time (e.g. Azua-Ulloa cheese) has been reported to result in higher levels of proteolytic products and higher sensory quality scores in many studies [120, 124, 134, 175]. However, some adjunct cultures may cause high levels of acidity, bitterness, off flavours and open and crumbly textures, clearly demonstrating the importance of culture selection [46, 69, 101]. A number of studies have recently evaluated the suitability of probiotic cultures as adjunct cultures in various cheeses: *Lb. acidophilus* and *Lb. casei* in Argentinian Fresco cheese [191], *Lb. paracasei* in Cheddar cheeses [76, 172], and *Lb. acidophilus* in goat's milk cheeses [85].

1.2. Yoghurts and fermented milks

Lactobacillus delbrueckii ssp. *bulgaricus* is one of the two bacteria necessary for the production of yoghurts, and *Lb. kefir* is essential for the production of Caucasian sour milk kefir [111]. A recent trend is to add probiotic lactobacilli to fermented milks to generate health benefits. The bacteria generally added are *Lb. acidophilus*, *Lb. rhamnosus, Lb. reuteri, Lb. casei, Lb. plantarum, Lb. johnsonii, Lb. crispatus, Lb. paracasei* and *Lb. gasseri* [88, 94, 113, 142, 159]. For these products, careful strain selection is necessary. Klein et al. [113] recently observed that the identity of most of the lactobacilli used as probiotics differs from that marked on the packaging. Shah [162] reported that it is important to monitor the survival of probiotic lactobacilli because a number of products have been found to contain only a few viable bacteria by the time they reach the market [87, 163, 167].

Given the great potential economic value of lactobacilli, one of the main objectives of microbiologists is to develop a clear picture of the microflora present in the various dairy products, and the way in which it changes during processing. For example, during cheese manufacture and ripening, complex interactions occur between individual components of the cheese microflora, and identification of these bacteria is essential for understanding their individual contribution to cheese manufacture. This allows the development of a more targeted approach to starter/adjunct selection for the improvement of cheese quality [14]. Quality assurance programmes associated with research, development, production and validation of the health or technological benefits of these bacteria require the relevant isolation, counting and identification of bacteria.

Depending on the taxonomic level desired, several phenotypical or molecular methodologies (polyphasic analysis) can be used for isolation, characterisation and identification of lactobacilli. Recent methodologies, which are culture-independent such as single strand conformation polymorphism (SSCP), temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) have also been proposed for characterisation of microbial diversity. This paper reviews how the literature proposes characterisation of lactobacilli from dairy products at genus, species or strain level.

2. STUDY OF LACTOBACILLI BY CULTURE-DEPENDENT METHODS

2.1. Isolation of lactobacilli: from crude sample to pure culture

Guidelines have been published for the extraction of lactobacilli from milk or dairy products [104]. It is often difficult to count microorganisms from particulate or solid samples. In such cases, the cells must be separated from the particles and the extraction efficiency must be assessed. The separation methods currently used to extract microorganisms from solid food matrices such as hard cheese and from dry starters and other types of cheese are based on an initial crude homogenisation in a blender and/or a stomacher [165]. For hard cheeses, an ultra-turrax or a stomacher is recommended.

2.1.1. Resuspension medium

Callichia et al. [28] increased the rate of recovery of dried microorganisms to a level similar to that of wet cultures that had never been dried by resuspending the dried microorganisms in a medium containing phosphate, cysteine, antifoaming agent and agar before plating. McCann et al. [130] analysed a number of commercial dairy cultures, comparing methods of sample preparation: duplicate samples of probiotic products were resuspended and diluted in both peptone and CRM (Calicchia resuspension medium). Following resuspension in peptone and incubation for 30 min at 23 °C before plating, the rate of CFU recovery for each product was approximately 50% of that obtained if the microorganisms were resuspended in CRM and incubated for 30 min at 37 °C before plating. For cheeses, dilution in Trisodium citrate (2% w/v) is generally recommended, and peptone salt or phosphate buffer salt is generally used for dairy products such as yoghurts and fermented milks.

2.1.2. Selective media

Several elective and selective media have been developed for the isolation and counting of *Lactobacillus* species and for the differential counting of mixed populations of lactic acid bacteria (Tab. I). Oxygen tolerance, nutritional requirements, antibiotic susceptibility, colony morphology and colour are used to differentiate strains in these methods. The differential detection and counting of lactobacilli can currently be achieved in a number of ways. However, only a few media are useful for the differential counting of lactobacilli because numerous other microorganisms including *Lactococcus, Enterococcus, Leuconostoc, Weissella, Bifidobacterium* and *Pediococci* grow on media similar to those used for lactobacilli. No medium has yet been described on which only lactobacilli are able to grow.

Lactobacilli are generally isolated on rich media such as MRS [54], which is routinely used for the isolation and counting of lactobacilli from most (fermented) food products. The addition of a reducing agent such as cysteine 0.05% to MRS improves the specificity of this medium for *Lactobacillus* isolation [90, 116, 162]. MRS and M17 are, respectively, the medium of choice for the differential counting of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in yoghurt [105]. Birollo et al. [18] proposed the use of a cheaper medium – skim milk agar – for counting and differentiating colonies of these two bacteria. When yogurt microflora was supplemented by other lactobacilli, specific methods were needed. Enumeration of *Lb. delbrueckii* ssp. *bulgaricus* in the presence of *Lb. acidophilus* was possible, on acidified MRS at pH 5.2 or on reinforced clostridial agar (RCA) at pH 5.3, at 45 °C for 72 h [162]. Selective counting of *Lb. acidophilus* has been developed using several MRS media in which the dextrose is replaced by maltose, raffinose, melibiose, trehalose, arabinose, galactose, sorbitol, ribose, gluconate or salicin or cellobiose-

Table I. Differential plating media for detection and counting of *Lactobacillus* species.

Product	Population	Media	Incubation conditions	Isolation	Notice	Ref.
Yogurts	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus*	Acidified MRS	37 °C, 3 d, Ana	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus*	*S. thermophilus* = circular opalescent white colonies with well defined borders *Lb. delb.* ssp. *bulgaricus* = rounded colonies, duller, flat with non defined borders	[104]
		Skim milk agar	37 °C, 3 d, Ana	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus*	Impossibility to differentiate between both types of colonies	[18, 190]
		Acidified MRS Skim milk agar	37 °C, 3 d, Aer	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus*	*S.thermophilus* = circular opalescent white colonies with well defined borders *Lb. delb.* ssp. *bulgaricus* = bigger irregular translucent colonies with non defined borders	[104] [18] [190]
		Acidified skim millk agar	47 °C, 2 d, Ana	*Lb. delb.* ssp. *bulgaricus*		[32]
Fermented milks and probiotic products	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus* *Lb. casei*	CLBS (Cellobiose *Lactobacillus* selection agar)	37 °C, 2 d, Ana	*Lb. acidophilus* *Lb. casei*		[142]
		MRS Salicin				[162]
		MRS Sorbitol		*Lb. casei*		[151]
		LC (Lb. casei agar)				
	S.thermophilus *Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus* *Bifidobacterium* spp.	HHD (Homofermentative-heterofermentative Differential medium)	37 °C, 2 d, Ana	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus* *Bifidobacterium* sp.	*S. thermophilus* = blue colonies *Lb. delb.* ssp. *bulgaricus* = blue colonies *Lb. acidophilus* = blue colonies with white surrounding *Bifidobacterium* spp. = white colonies	[29]
		LA agar Bifidus blood agar			*S. thermophilus* = pin point colonies *Lb. delb.* ssp. *bulgaricus* = elevated and white colonies *Lb. acidophilus* = flat and grey colonies *Bifidobacterium* sp. = elevated and chocolate brown colonies	[32]
		TPPY (Tryptose Proteose Peptone yeast extract-eriochrome T agar)	37 °C, 2 d, Aer	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus*	*S. thermophilus* = circular or semi circular colonies, convex, opaque, white-violet, often with a dark centre *Lb. delb.* ssp. *bulgaricus* = flat, transparent, diffuse colonies, of undefined shape, with an irregular edge *Lb. acidophilus* = small colonies, white to violet, slightly elevated and somewhat fuzzy	[23]

Table I. Differential plating media for detection and counting of *Lactobacillus* species.

		TPPYPB (TPPY with Prussian blue)	37 °C, 2 d, Aer	*S.thermophilus* *Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus*	*S. thermophilus* = circular or semi circular colonies, convex, opaque, white-violet, often with a dark centre *Lb. delb.* ssp. *bulgaricus* = small shiny white colonies surrounded by a wide royal blue zone *Lb. acidophilus* = large pale colonies surrounded by a wide royal blue zone	[78]
		MRS maltose	37 °C, 3 d, Ana	*Lb. acidophilus* *Bifidobacterium* spp.		[117]
		MRS arabinose		*Lb. acidophilus* *Bifidobacterium* spp.	Growth of *Bifidobacterium bifidum, B. infantis* and *B. breve* is inhibited, but not for *B. longum* and *B. pseudolongum*	
		Bile-MRS		*Lb. acidophilus* *Bifidobacterium* spp.	All *bifidobacterium* species do not grow on this media and some *Lb. delb.bulg* can grow.	
		RCA pH 5.5		*Lb. delb.bulgaricus* *Bifdobacterium* spp. *Lb. acidophilus*	Only some strains of *Lb. acidophilus* can grow on this media	
		T-MRS (Trehalose MRS)	37 °C, 3 d, Aer	*Lb. acidophilus*	*Lb. acidophilus* = round creamly colonies *Lb. delb.* ssp. *bulgaricus* = irregular white colonies	[103] [190]
		Bile-MRS				
		OG-MRS (Oxgall, Gentamycin MRS)				
		G-MRS (Galactose MRS)	37 °C, 3 d, Aer	*Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus*		
		X-Glu	37 °C, 3 d, Ana	*Lb. delb.* ssp. *bulgaricus* *Lb. acidophilus*	*Lb. delb.* ssp. *bulgaricus* = white colonies *Lb. acidophilus* = blue colonies	[114]
	S.thermophilus *Lb. acidophilus* *Bifidobacterium* spp.	LBS (*Lactobacillus* selection agar)	37 °C, 2 d, Ana	*Lb. acidophilus*		[159]
	E.faecium *Lb. acidophilus* *Bifidobacterium* spp.	Brigg's agar	37 °C, 2 d, Aer	*E. faecium* *Lb. acidophilus*	*E. faecium* (24H) *Lb. acidophilus* (48H)	[28]
		Modified Brigg's agar (Brigg's agar, plus Streptomycin sulfate)	37 °C, 2 d, Aer	*Lb. acidophilus*	All the strains are not Streptomycin resistant	
		3 BLA	37 °C, 2 d, Aer	*E. faecium* *Lb. acidophilus*	*E. faecium* (24H) *Lb. acidophilus* (48H)	[130]
Cheeses		MRS	37 °C, 3 d, Ana	Lactobacilli, and other lactic acid bacteria		[105]
		MRS	42 °C, 3 d, Ana	Thermophilic Lactobacilli		[21] [58]
		LBS medium plus Cycloheximide	30 °C, 3 d, Ana	Lactobacilli		[120]

Table I. Differential plating media for detection and counting of *Lactobacillus* species.

		FH agar (Facultativ Heterofermentativen Laktobazillen agar)	38 °C, 3 d, Ana	Facultative heterofermentative Lactobacilli		[106] [58]
		Facultativ Heterofermentativen Laktobazillen agar plus nalidixic acid				[21]
		MRS plus Cycloheximide	30 °C, 2 d, Ana	Mesophilic Lactobacilli		[45]
			44 °C, 2 d, Ana	Thermophilic Lactobacilli		
		LBS (*Lactobacillus* selection agar)	30 °C, 3 d, Ana	Lactobacilli		[70]
		Facultativ Heterofermentativen Laktobazillen agar plus Vancomycin	37 °C, 3 d, Ana	Facultative heterofermentative Lactobacilli		
		OH medium (Obligate Heterofermentative Lactobacilli medium)	37 °C, 3 d, Aer	Obligate heterofermentative Lactobacilli		
		HHD (homofermentative and heterofermentative differential medium)	30 °C, 3 d, Aer	Lactic acid bacteria	Homofermentative LAB = blue to green colonies Heterofermentative LAB = white colonies	[132]
		MRS agar pH 6.5 FH agar	20 °C, 5 d, Ana 37 °C, 3 d, Ana	Mesophilic Lactobacilli		[17]
Probiotic cheeses	*with Bifidobacterium* spp., *Lb. acidophilus, Lb. casei*	LP-MRS agar	37 °C, 3 d, Aer	*Bifidobacterium* spp., *Lb. casei*		[190]
		Bile-MRS agar	37 °C, 3 d, Ana	*Lb. acidophilus*		
	With Lb. paracasei	LBS (*Lactobacillus* selection agar)	30 °C, 5 d, Ana	Lactobacilli		[76]
Miscellanous		LAMVAB	37 °C, 3 d, Ana	Lactobacilli		[90]

Aer: Aerobic conditions; Ana: Anaerobic conditions; d: day.

esculine [52, 117, 162, 190]. An agar medium based on X-Glu [114] has also been used. MRS with trehalose (T-MRS) is recommended by the International Dairy Federation [104] for the counting of *Lb. acidophilus* if this organism occurs in mixed populations with yoghurt bacteria and bifidobacteria. Dave and Shah [52] reported that Rogosa acetate or media containing bile salts, oxgall or NaCl strongly inhibited the growth of *Lb. acidophilus*. In probiotic products containing *Bifidobacterium bifidum, Enterococcus faecium* and *Lb. acidophilus,* lactobacilli can be counted on modified Brigg's agar supplemented with streptomycin sulphate [28], or on 3BLA medium [130].

However, the use of such specific media is limited by the target species. For example, MRS-salicin or MRS-sorbitol agar can be used for the selective counting of *Lb. acidophilus* provided that *Lb. casei* is not present in the product (in which case a total count for both species is obtained). If *Lb. casei* is present, a second medium, such as *Lactobacillus casei* agar (LC agar) [151], must be used. The selective counting of

other lactobacilli used in probiotic and dairy products, such as *Lb. plantarum, Lb. reuteri* and *Lb. rhamnosus* has not been studied extensively. Moreover, all the members of the so-called *Lb. acidophilus* group: *Lb. crispatus, Lb. gasseri, Lb. johnsonii, Lb. gallinarum* and *Lb. amylovorus,* are hardly discriminated by plating.

For the lactobacilli found in cheeses, HHD medium (homofermentative and heterofermentative differential medium) can be used for the differential counting of homofermentative and heterofermentative lactobacilli [72]. Mesophilic and thermophilic lactobacilli are separated by the use of different incubation temperatures: 30 °C or 42 °C to 45 °C. FH medium (facultative heterofermentative *Lactobacillus* agar) contains vancomycin (50 $mg \cdot L^{-1}$), and has been used for the isolation of NSLAB [13, 22, 127, 150]. LBS (*Lactobacillus* selection agar) medium, also known as Rogosa medium [156], incubated at 30 °C has been used to isolate lactobacilli from hand-made cheeses such as Bergkäse [70, 83, 120, 150] but MRS remains the most commonly used medium for the isolation of lactobacilli.

Hartemink et al. [90] developed a new selective medium, LAMVAB (*Lactobacillus* anaerobic MRS with vancomycin and bromocresol green), for the isolation of *Lactobacillus* species. Firstly, they used it to isolate lactobacilli from faeces (in which they are present in small numbers), and then successfully for various species of lactobacilli from dairy products. The medium is highly selective, due to its low pH and the presence of vancomycin (20 $mg \cdot L^{-1}$). Unlike other Gram-positive bacteria, most lactobacilli are resistant to vancomycin and Gram-negative bacteria are generally sensitive. Vancomycin cannot be used to select lactobacilli in products also containing leuconostocs and pediococci because these bacteria are also vancomycin-resistant, and careful morphological examination is required in such cases for differentiation. However, this medium remains the most specific medium to date described for lactobacilli. Unfortunately, some *Lactobacillus* species, such as *Lactobacillus delbrueckii* spp. *bulgaricus,* and some strains of *Lb. acidophilus* are vancomycin-sensitive [32, 90]. Hartemink et al. [90] proposed the use of this medium for the isolation of lactobacilli from probiotics containing mixed populations of lactobacilli, bifidobacteria and enterococci. Bifidobacteria are susceptible to vancomycin and only a few vancomycin-resistant enterococci have been isolated.

The use of coloured indicators facilitates differentiation between microorganisms. For example, in LAMVAB and HHD [132], bromocresol green (pH indicator) is used as an indicator of acid production. Ghoddusi and Robinson [78] added Prussian blue to TPPY agar, Tryptose Proteose Peptone Yeast extract agar (giving TPPYB medium), to distinguish *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. acidophilus* from *Bifidobacterium* and *Streptococcus thermophilus*. Kneifel and Pacher [114] developed an agar medium, X-Glu agar, for the selective counting of *Lb. acidophilus* in yoghurt-related milk products containing a mixed flora of lactobacilli, streptococci and bifidobacteria. In this medium, *Lb. acidophilus* is identified by testing for its β-D-glucosidase activity by means of a chromogenic reaction involving X-Glu, which is incorporated into Rogosa agar medium. Based on a similar principle for β-D-galactopyranosidase activity, Kneifel et al. [115] used X-Gal medium to differentiate blue colonies of lactobacilli from white colonies of pediococci and enterococci in silage inoculants. The use of TTC (triphenyl tetrazolium chloride) may also be helpful for strain differentiation [140, 160].

2.2. Characterisation and identification of lactobacilli from genus level to strain level

For decades, differentiation between genera has been based on phenotypic characters. Under a light microscope, lactobacilli

are generally regularly shaped, non-motile, non-spore-forming, Gram-positive rods. However, cell morphology varies widely, from long, straight or slighty crescent-shaped rods to coryneform coccobacilli. Numerous genera display such morphological features. However, we can separate by simple tests such as tests for the oxygen tolerance, presence of catalase and growth on acidified MRS *Carnobacterium, Lactobacillus* and *Weissella* (non-obligate aerobe, catalase (-), growth on acidified MRS) from *Brochothrix, Caryophanon, Erysipelothrix, Kurthia, Listeria* and *Renibacterium* [111]. *Carnobacterium* resembles lactobacilli but does not grow on acetate media. The establishment of a new genus, *Weissella* [41], encompassing the *Paramesenteroides* group, which includes *Leuconostoc paramesenteroides* and some heterofermentative *Lactobacillus* species, seems to be justified on the basis of phylogenetic analysis. A cell wall murein, based on lysine with an interpeptide bridge containing alanine or alanine plus serine or glycine, can be used to distinguish *Weissella* from heterofermentative lactobacilli [111]. Classical phenotypic tests for identification of lactobacilli are based on physiological characteristics such as respiratory type, motility, growth temperature and growth in NaCl, and on biochemical characteristics such as homo/hetero-fermentative, production of lactic acid isomers, metabolism of carbohydrate substrates, coagulation of milk and presence of particular enzymes (e.g. arginine dihydrolase, antibiotic susceptibilities, and so on). Lactobacilli are typically chemoorganotrophic and ferment carbohydrates, producing lactic acid as a major end product. In 1919, Orla-Jensen divided them into three subgenera – *Thermobacterium*, *Streptobacterium* and *Betabacterium* – according to optimal growth temperature and hexose fermentation pathways. This classification was given up by Kandler and Weiss [111], who proposed a classification into three groups – I (obligate homofermentative), II (facultative heterofermentative) and III (obligate heterofermentative) – which is still used today for phenotypical analysis.

The most recent version of Bergey's Manual of Systematics [111] included about 50 species of *Lactobacillus*. This manual reported taxonomic changes at species level (e.g. *Lb. bavaricus* became *Lb. sake*) and at subspecies level (e.g. *Lb. casei* ssp. *rhamnosus* became *Lb. rhamnosus*, *Lb. bulgaricus* became *Lb. delbrueckii* ssp. *bulgaricus,* etc.). Moreover, some lactobacilli became members of the new genus *Weissella* (e.g. *Lb. kandleri* became *W. kandleri*), which also includes former members of the genus *Leuconostoc* (e.g. *Leuconoctoc paramesenteroides* became *W. paramesenteroides*), whereas other lactobacilli were assigned to another new genus, *Carnobacterium* (e.g. *Lb. divergens* became *Cb. divergens*). Today, the genus *Lactobacillus* contains 88 species and 15 subspecies according to a recent listing (Tab. II, www.bacterio.cict.fr, 6 January 2003). Protein analysis such as protein fingerprinting (analysis of total soluble cytoplasmic proteins), or multilocus enzyme electrophoresis (analysis of electrophoretic mobilities of certain enzymes) are advanced phenotypic methods in current use. Such analysis can discriminate between bacteria to the species level and beyond. Lipid profiling has also been used. However, the identification of isolates to species level can be difficult because of the considerable variations in biochemical attributes (fermentation profiles) that seem to occur between strains currently considered to belong to the same species, and some species are not readily distinguishable in terms of phenotypic characteristics. This is especially true for the so-called *Lactobacillus plantarum* group (*Lb. plantarum, Lb. paraplantarum* and *Lb. pentosus*), the *Lactobacilllus casei* and *Lactobacilllus paracasei* group (*Lb. casei, Lb. rhamnosus, Lb. zeae* and *Lb. paracasei*), *Lb. brevis* and *Lb. buchneri*. Recently Dellaglio et al. [57] proposed, on the basis of considerable published evidence, that the name of *Lactobacillus*

Table II. *Lactobacillus* species (www.bacterio.cict.fr, 6 January 2003).

Lactobacillus acetotolerans	*Lactobacillus equi*	***Lactobacillus paracasei*** ssp.
Lactobacillus acidipiscis	**Lactobacillus farciminis*	***tolerans***
****Lactobacillus acidophilus***	*Lactobacillus ferintoshensis*	*Lactobacillus parakefiri*
Lactobacillus agilis	***Lactobacillus fermentum***	*Lactobacillus paralimentarius*
Lactobacillus algidus	*Lactobacillus fornicalis*	*Lactobacillus paraplantarum*
Lactobacillus alimentarius	*Lactobacillus fructivorans*	*Lactobacillus pentosus*
Lactobacillus amylolyticus	*Lactobacillus frumenti*	*Lactobacillus perolens*
Lactobacillus amylophilus	*Lactobacillus fuchuensis*	****Lactobacillus plantarum***
Lactobacillus amylovorus	*Lactobacillus gallinarum*	*Lactobacillus pontis*
Lactobacillus animalis	****Lactobacillus gasseri***	*Lactobacillus psittaci*
Lactobacillus arizonensis	*Lactobacillus graminis*	****Lactobacillus reuteri***
Lactobacillus aviarius ssp.	*Lactobacillus hamsteri*	****Lactobacillus rhamnosus***
araffinosus	***Lactobacillus helveticus***	*Lactobacillus rogosae*
Lactobacillus aviarius ssp.	*Lactobacillus heterohiochii*	*Lactobacillus ruminis*
aviarius	*Lactobacillus hilgardii*	*Lactobacillus sakei* ssp.
Lactobacillus bifermentans	*Lactobacillus homohiochii*	*carnosus*
Lactobacillus brevis	*Lactobacillus iners*	*Lactobacillus sakei* ssp.
Lactobacillus buchneri	*Lactobacillus intestinalis*	*sakei*
****Lactobacillus casei***	*Lactobacillus jensenii*	*Lactobacillus salivarius* ssp.
Lactobacillus catenaformis	**Lactobacillus johnsonii*	*salicinius*
Lactobacillus cellobiosus	*Lactobacillus kefiranofaciens*	*Lactobacillus salivarius* ssp.
Lactobacillus coleohominis	*Lactobacillus kefirgranum*	*salivarius*
Lactobacillus collinoides	***Lactobacillus kefiri***	*Lactobacillus sanfranciscensis*
Lactobacillus coryniformis ssp.	*Lactobacillus kimchii*	*Lactobacillus sharpeae*
coryniformis	*Lactobacillus kunkeei*	*Lactobacillus suebicus*
Lactobacillus coryniformis ssp.	*Lactobacillus leichmannii*	*Lactobacillus trichodes*
torquens	*Lactobacillus lindneri*	*Lactobacillus vaccinostercus*
**Lactobacillus crispatus*	*Lactobacillus malefermentans*	*Lactobacillus vaginalis*
Lactobacillus curvatus ssp.	*Lactobacillus mali*	*Lactobacillus vitulinus*
curvatus	***Lactobacillus maltaromicus***	*Lactobacillus zeae*
Lactobacillus curvatus ssp.	*Lactobacillus manihotivorans*	
melibiosus	*Lactobacillus mucosae*	
Lactobacillus cypricasei	*Lactobacillus murinus*	
Lactobacillus delbrueckii ssp.	*Lactobacillus nagelii*	
bulgaricus	*Lactobacillus oris*	
**Lactobacillus delbrueckii* ssp.	*Lactobacillus panis*	
delbrueckii	*Lactobacillus pantheris*	
Lactobacillus delbrueckii ssp.	***Lactobacillus parabuchneri***	
lactis	****Lactobacillus paracasei*** ssp.	
Lactobacillus diolivorans	***paracasei***	
Lactobacillus durianis		

In bold: *Lactobacilli* used in dairy products; with an *: *Lactobacilli* used in probiotic product.

paracasei had to be rejected by the judicial commission and that the species *Lactobacillus casei* is not correctly represented by the strain ATCC 393.

Studies based on 16S rDNA have led to the classification of *Lactobacillus* species into three major groups: the *Leuconostoc* group, the *Delbrueckii* group, and the *Lb. casei-Pediococcus* group [40, 174, 188]. Recently *Lb. fructosus* (the only lactobacilli member of the *Leuconostoc* group) was reclassified as *Leuconostoc fructosum* [8]. Closely related species and strains with similar phenotypic features may now be reliably differentiated from each other by DNA-based techniques. Molecular methods can be used for taxonomic analyses to firstly determine the species to which a bacterium belongs, by DNA/DNA hybridisation, sequencing, polymorphism chain reaction (PCR), ribotyping, polymorphism chain reaction-restriction fragment length polymorphism (PCR-RFLP), and secondly permit strain differentiation by the use of techniques such as restriction enzyme analysis (REA), randomly amplified polymorphic DNA (RAPD), repeated sequence extragenic palindrom PCR (REP-PCR), amplified fragment length polymorphism (AFLP), plasmid profiling and pulsed field gel electrophoresis (PFGE). However, identification can be done with a high degree of confidence only if a correct validation of the method or probes or primers have been checked using close genera, species or strains.

Polyphasic taxonomy is increasingly used [4, 75, 118, 167, 174, 188]. Lawson et al. [118] described, on the basis of phylogenetic and phenotypic evidence, a new species of *Lactobacillus*, *Lb. cypricasei,* which was isolated from Halloumi cheese, a semi-hard cheese from Cyprus.

2.2.1. Analysis at genus level

The genus *Lactobacillus* is heterogeneous, with the G+C content of the DNA of its species varying from 33 to 55% [40, 89]. However, it is generally thought that G+C content should vary by no more than a 10% range within a well-defined genus [188]. The nucleotide sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) provide an accurate basis for identification. The sequence obtained from an isolate can be compared with those of *Lactobacillus* species held in databases. Recently, Dubernet et al. [62] defined a genus-specific primer by analysing similarities between the nucleotide sequences of the spacer region between the 16S and 23S ribosomal RNA genes of *Lactobacillus*. The specificity of this genus-specific primer combined with a universal primer was tested against 23 strains of lactobacilli of varied origin (corresponding to 21 species) *Escherichia coli*, two leuconoctocs species, *Carnobacterium piscicola, Pediococcus pentosaceus, Bifidobacterium bifidum, Weissella confusa, Enterococcus faecalis, Staphylococcus aureus* and *Listeria monocytogenes*. Positive amplification was only obtained with the lactobacilli strains.

2.2.2. Analysis at species level

Phenotypical micro methods

Several combinations of tests and ready-to-inoculate identification kits such as API 50 CH, LRA Zym and API Zym enzymatic tests can be used for the rapid and theoretically reproducible phenotypic identification of pure cultures. They have been used for the characterisation and identification of lactobacilli in milks [133], yoghurts and other fermented milks [6] and in cheeses [6, 21, 53, 58, 92, 118, 120, 133, 175]. However, the reliability of these tests has been questioned, especially for API 50 CH, initially developed for the identification of medical *Lactobacillus* strains. In addition, the manufacturer's database is not updated and some *Lactobacillus* species are missing. Andrighetto et al. [6] used API 50 CH to analyse 25 strains of thermophilic lactobacilli isolated from yoghurt and from semi-hard and hard cheeses (*Lb. delbrueckii* ssp. *lactis* and ssp. *bulgaricus,*

Lb. helveticus and *Lb. acidophilus*). For most of the strains, clear assignment to a particular species or subspecies was not possible because ambiguous results were obtained for the sugar fermentation profile. Nigatu [141] also reported a lack of agreement between the API 50 CH grouping pattern of isolates and RAPD clusters. Tynkkynen et al. [184] used API 50 CH for identifying strains of the *Lb. casei* group (*Lb. rhamnosus, Lb. zeae* and *Lb. casei*). The exact identifications of these closely related species were not reliable; some were doubtful or unacceptable and some strains were misidentified with a good identification level. Furthermore, variability may be observed within a single strain. For example, the *Lb. rhamnosus* GG strain has traditionally been detected, counted and identified on the basis of cultures in selective anaerobic conditions on MRS or Rogosa agar (37 °C for 78 h), colony morphology (large, white, creamy and opaque), Gram staining and cell morphology (Gram-positive and uniform rods in chains) and the carbohydrate fermentation profile in the API 50 CHL test. However, it has been pointed out that the colony morphology and the carbohydrate fermentation pattern of strain GG are not always typical, due to variation [32]. This variation may result from the loss or gain of plasmids, leading to inconsistency in the metabolic traits of a strain, as most of the proteins involved in carbohydrate fermentation are plasmid-encoded [9].

Protein fingerprinting

A bacterial strain always produces the same set of proteins if grown under standardised conditions. The electrophoregrams produced by zone electrophoresis of these proteins under well-defined conditions can be considered as a sort of fingerprint of the bacterial strains from which they are obtained. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins is one of the techniques used. It is a relatively simple and inexpensive method that has already been used for the identification and classification of lactobacilli. The entire procedure consists of several experimental steps, from the growth of bacteria to the scanning of the electrophoregram. SDS-PAGE separates proteins exclusively according to molecular weight. Native (non-denaturing) PAGE can be used as a complementary technique, separating cell proteins according to their charge and size, providing high resolution and good band definition. In highly standardised conditions, reproducible patterns can be obtained that are amenable to rapid, computer-based digital analysis. Protein profiles can be stored in database format and may be routinely used to confirm the identity of *Lactobacillus* strains, to differentiate between unknown isolates and to evaluate classification schemes, at species level or below [53, 75, 92, 118, 122, 146, 147]. De Angelis et al. [53] isolated NSLAB from 12 Italian ewe's milk cheeses. Most of the species studied gave specific protein profiles, except *Lb. plantarum* and *Lb. pentosus,* which were grouped in the same cluster, confirming the results previously obtained by Van Reenen and Dicks [187]. Gancheva et al. [75] used SDS-PAGE to analyse the cellular proteins of a set of 98 strains belonging to nine species of the *Lactobacillus acidophilus* rRNA-group of varied origin (*Lb. acidophilus, Lb. amylolyticus, Lb. crispatus, Lb. johnsonii, Lb. gasseri, Lb. gallinarum, Lb. helveticus, Lb. iners* and *Lb. amylovorus*). Most of these species can be differentiated by SDS-PAGE, but poor discrimination was obtained between *Lb. johnsonii* and *Lb. gasseri* strains, and between some strains of *Lb. amylovorus* and *Lb. gallinarum*.

Multilocus enzyme electrophoresis

About 50% of all enzymes investigated to date exist in multiple molecular forms. These isoenzymes usually differ in electrophoretic mobility and catalytic parameters. Enzyme multiplicity may depend on genetic

factors directly (primary isoenzymes and allozymes) or indirectly (secondary isoenzymes, generated by post-translational modifications). Isoenzymes may be distributed between different cell compartments and may be encoded by at least two different genes. Multiple loci encoding enzymes of identical substrate specificities are usually thought to result from gene duplications. Point mutations then lead to divergence in the amino acid sequences of the proteins encoded by the duplicated genes, resulting in the production of different enzyme forms, separable by electrophoresis. Differences in electrophoretic mobility may result from differences in charge and/or size. Multilocus enzyme analysis has been shown to be of great potential [161] in the differentiation of LAB species [182]. The electrophoretic mobility of LDH (lactate dehydrogenase) in starch gels [77] and in polyacrylamide gels [95] has been used to discriminate between phenotypically very similar species: *Lb. acidophilus, Lb. crispatus, Lb. gallinarum, Lb. gasseri* and *Lb. johnsonii*. Lortal et al. [122] studied peptidoglycan hydrolases of industrial starters as a new tool for bacterial species identification. The peptidoglycan hydrolase patterns of 94 strains of lactobacilli belonging to 10 different species were determined (*Lb. helveticus, Lb. acidophilus, Lb. delbrueckii, Lb. brevis, Lb. fermentum, Lb. jensenii, Lb. plantarum, Lb. sake, Lb. curvatus* and *Lb. reuteri*). Each species gave its own specific pattern, with differences observed even between closely related species. It is also possible to type strains of *Lactobacillus acidophilus* [146], or clinical isolates and biotechnologically-used strains of *Lactobacillus rhamnosus* [112], or strains of *Lactobacillus casei* [55] isolated from ensiled high-moisture corn grain.

Lipid profiling

Lipid profiling by gas chromatography is more useful for the grouping of strains than for the identification of individual strains [47, 66, 154]. Moreover, the reliability of lipid and polysaccharide profiling for discriminating between *Lactobacillus* species has been questioned and fatty acid methyl ester (FAME) analysis does not seem to be reliable for LAB [113].

Hybridisation

The use of probes for hybridisation with nucleic acid fragments is a technique with great potential for the future. A nucleic acid probe is a fragment (20-30 pb) of a single-stranded nucleic acid fragment that specifically hybridises to complementary regions of a target nucleic acid. It can be used directly on a colony, or after DNA/RNA extraction. The target nucleic acid consists of single-stranded DNA or RNA molecules. Molecular probes may be labelled radioactively or non-radioactively. Radioactive labelling involves the phosphorylation of the 5' terminus of the probe with [^{32}P] ATP. Non-radioactive labelling may be direct, using alkaline phosphatase or peroxidase, or indirect, by attachment of a ligand-protein or a hapten-antibody. Fluorescent probes (FISH: fluorescent in situ hybridisation) may also be used. The extensive use of multiple oligonucleotide probes has become possible following major developments in the sequencing of rRNA genes. Depending on the level of detection required (genus or species), different regions of the genome might be used as targets. The sequences of 16S and 23S rRNA molecules contain highly conserved regions common to all eubacteria, and highly variable regions unique to a particular species [32, 199]. Thus, nucleic acid probes, in particular probes targeting rRNA sequences, can be used for the reliable identification of bacteria, for monitoring population changes during fermentation and detecting the presence of bacterial contamination or spoilage bacteria [60]. Such probes have been extensively used in the analysis of dairy products [6, 98]. Oligonucleotide DNA probes, mostly targeting variable regions of the 16S or 23S

Table III. Oligonucleotide probes for the identification of Lactobacilli.

Probe	5'Sequence3'	Target	Specificity	Ref.
Lba	TCTTTCGATGCATCCACA	23S	*Lb. acidophilus*	[193]
Lba	AGCGAGCUGAACCAACAGAUUC	16S	*Lb. acidophilus*	[96]
Lbam	GTAAATCTGTTGGTTCCGC	16S	*Lb. amylovorus*	[68]
Lbb	TGTTGAAATCAAGTGCAAG	16S	*Lb. brevis*	[193]
Lbc	ATGATAATACCCGACTAA	23S	*Lb. curvatus*	[97]
Lbco	AGCACTTCATTTAACGGG	16S	*Lb. collinoides*	[68]
Lbcp	CAATCTCTTGGCTAGCAC	23S	*Lb. crispatus*	[67]
Lbcr	GCAGGCAATACACTGATG	23S	*Lb. casei /Lb. rhamnosus*	[98]
Lbcrp	CTGATGTGTACTGGGTTC	23S	*Lb. casei / Lb. paracasei / Lb. rhamnosus*	[98]
Lbd	AAGGATAGCATGTCTGCA	23S	*Lb. delbrueckii*	[98]
Lbdb	ATCCGAAAACCTTCT	16S	*Lb. delbrueckii* ssp. *bulgaricus*	[119]
Lbdl	ATCCGAAGACCTTCT	16S	*Lb delbrueckii* ssp. *lactis/delbrueckii*	[119]
33/2	CATCAACTGGCGCCTT	730pb *EcoR*I/*Pst*I DNA fragment	*Lb. delbrueckii* ssp. *lactis*	[119]
34B	CATCAACCGGGGCTTT	730pb *EcoR*I/*Pst*I DNA fragment	*Lb. delbrueckii* ssp. *bulgaricus*	[119]
Lbfe	GCGACCAAAATCAATCAGG	16S	*Lb. fermentum*	[193]
Lbfr	CTCGCTGCTAACTTAAGTC	16S	*Lb. fructivorans /Lb. homohiochii*	[193]
Lbg	TCCTTTGATATGCATCCA	23S	*Lb. gasseri*	[146]
Lbh	ACTTACGTACATCCACAG	23S	*Lb. helveticus*	[98]
Lbhi	CTCAACTTCATTGACCAAG	16S	*Lb. hilgardii*	[68]
Lbj	ATAATATATGCATCCACAG	23S	*Lb. johnsonii*	[146]
Lbk	GTTTCATGTTAAATCATTCA	16S	*Lb. kefir*	[68]
Lbkf	TGCGGCTAGCCCTTCCGG	23S	*Lb. kefiranofaciens*	[68]
Lbl	TCGGTCAGATCTATCGTC	16S	*Lb. lindneri*	[68]
Lbma	CAAAAGCGACAGCTCGAAAG	16S	*Lb. manihotivorans*	[3]
Lbp	ATCTAGTGGTAACAGTTG	23S	*Lb pentosus / Lb plantarum*	[97]
Lbpa	CACTGACAAGCAATACAC	23S	*Lb paracasei*	[98]
Lbpa	TAACTCATTGACTGACTCG	23S	*Lb parabuchneri*	[68]
Lbpe	TCAAATGTAAATCATGATG	16S	*Lb pentosus / plantarum*	[68]
Unamed	GGTATTGGTGATGCAAG	16S	*Lb. perolens*	[11]
Lbpp	ATCTAGTCGTAACAGTTG	23S	*Lb plantarum / pentosus*	[97]
Lbpo	GGTAATCCATCGTCAAATC	16S	*Lb pontis*	[193]
Lbre	GATCCATCGTCAATCAGG	16S	*Lb reuteri*	[68]
Lbru	TTCGGTGAAAGAAAGCTTG	16S	*Lb ruminis*	[96]
Lbs	TTAATGATAATACTCGATT	23S	*Lb sake*	[97]
Lbsa	TAAGAATCAATTGGGCGAC	16S	*Lb sanfransiscensis*	[193]

rRNA genes, have been widely used for species identification and strain detection (Tab. III). However, such rRNA probes cannot be used for closely related species due to the high level of similarity between their rRNA gene sequences. For example, such probes cannot distinguish *Lb. plantarum* from *Lb. pentosus* or *Lb. paraplantarum*

[25]. These species are currently distinguished by probing Southern blots with a pyrDFE gene fragment from *Lb. plantarum* or by DNA/DNA hybridisation. Particular attention had to be done to their specificity since Roy et al. [158] demonstrated that the probe defined by Hertel et al. [98] for *Lb. helveticus* also hybridise with *Lb. gallinarum* strains.

In colony hybridisation, bacteria are plated on membranes that are then placed on nutrient agar, allowing the bacteria to form colonies. The colonies are lysed. Hybridisation with a labelled probe can be used for both qualitative and quantitative analyses, and has been used for LAB [37].

Sequencing

Comparison of rRNA gene sequences is currently considered to be the most powerful and accurate method for determining the degree to which microorganisms are phylogenetically related [199]. Advances in molecular biological techniques have made it possible to sequence long stretches of rRNA genes. Initially, reverse transcriptase was used to generate DNA from rRNA, and this DNA was then sequenced. It is now possible to sequence 16S or 23S rDNA molecules by direct PCR sequencing, and this method has generated large sequence databases. Although the species-specific sequences are located in the first half of the 16S rRNA gene (V1-V3 region), identification is more accurate if the whole gene is sequenced [171]. This requires the sequencing of about 1.5 kb of DNA. Tannock et al. [177] showed that comparison of the16S-23S spacer region sequences of lactobacilli can be used in practical situations for strain identification. The spacer region sequences is sequencing rapidly and accurately identifies *Lactobacillus* isolates obtained from gastrointestinal, yoghurt and silage samples. The 16S-23S spacer sequences of lactobacilli are small, only about 200 bp in length. These short sequences are easy to sequence on both strands and provide reliable information for comparative work. The spacer region method has the advantage of distinguishing between *Lb. rhamnosus* and *Lb. casei* strains [177]. It can be used to distinguish *Lb. plantarum,* from *Lb. paraplantarum,* these two closely related species belonging to the *Lb. plantarum* group [12]. Chen et al. [33] analysed the 5S-23S rRNA intergenic spacer regions (ISRs) of the *Lactobacillus* group. This method was found to be an effective way of discriminating *Lb. rhamnosus* from *Lb. casei/Lb. paracasei* because spacer length polymorphism results in a 76/80 bp insertion with respect to the 16S V2-V3 sequences.

Polymerase chain reaction (PCR)

This method developed by Mullis [139] uses primers, which are about 20 to 30 pb. Berthier and Ehrlich [15] studied the 16S/23S SRs DNA from six closely related species of lactobacilli (*Lb. curvatus, Lb. graminis, Lb. sake, Lb. plantarum, Lb. paraplantarum* and *Lb. pentosus*). Only the larger fragment displayed differences in sequence between species, and primers derived from this region were defined for the six species. SR sequences could not be used to type strains within the two groups of *Lactobacillus* species.

Numerous species-specific primers have been derived from spacer regions [15, 170, 177, 179]. The species-specific primers currently available are listed in Table IV.

Mannu et al. [126] used seven pairs of specific primers designed by Berthier and Ehrlich [15], Drake et al. [60] and Ward and Timmins [196] to analyse 457 isolates from Fiore Sardo cheese, a traditional hard cheese from Sardinia. Only seven isolates were not successfully identified with this method; 31 were obligate homofermentative lactobacilli, 419 isolates were facultative heterofermentative lactobacilli (*Lb. plantarum, Lb. paracasei* and *Lb. curvatus*).

Many recent studies have been carried out by multiplex PCR (Tab. V), in which several primers are added to the same sample,

Table IV. PCR primers used for lactobacilli identification.

Primers	5'–3'	Target	Ref.
LbLMA1-rev	CTCAAAACTAAACAAAGTTTC	16S/23S spacer region Lactobacilli	
R16-1	CTTGTACACACCGCCCGTCA	16S rRNA gene	[62]
(/LbLMA1-rev)	(/LbLMA1-rev)		
Y2	CCCACTGCTGCCTCCCGTAGGAGT	16S rRNA gene	
casei(/Y2)	TGCACTGAGATTCGACTTAA (/ Y2)	16S *Lb. casei*	[196]
para(/Y2)	CACCGAGATTCAACATGG (/Y2)	16S *Lb. paracasei*	
rham(/Y2)	TGCATCTTGATTTAATTTTG (/Y2)	16S *Lb. rhamnosus*	
16	GCTGGATCACCTCCTTTC	16S rRNA gene	
paracaseiITS/16	CGATGCGAATTTCTTTTTC /16	16S/23S spacer region *Lb. paracasei*	
rhamnosusITS/16	CGATGCGAATTTCTATTATT /16	16S/23S spacer region *Lb. rhamnosus*	
zeaeITS/16	CGATGCGAATTTCTAAATT /16	16S/23S spacer region *Lb. zeae*	
16 reverse	GAAAGGAGGTGATCCAGC	16S rRNA gene	
paracasei 16S/16 reverse	CACCGAGATTCAACATGG/16 reverse	16S rRNA gene. *Lb. paracasei*	[17]
rhamnosus 16S/16 reverse	TTGCATCTTGATTTAATTTTG/16 reverse	16S rRNA gene. *Lb. rhamnosus*	
zeae 16S/16 reverse	GCATCGTGATTCAACTTAA/16 reverse	16S rRNA gene. *Lb. zeae*	
Ala	CTGCTGGGACGATTTG		
Ala'(/Ala)	CTGCTGGGACCATGTG (/Ala)	*Lb. curvatus* (1840 pb)	
Alb	CTGCTGGGACCATTATTG		
Alb'(/Alb)	CTGCTGGGACACAATATG (/Alb)	Not tested (1470 pb)	
Alc	GGAGGGTGTTCAGGAC		
Alc'(/Alc)	GGAGGGTGTTGATAGG (/Alc)	*Lb. curvatus* (260 pb)	
Bla	CTGCTGGGACCAATT		[16]
Bla'(/Bla)	CTGCTGGGACGAAAAG (/Bla)	*Lb. sake* B1 (750 pb)	
B2a	CTGCTGGGACCTTAA		
B2a'(/B2a)	CTGCTGGGACTGAAG (/B2a)	*Lb. sake* B2 (1700 pb)	
16	GCTGGATCACCTCCTTTC		
Lc(/16)	TTGGTACTATTTAATTCTTAG (/16)	*Lb. curvatus* (220 pb)	
Ls(/16)	ATGAAACTATTAAATTGGTAC (/16)	*Lb. sake* (220 pb)	
16	GCTGGATCACCTCCTTTC	16S rRNA gene	
23(/16)	AGTGCCAAGGCATCCACC (/16)	23S rRNA gene(/16S)	
Lc(/16)	TTGGTACTATTTAATTCTTAG (/16)	16S/23S spacer region *Lb. curvatus*	
Lg(/16)	GTTGGTACATTTAATTCTTGA (/16)	16S/23S spacer region *Lb. graminis*	
Lpapl(/16)	ATGAGGTATTCAACTTATT (/16)	16S/23S spacer region *Lb. paraplantarum/plantarum*	[15]
Lpe(/16)	GTATTCAACTTATTAGAACG (/16)	16S/23S spacer region *Lb. pentosus*	
Lpl(/16)	ATGAGGTATTCAACTTATG (/16)	16S/23S spacer region *Lb. plantarum*	
Ls(/16)	ATGAAACTATTAAATTGGTAC (/16)	16S/23S spacer region *Lb. sake*	
LB1	AAAAATGAAGTTGTTTAAAGTAGGTA	*Lb. delbrueckii bulgaricus* (1065 pb)	[181]
LLB1 (/LB1)	AAGTCTGTCCTCTGGCTGG (/LB1)	*Lb. delbrueckii lactis* (1600 pb)	
20A	AATTCCGTCAACTCCTCATC		
23B (/20A)	TGATCCGCTGCTTCATTTCA(/20A)	*Lb. delbrueckii* ssp. (715 pb)	
34/2	CGTCAACTCCTCATCAACCGGGGCT		
37/1(/34/2)	CGCCGCCCGGGTGAAGGTG(/34/2)	*Lb. delbrueckii* ssp. *bulgaricus* (678 pb)	[119]
33	CCTCATCAACTGGCGCC		
37(/33)	CGCCCGGGTAAAGGTA(/33)	*Lb. delbrueckii* ssp. *lactis* (670 pb)	

Table IV. PCR primers used for lactobacilli identification.

LbP11	AATTGAGGCAGCTGGCCA		
LbP12 (/LbP11)	GATTACGGGAGTCCAAGC (/LbP11)	RAPD derived primer. *Lb. plantarum*	[148]
Lb1	AGAGTTTGATCATGGCTCAG		
Lb2 (/Lb1)	CGGTATTAGCATCTGTTTCC (/Lb1)	semi-universal primer. 16S rRNA	
Aci I	TCTAAGGAAGCGAAGGAT		
Aci II (/Aci I)	CTCTTCTCGGTCGCTCTA (/Aci I)	16S-23S SR. *Lb. acidophilus*	
Pr I	CAGACTGAAAGTCTGACGG		
PrII 5/PrI)	GTACTGACTTGCGTCAGCGG (/PrI)	16S-23S SR *Lb. paracasei/rhamnosus*	
Pcas I (/PrI)	GCGATGCGAATTTCTTTTTC (/PrI)	16S-23S SR *Lb. paracasei*	[179]
Rha II(/PrI)	GCGATGCGAATTTCTATTATT (/PrI)	16S-23S SR *Lb. rhamnosus*	
Del I	ACGGATGGATGGAGAGCAG		
Del II (/Del I)	GCAAGTTTGTTCTTTCGAACTC (/Del I)	16S-23S SR *Lb. delbrueckii*	
Hel I	GAAGTGATGGAGAGTAGAGATA		
Hel II (/Hel I)	CTCTTCTCGGTCGCCTTG (/Hel I)	16S-23S SR *Lb. helveticus*	
DB1	ACCTATCTCTAGGTGTAGCGCA		
SS1 (/DB1)	GTGCTGCAGAGAGTTTGATCCTGGCT-CAG (/DB1)	16S *Lb. delbrueckii* (1100 pb)	
HE1	AGCAGATCGCATGATCAGCT		
SS2 (/HE1)	CACGGATCCTACGGGTACCTTGTTAC-GACTT(/HE1)	16S *Lb. helveticus/Lb. acidophilus* (1400 pb)	[60] [6]
CA1 (/SS1)	TGATCTCTCAGGTGATCAAAA (/SS1)	16S *Lb. casei, Lb. rhamnosus*	
16 SII	ACTACCAGGGTATCTAATCC		
Aci 16SI (/16SII)	AGCTAACCAACAGATTCAC (/16 SII)	16S *Lb. acidophilus*	
Cri 16SI(/16SII)	GTAATGACGTTAGGAAAGCG (/16 SII)	16S *Lb. crispatus*	
GasI	GAGTGCGAGAGCATAAAG		
GasII (/GasI)	CTATTTCAAGTTGAGTTTCTCT (/GasI)	16S-23S SR *Lb. gasseri*	
Joh 16SI(/16SII)	GAGCTTGCCTAGATGATTTTA (/16 SII)	16S-23S SR *Lb. johnsonii*	
Lpfr	GCCGCCTAAGGTGGGACAGAT		
PlanII (/Lpfr)	TTACCTAACGGTAAATGCGA (Lpfr)	16S-23S SR *Lb. plantarum*	
PrI	CAGACTGAAAGTCTGACGG		[194]
CasII (/PrI)	GCGATGCGAATTTCTTTTTC (/PrI)	16S-23S SR *Lb. casei*	[184]
ZeaeI	TGTTTTGAGGGGACG		
ZeaeII (/ZeaeI)	ATGCGATGCGAATTTCTAAATT (/ZeaeI)	16S-23S SR *Lb. zeae*	
RhaII(/PrI)	GCGATGCGAATTTCTATTATT (/PrI)	16S-23S SR *Lb. rhamnosus*	
Reu(/lpfr)	AACACTCAAGGATTGTCTGA (/lpfr)	16S-23S SR *Lb. reuteri*	
FermII(/lpfr)	CTGATCGTAGATCAGTCAAG (/lpfr)	16S-23S SR *Lb. fermentum*	
ShaI	GATAATCATGTAAGAAACCGC		
ShaII(/ShaII)	ATATTGTTGGTCGCGATTCG (/ShaII)	16S-23S SR *Lb. sharpae*	
SAL1	ATTCACTCGTAAGAAGT	16S	[30]
LOWLAC(/SAL1)	CGACGACCATGAACCACCTGT(/SAL1)	16S *Lb. salivarius*	
CbsA2F	GTACCAAGCCAAAGCAAGAC		
CbsA2R(/CbsA2F)	GTTTGAAGCCTTTACGTAAGTC (/CbsA2F)	CbsA (S-Layer protein gene) *Lb. perolens*	[99]
97K	CTGCTGCCTCCCGTA	16S Universal	[27]
Lpacaf(/97K)	CCGAGATTCAACATGG(/97K)	16S *Lb. paracasei*	

making it possible to detect several microorganisms or species at the same time. Multiplex PCR has been used to detect *Lb. pontis* and *Lb. panis* in sourdough fermentation [138], and *Lactobacillus* in faecal samples [123] and in meat spoilage [200]. Song et al. [170] used multiplex PCR as a rapid, simple and reliable method for the identification of common *Lactobacillus* isolates from human stool samples, and

Table V. Multiplex PCR primers used for lactobacilli identification.

Primers	5'–3'	Target	Ref.
Lac-2	CCTCTTCGCTCGCCGCTACT		
Ldel-7 (/lac-2)	ACAGATGGATGGAGAGCAGA (/lac-2)	ISR/23S PCR-G Group I lactobacilli (450pb)	
LU-1'(/lac-2)	ATTGTAGAGCGACCGAGAAG (/lac-2)	ISR/23S PCR-G Group II lactobacilli (300 pb)	
LU-3'(/lac-2)	AAACCGAGAACACCGCGTT (/lac-2)	ISR/23S PCR-G Group IV lactobacilli (350 pb)	
LU-5(/lac-2)	CTAGCGGGTGCGACTTTGTT (/lac-2)	ISR/23S PCR-G Group III lactobacilli (400 pb)	
23-10C	CCTTTCCCTCACGGTACTG		
Laci-1 (23-10C)	TGCAAAGTGGTAGCGTAAGC (/23-10C)	ISR/23S PCR-II-1 Group II, *Lb. acidophilus* (210 pb)	
Ljen-3 (23-10C)	AAGAAGGCACTGAGTACGGA (/23-10C)	ISR/23S PCR-II-1 Group II, *Lb. jensenii* (700 pb)	
Lcri-1	AGGATATGGAGAGCAGGAAT		
Lcri-2(/Lcri-1)	CAACTATCTCTTACACTGCC (Lcri-1)	ISR/23S PCR-II-2 Group II, *Lb. crispatus* (522 pb)	
Lgas-1	AGCGACCGAGAAGAGAGAGA		
Lgas-2 (/Lgas-1)	TGCTATCGCTTCAAGTGCTT (/Lgas-1)	ISR/23S PCR-II-2 Group II, *Lb. gasseri* (360 pb)	[170]
Lfer-3	ACTAACTTGACTGATCTACGA		
Lfer-4 (/Lfer-3)	TTCACTGCTCAAGTAATCATC (/Lfer-3)	ISR/23S PCR-IV Group IV, *Lb. fermentum* (192 pb)	
Lpla-3	ATTCATAGTCTAGTTGGAGGT		
Lpla-2 (Lpla-3)	CCTGAACTGAGAGAATTTGA (/Lpla-3)	ISR/23S PCR-IV Group IV, *Lb. plantarum* (248 pb)	
Lreu-1	CAGACAATCTTTGATTGTTTAG		
Lreu-4 (/Lreu-1)	GCTTGTTGGTTTGGGCTCTTC (/Lreu-1)	ISR/23S PCR-IV Group IV, *Lb. reuteri* (303 pb)	
Lsal-1	AATCGCTAAACTCATAACCT		
Lsal-2 (/lsa-2)	CACTCTCTTTGGCTAATCTT (/lsa-2)	ISR/23S PCR-IV Group IV, *Lb. salivarius* (411 pb)	
Lpar-4 (/LU-5)	GGCCAGCTATGTATTCACTGA (/LU-5)	ISR/23S PCR-III Group III, *Lb. paracasei* (312 pb)	
RhaII (/LU-5)	GCGATGCGAATTTCTATTATT (/LU-5)	ISR/23S PCR-III Group III, *Lb. rhamnosus* (113 pb)	
Y1	TGGCTCAGAACGAACGCTAGGCCCG	16S rRNA	
Y2 (/Y1)	CCCACTGCTGCCTCCCGTAGGAGT (/Y1)	16S rRNA PCR A (350 pb)	
16	GCTGGATCACCTCCTTTC	16S rRNA gene	
Ls (/16)	ATGAAACTATTAAATTGGTAC (/16)	16S/23S SR *Lb. sake* PCR A (220 pb)	
Lc (/16)	TTGGTACTATTTAATTCTTAG (/16)	16S/23S SR *Lb. curvatus* PCR B (220 pb)	
Lu1r	CCACAGCGAAAGGTGCTTGCAC	*Leuconostoc* 16S rRNA gene	[200]
Lu2 (/lu1r)	GATCCATCTCTAGGTGACGCCG (/lulr)	*Leuconostoc* PCR B (175 pb)	
Lw5 (/Y1)	ACTAGAATCATTCCCTATTCTAGC (/Y1)	*Leuconostoc* PCR C (470 pb)	
Cb1	CCGTCAGGGGATGAGCAGTTAC	*Carnobacterium* 16S rRNA gene	
Cb2r (/Cb1)	ACATTCGGAAACGGATGCTAAT (/Cbl)	*Carnobacterium* PCR D (340 pb)	
616V	AGAGTTTGATYMTGGCTCAG	universal	
609R (/616V)	ACTACYNGGGTATCTAAKCC (/616V)	universal (800 pb)	
LaponR (/616V)	AGCCATCTTTGAAAT (/616V)	*Lb. pontis* (236 pb)	[138]
LapanR (/616V)	AACCATCTTTTATAC (/616V)	*Lb. panis*(236 pb)	
LaspecR (/616V)	AGCCTTCTTTTATAC (/616V)	*Lb. species*(236 pb)	

established a two-step PCR method. In this method, lactobacilli are first classified into four groups, and then one or two multiplex PCR assays are carried out for each group, for species identification. *Lb. delbrueckii* was identified in the first grouping multiplex PCR and 10 species were identified in the second multiplex PCR for each group.

Ribotyping

Southern blotting is carried out after the restriction digestion of chromosomal DNA and agarose electrophoresis. In this process, DNA is transferred to a membrane for hybridisation with a labelled 23S, 16S or 5S rRNA gene probe. As bacteria have multiple copies of rRNA operons in their

chromosome, several fragments in the restriction digest mixture hybridise with the probe. In general, the fingerprint patterns obtained by this method are more stable and easier to interpret than those obtained by restriction enzyme analysis (REA). Ribotyping has been used with some success to characterise strains of various *Lactobacillus* species [155], strains of *Lb. helveticus* [80] and strains of *Lb. delbrueckii* [135]. Miteva et al. [135] successfully differentiated between the three subspecies of *Lb. delbrueckii* by *Eco*RI ribotyping. Zhong et al. [202] used ribotyping for species discrimination (*Lb. jensenii, Lb. casei, Lb. rhamnosus, Lb. acidophilus, Lb. plantarum* and *Lb. fermentum).* In general, ribotyping has a greater discriminatory power at species level than at strain level. Tynkkynen et al. [184] analysed 24 lactobacilli strains biochemically identified as members of the *Lactobacillus casei* group (*Lb. rhamnosus* and *Lb. casei*): ribotyping by *Eco*RI digestion and southern blotting with a chemiluminescent probe corresponding to the *rrnB* rRNA operon from *E. coli* resulted in the detection of a triplet, which seems to be typical for most *Lb. rhamnosus* strains.

A fully automated ribotyping system, the RiboPrinter microbial characterisation system, has been developed for identification at the genus, species and strain levels [26]. This method is automated and based on a standardised protocol, maximising interlaboratory reproducibility. It is easy to carry out but the equipment is rather expensive. In the database supplied by the manufacturer (Qualicon), reference is made to several bacterial genera: lactic acid bacteria (lactobacilli included), *Salmonella, Listeria, Escherichia, Pseudomonas* and others.

PCR – Restriction Fragment Length Polymorphism analysis (PCR-RFLP)

PCR-restriction fragment length polymorphism analysis involves the amplification of a specific region, followed by restriction enzyme digestion and conventional gel electrophoresis (CGE). RFLP analysis of the 16S rRNA gene, chromosomal DNA cleaved with *Eco*RI and *Hin*dIII, gave identical patterns for most of the strains of *Lb. plantarum* [109]. When the specific region corresponds to rDNA, then this method is called PCR-ARDRA (amplified rDNA restriction analysis), and is derived from ribotyping. Andrighetto et al. [6] analysed a 1500 bp polymorphism in *Eco*RI-digested 16S rDNA fragments from 25 strains of 4 species of lactobacilli isolated from cheeses and yoghurts*: Lb. delbrueckii* ssp. *lactis* and *Lb. delbrueckii* ssp. *bulgaricus, Lb. helveticus* and *Lb. acidophilus*. Different patterns were observed, making it possible to distinguish between the various *Lactobacillus* species and subspecies. Giraffa et al. [80] used PCR-ARDRA to identify *Lb. delbrueckii* isolates to subspecies level and to differentiate this species from *Lb. helveticus* and *Lb. acidophilus.* As these species were present in the same ecological niches, and displayed similar phenotypic characteristics and a close genetic relationship, PCR-ARDRA was efficient. Bouton et al. [22] confirmed by PCR-ARDRA strains isolated from Comté cheese belonged to *Lb. delbrueckii* ssp. *lactis*. For six presumed strains of *Lb. helveticus,* no cutting by *Eco*RI was obtained, even if fermentation profiles suggested that all strains belonged to *Lb. helveticus* rather than *Lb. acidophilus*. Chromosomal rearrangements [82] or cross-protection by methylation could explain the loss of a cleavage site.

2.2.3. Analysis at strain level

Restriction Enzyme Analysis (REA)

Restriction enzyme analysis (REA) involves the extraction and digestion of chromosomal DNA with restriction endonucleases and separation of the fragments by conventional gel electrophoresis (CGE). The number of bands obtained, generally ranging between 1000 and 20 000 bp in size, are dependent on the restriction

enzymes used. The complexity of the banding pattern makes visual evaluation difficult and necessitates the use of computer-assisted multivariate analysis [32]. Electrophoretic separation of the DNA fragments obtained after restriction endonuclease digestion has been achieved for many bacterial species of the genus *Lactobacillus*. REA has been successfully used to differentiate between strains of *Lb. acidophilus* [157]. Zhong et al. [202] used *Bcl*I and *Dra*I to separate 64 strains of lactobacilli; this method allowed discrimination between the strains, but the patterns produced were very complex.

RAPD/ AP-PCR

Polymerase chain reaction (PCR)-based DNA fingerprinting methods using arbitrary primers (AP) have been developed for studying genomic DNA polymorphism. Arbitrarily primed PCR and randomly amplified polymorphic DNA (RAPD) methods were first described in 1990 [197, 198]. In these similar techniques, the primers are generally about 10 nucleotides long and are not directed at any known sequence of the bacterial genome, as the primers are selected arbitrarily. A single arbitrary oligonucleotide primer directs the amplification of random segments of genomic DNA. It generates a characteristic spectrum of short DNA products of various complexities. RAPD techniques have been extensively used in the typing of lactic acid bacteria [176]. Some of the primers used are listed in Table VI. Randomly amplified polymorphic DNA analysis has been used to monitor population dynamics in food fermentation and to estimate the diversity of *Lactobacillus* strains in cheeses [12, 17, 22] whey culture [38], sausage fermentation [137, 152] and maize dough [91]. It can also be used to distinguish a particular strain from the natural flora, such as distinguishing *Lactobacillus* probiotic adjunct from the natural NSLAB population in Cheddar cheese [172]. Du Plessis and Dicks [61] used RAPD to separate species of the *Lactobacillus acidophilus* group (*Lb. acidophilus, Lb. crispatus, Lb. amylovorus, Lb. gallinarum, Lb. gasseri* and *Lb. johnsonii*), which are difficult to distinguish on the basis of simple physiological and biochemical tests. Johansson et al. [109] evaluated the typing potential of RAPD for *Lb. plantarum* strains from various sources: 50% of the strains could be individually separated from all the other strains tested and REA could separate the rest.

Cocconcelli et al. [38] demonstrated that the community of thermophilic lactobacilli that dominates in Parmesan cheese is composed of a limited number of bacterial strains belonging to the *Lb. helveticus* and *Lb. delbrueckii* ssp. *lactis* species. Baruzzi et al. [12] reported strains of the following species: *Lb. acetotolerans*, *Lb. alimentarius, Lb. brevis, Lb. gasseri, Lb. kefiri, Lb. paracasei, Lb. plantarum* and *Lb. zeae* in Ricotta forte cheese. RAPD analysis was used to separate the strains, which were previously grouped into one protein profile cluster as *Lb. plantarum* and *Lb. pentosus* [187]. Quiberoni et al. [149] used primers P1 and P2 to discriminate between 25 isolates obtained from Sardo and Reggianito cheeses. Giraffa et al. [81] characterised 23 strains of *Lb. helveticus* isolated from natural whey starter cultures used for Italian hard cheese. Sohier et al. [169] used RAPD primers (and REP-PCR) to separate isolates of *Lb. brevis* and *Lb. hilgardii*. The two fingerprinting methods were equally suitable for revealing species-specific genetic profiles. RAPD analysis may have the advantage of facilitating simultaneous strain typing, species affiliation determination and individual strain differentiation [16]. RAPD-derived probes and primers have been described for the identification of lactobacilli to species level, and even to strain level [148]. Tilsala-Timisjarvi and Alatossava [180] have also developed strain-specific DNA-derived PCR primers for a probiotic strain of *Lb. rhamnosus*.

Table VI. RAPD primers.

RAPD primers (5'–3')	Used for	Ref.
1254 CCGCAGCCAA **M13** GAGGGTGGCGGTTCT	*Lb. delb. bulgaricus, Lb. acidophilus, Lb. kéfiranofasciens, Lb. helveticus, Lb. delb. lactis, Lb. casei, Lb. rhamnosus, Lb. maltoromicus, Lb. buchneri, Lb. kéfir.*	[181]
9898 GCAGCCGG	*Lb. brevis, Lb. hilgardi*	[169]
AGTCAGCCAC	*Lb. casei, Lb. rhamnosus, Lb. zeae*	[184]
P1 GCGGCGTCGCTAATACATGC **P4** ATCTACGCATTTCACCGCTAC	*Lactobacillus*	[38] [152]
CTGCTGGGAC GGAGGGTGTT	*Lb. curvatus, Lb. graminis, Lb. sakei*	[16]
OPL-01 GGCATGACCT **OPL-04** GACTGCACAC	*Lb. acidophilus, Lb. crispatus, Lb. amylovorus, Lb. gallinarum, Lb. gasseri, Lb. johnsonii*	[61]
UNAMED ACGCGCCCT	*Lb. plantarum* *Lactobacillus*	[109] [7]
UNAMED AGCAGCGTGG	*Lb. acidophilus, Lb. helveticus, Lb. casei, Lb. reuteri, Lb. plantarum.*	[36] [12]
OPL-01 GGCATGACCT **OPL-04** GACTGCACAC **OPL-02** TGGGCGTCAA **OPL-05** ACGCAGGCAC	*Lb. pentosus, Lb. casei, Lb. sake, Lb. curvatus, Lb. plantarum*	[187]
OPB-06 TGCTCTGCCC **OPB-10** CTGCTGGGAC	*Lb. helveticus*	[80] [22]
P1 TGCTCTGCCC **P2** CTGCTGGGAC	*Lb. helveticus*	[149]
RP CAGCACCCAC	*Lb. paracasei, Lb. rhamnosus*	[196]
CRA 23 GCGATCCCCA **CRA25** AACGCGCAAC	*Lactobacillus* sp.	[51]
OPA-02 TGCCGAGCTG **OPM-05** GGGAACGTGT **OPL-07** AGGCGGGAAC **OPL-16** AGGTTGCAGG	*Lb. acidophilus* group	[75]

Table VII. REP/ERIC primers.

Primers (5'–3')	Used for	Ref.
REP1R-I IIIICGICGICATCIGGC **REP2-I** ICGICTTATCIGGCCTAC	*Lb. hilgardii, Lb. brevis*	[169]
REP-1R-Dt IIINCGNCGNCATCNGGC **REP2-D** NCGNCTTATCNGGCCTAC	*Lactobacillus* sp.	[17] [22]
REP-1R-Dt IIINCGNCGNCATCNGGC **REP2-Dt** NCGNCTTATCNGGCCTAC **BOXA1R** CTACGGCAAGGCGACGCTGACG **RW3A** TCGCTCAAAACAACGACACC	*Lb. sakei*	[102]

It is also possible to use a combination of two or more 10-mer oligonucleotides (multiplex RAPD) in a single PCR to generate RAPD profiles, making it possible to discriminate between *Lactobacillus* strains [51].

RAPD analysis has been shown to be less effective than other molecular methods, although in some cases, it allowed the separation of strains indistinguishable by other techniques. The screening of new RAPD primers might increase the specificity of this technique for strain typing. RAPD analysis is a rapid and cheap method, but careful optimisation is required to obtain reproducible results.

REP-PCR/ERIC-PCR

Repeated sequences are present in the genomes of all organisms. The first described and most extensively studied repeated sequence is the extragenic palindrome (REP) or palindromic unit (PU), initially identified in *Salmonella typhimurium* and *Escherichia coli* [79]. This sequence has a copy number of 500–1000 and consists of a 35-40 bp inverted repeat. It is found in clusters, with successive copies arranged in opposite orientations. A second family of repetitive elements, called IRU (intergenic repeat units) or ERIC (enterobacterial repetitive intergenic consensus) has been described; IRU are 124-127 bp long and have a copy number of about 30-50 in *E. coli* and 150 in *S. typhimurium* [100, 166]. The members of both the PU and IRU families are located in non-coding but probably transcribed regions of the chromosome and both have a potential stem-loop structure. These sequences have been searched for and studied in lactobacilli [17, 22, 102, 169], and repetitive primers were designed for PCR. Some of the primers used are listed in Table VII.

Berthier et al. [17] analysed 488 isolates of mesophilic lactobacilli isolated from Comté cheese. These isolates were identified to species and strain level with a combination of two PCR-based methods: amplification with pairs of repetitive primers (ERIC and REP), and amplification with specific primers. REP-PCR fingerprints were used to assign strains to species. Combined REP and ERIC fingerprints have the advantage over RAPD analysis that the sequences considered are longer and are therefore less sensitive to minor changes in reaction conditions [17]. Hyytiä-Trees et al. [102] concluded that REP-PCR has a discriminatory power similar to that of RAPD analysis, but

weaker than that of pulsed-field gel electrophoresis (PFGE). However, if the results of REP-PCR and RAPD analysis were combined, the discriminatory power in some cases equalled that of PFGE.

Amplified fragment length polymorphism (AFLP)

Another method, although not widely used except in a systematic approach, is called amplified fragment length polymorphism (AFLP). This method is sophisticated and combines PCR and restriction enzyme techniques. AFLP templates are first prepared by cutting with two enzymes (for example, the six-base cutter *Hin*dIII and the four-base cutter *Mse*I, for *Lactobacillus*), resulting in DNA fragments with two different types of sticky ends. Appropriate adaptors (short oligonucleotides) are ligated to these ends to form templates for PCR, using two different primers containing the adaptor sequence extended to include one or more selective bases next to the restriction site of the primer. Only fragments that completely match the primer sequence are amplified, resulting in selective amplification according to the initial DNA structure and cutting. The amplification process results in an array of 30 to 40 DNA fragments that are group- and/or species- and/or strain-specific [108]. However, AFLP analysis, which involves a large number of experimental steps, has to be carefully monitored by a specialist to ensure a high level of reproducibility, even if an automated AFLP-fingerprinting system is used [75]. RAPD-PCR and AFLP analyses have been used for the rapid typing of strains of *Lb. acidophilus* and related species, using at least 3 different primers, and digital analysis of the combined pattern for all the primers. These techniques were found to discriminate between the strains at a much finer taxonomic level than SDS-PAGE fingerprinting, although their reproducibility was found to be a matter of debate [75].

Pulsed-field gel electrophoresis (PFGE)

Like REA, this method involves restriction enzyme digestion, but the enzymes used for PFGE must have a low cutting frequency, as is the case for *Sma*I and *Sgr*AI. However, in this case, the restriction fragments are resolved by pulsed-field gel electrophoresis. This technique, involving the application of an alternating electric field in two defined directions, is used to separate very large fragments, from 5×10^4 pb to 2×10^6 pb. This method is highly discriminatory and reproducible, and generates a banding pattern that is easy to interpret.

PFGE analysis alone, with two or three appropriate enzymes, can be used for reliable strain typing. In several *Lactobacillus* studies, PFGE has been shown to be a powerful method for strain typing (Tab. VIII). However, one drawback of this method is that only a limited number of samples can be analysed. Tynkkynen et al. [184] identified, by PCR with specific primers, 24 strains of *Lactobacillus* biochemically related to the *Lactobacillus casei* group. These strains were typed by RAPD, ribotyping and PFGE, to make possible comparison of the discriminatory power of the methods. Twelve RAPD genotypes were detected among the 24 *Lactobacillus* strains; ribotyping with *Eco*RI produced 15 different fingerprint patterns and PFGE revealed 17 different genotypes.

Blaiotta et al. [20] used PFGE to monitor the addition of *Lactobacillus,* used as starter, to Cacioricotta cheese. This technique made it possible to analyse the growth kinetics of each starter strain during the process. Similarly, Jacobsen et al. [107] monitored the survival of probiotic strains of *Lb. rhamnosus*, *Lb. reuteri* and *Lb. delbrueckii* ssp. *lactis* in faeces.

Bouton et al. [22] used a PCR-based method and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. Isolates, which exhibited unique patterns by RAPD or

Table VIII. PFGE Restriction enzymes and migration conditions used for lactobacilli.

Lactobacilli species	Restriction enzyme	Running conditions	Ref.
Lb. acidophilus	*Sma*I, *Apa*I	(0.5–15) s, 24 h, 6 V·cm^{-1} (5–25) s, 24 h, 6 V·cm^{-1}	[157]
Lb. sake	*Sma*I, *Avr*II	(0.3 s: 1 h; 0.5 s: 1 h; 0.7 s: 1 h; 2 s: 5 h; 4 s: 6 h) 200 V	[19]
Lb. casei/rhamnosus *Lb. paracasei*	*Sma*I, *Bgl*I, *Sfi*I	(1–5) s, 20 h, 6 V·cm^{-1} (1–20) s, 24 h, 4.5 V·cm^{-1} (40–80) s, 20 h, 6V·cm^{-1}	[71]
Lactobacilli	*Apa*I	(1–12) s, 17 h, 5 V·cm^{-1}	[131]
Lb. sanfranciscensis	*Apa*I, *Sac*II, *Sgr*AI	(0.5–8) s, 17 h, 6 V·cm^{-1} (3–40) s, 24 h, 6 V·cm^{-1}	[201]
Lb. acidophilus *Lb. gallinarum* *Lb. gasseri* *Lb. helveticus*	*Apa*I, *Sma*I, *Sgr*AI	(2 s, 6 h, 350 mA / 5 s, 6 h, 370 mA / 10 s, 4 h, 390 mA / 15 s, 4 h, 410 mA / 30 s, 4 h 430 mA / 60 s, 3 h, 450 mA)	[158]
Lb. helveticus	*Sma*I, *Sgr*AI	(1–13) s, 20 h, 200 V	[121]
Lb. plantarum	*Apa*I, *Sma*I, *Not*I, Sfi*I*, Swa*I*	5 s, 16 h, 140 mA 10 s, 16 h, 140 mA	[50]
Lb. casei *Lb. rhamnosus* *Lb. zeae*	*Not*I, *Sfi*I	(1–15) s, 22 h, 5 V·cm^{-1}	[184]
Lb. delbrueckii ssp. *bulgaricus*	*Not*I		[20]
Lb.rhamnosus *Lb. reuteri* *Lb. delbrueckii* ssp. *lactis*	*Apa*I	(1–15) s, 20 h, 200 V	[107]
Lb. helveticus *Lb. delbrueckii*	*Sgr*A1, *Xho*I	(2–13) s, 22 h, 200 V	[22]

REP-PCR, were distinguishable by PFGE, but some strains which were distinguishable by RAPD or REP-PCR were related by PFGE; these discrepancies were explained by the different exploration of DNA polymorphism (the whole DNA chromosome for PFGE, and region amplified by primers for RAPD and the REP-PCR). The use of second restriction enzymes would certainly be useful in this case.

Plasmid profiling

Plasmid-based typing methods are useful, particularly if large numbers of strains are to be examined. Plasmids are not evenly distributed among the various isolates belonging to different species of *Lactobacillus*. A rapid mechanical lysis method for the routine analysis of plasmids from *Lactobacillus* isolated from sourdough has been reported [153].

The plasmid profile of a particular LAB or *Lactobacillus* strain may be used as an identification marker. It has been performed on *Lactobacillus* strains [63, 64, 136]. However, a plasmid DNA replicates independently from the chromosome, and plasmidic genes are usually more unstable than chromosomal gene function [65]. Although the plasmid profile of a strain remains stable under laboratory conditions, plasmids may be lost during fermentation in unfavourable growth conditions, or may undergo rearrangements by conjugative transfer. Therefore, plasmid profiling techniques have several potential disadvantages, such as the ability of a strain to gain, lose or modify its plasmids [192], and

there is often no correlation between plasmid content and species identification [202].

Phage-related DNA polymorphism

Bacterial lysogeny, that is, the presence of prophages as genetic elements in the bacterial chromosome, is common in lactobacilli, especially in the *Lb. casei* group [72]. Brandt et al. [24] investigated whether further intraspecies DNA polymorphism could be revealed by screening for the presence and distribution of phage-related DNA sequences among the strains of a single bacterial species. He studied 11 *Lb. rhamnosus* strains used as starter and probiotic cultures in the Finnish dairy industry. Six different PCR product patterns were obtained by amplification with primer pairs derived from the nucleotide sequence of a *Hin*dIII fragment of the phage Lc-Nu genome. Phage Lc-Nu DNA-derived PCR was found to be an effective tool for detecting polymorphism in the *Lb. rhamnosus* strains.

2.2.4. Global techniques

The recent development of whole bacterium analysis by FTIR (Fourier transform infrared spectroscopy) is of great potential for rapid identification of lactobacilli [1, 2, 48, 49, 128]. Bacterial spectra are usually recorded in the mid-infrared. They are specific to a bacterial strain and show the vibrational characteristics of the whole cellular components: fatty acids, intracellular and membrane proteins, polysaccharides and nucleic acids. The statistical treatment of spectral data makes it possible to discriminate between different genera, species and strains. The reproducibility problems initially encountered have been resolved, resulting in standardised conditions for cell growth and sample preparation. The principal advantage of this technique, as pointed out by almost all authors, is its simplicity with respect to genome analysis. Amiel et al. [1] established libraries of species used in the cheese industry. Wild strains of lactobacilli isolated from raw milk cheeses from Normandy were well identified. The spectral database makes it possible to identify new strains, with a high percentage of good results: 100% at the genus and species level for collection strains; and 100% at the genus level and 69% at the species level for wild isolates previously identified by RAPD and the phenotypic method. The results obtained were as reliable as those obtained by genomic methods such as RAPD analysis [2].

Another global technique, pyrolysis-mass spectrometry [125], is also very promising but has not yet been applied to lactobacilli.

3. CHARACTERISATION AND IDENTIFICATION OF LACTOBACILLI BY CULTURE-INDEPENDENT METHODS

Culture-independent methods involve extraction of nucleic acids (DNA or RNA) from raw samples and the use of probes for hybridisation and primers for denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and single strand conformation polymorphism (SSCP).

These techniques give a picture of the populations present in a complex matrix, bypassing problems related to injured, and viable but non-cultivable bacteria.

3.1. Hybridisation

Ehrmann et al. [68] developed a technique facilitating the direct identification of LAB, without prior culture, in cheese, yoghurt, sausages, sauerkraut and sourdough and based on a reverse dot-blot assay. Oligonucleotide probes specific to the various *Lactobacillus* species are extended by adding a polythymidine phosphate tail.

Table IX. DGGE/ TGGE primers.

Primers	5'–3'	Specificity	Ref.
HDA1/ HDA2	(GCclamp)ACTCCTACGGGAGGCAGCAGT/GTATTACCGCGGCTGCTGGCA	V2-V3/16S	[178] [194] [144]
V3F/ V3R	(GCclamp)CCTACGGGAGGCAGCA/ ATTACCGCGGCTGCTGG	V3/16S rDNA	[44]
gc338f/ 518r	(GCclamp)ACGGGGGGACTCCTACGGGAGGCAGCAG/ TCTGTGATGCCCTTAGATGTTCTGGG	V3/16S rDNA	[5]
P1/P2	(GCclamp)TACGGGAGGCAGCAG/ ATTACCGCGGCTGCTGG	V3/16S rDNA	[39]
Lac1/Lac2GC	AGCAGTAGGGAATCTTCCA/(GCclamp)ATTYCACCGCTACACATG	16S rDNA	[195]
Unnamed	CGCCGGGGGCGCGCCCCGGGCGGG/ GCGGGGGCACGGGGGG	16S rDNA	[150]

These extended oligonucleotides bind naturally to filter membranes and serve as species-specific capture probes for the labelled, PCR-amplified rRNA gene fragment [68].

A simple, rapid method for whole-cell hybridisation with fluorescent-labeled rRNA-targeted peptide nucleic acid (PNA) probes was recently developed for the detection and identification of thermophilic *Lactobacillus* cells growing in milk or present in industrial starter culture [129]. The protocol involves filtration of the samples, and epifluorescence microscopy is used for detection. Its detection limit is 10^4 to 10^6 cells per mL, and specific oligonucleotides are available for *Lb. delbrueckii, Lb. helveticus, Lb. pentosus* and *Lb. plantarum.* The rRNA-targeted oligonucleotide probes currently available for the identification of LAB are listed in Table III. Various types of assay can be used. In dot-blot assays the target nucleic acid must be extracted and immobilised on a membrane. Such assays have been used for the simultaneous identification of various lactobacilli, such as *Lb. curvatus*, *Lb. sake*, *Lb. pentosus*, *Lb. plantarum, Lb. delbrueckii* and *Lb. helveticus* [97, 143, 192].

3.2. PCR-DGGE, PCR-TGGE

Methods such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) have been developed for the analysis of microbial communities without culture, by the sequence-specific separation of amplified 16S rDNA fragments. Separation is based on the lower electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants, a mixture of urea and formamide (DGGE), or subjected to a linear temperature gradient (TTGE). The melting of fragments proceeds in discrete melting domain stretches of base pairs with an identical melting temperature. Once the domain with the lowest melting temperature reaches its melting temperature (Tm), in the denaturing or temperature gradient gel, the molecule undergoes a transition from a helical to a partially melted structure, and its migration stops. Optimal resolution occurs when amplicons are not completely denatured and when the region to be screened is in the lowest melting domain. This is achieved by adding a 30-40 bp GC-rich clamp to one of the PCR primers (Tab. IX). This results in sequence variants of particular fragments ceasing to migrate at different positions in the denaturing gradient, facilitating their effective separation by TGGE or DGGE.

The members of the bacterial community are often amplified using primers corresponding to the 16 S rDNA sequence [5, 39, 93, 144, 168, 186, 194, 195]. Species can then be distinguished by comparing the

migration distance of the PCR amplicons in gels with those of reference strains [3].

These techniques have recently been used in the evaluation of microbial diversity, particularly the diversity of lactobacilli in cheeses [44, 144, 150], sausages [39], starch fermentation [5], malt whisky fermentation [186], beer [183], faeces [168, 178, 195], and the gastrointestinal tract [194], and for the identification of *Lactobacillus* species [189]. Coppola et al. [44] used PCR 16S-23S rDNA spacer polymorphism and PCR-DGGE analysis of the V3 region of 16S rDNA to study the microbial diversity of unripened Pasta Filata cheeses. The number of bands in the PCR profiles obtained made it possible to distinguish between industrial, cottage industry and traditional dairy products.

Ogier et al. [144] identified by PCR-TGGE of the 16S rDNA V3 regions bacterial species (*Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Pediococcus, Streptococcus* and *Staphylococcus*) present in home-made or commercial dairy products. V3-TGGE sequences differentiate between bacteria belonging to the different genera. However, V3-TGGE did not distinguish between members of the *Lactobacillus casei* group (*Lb. casei, Lb. paracasei* and *Lb. rhamnosus*), or members of the *Lactobacillus acidophilus* group (*Lb. gallinarum, Lb. crispatus, Lb. amylovorus, Lb. acidophilus* and closely *Lb. helveticus*). *Lb. pentosus* and *Lb. plantarum* or *Lb. johnsonii* and *Lb. gasseri* have similar V3 sequences and co-migrate. Only *Lb. reuteri*, *Lb. brevis, Lb. fermentum, Lb. delbrueckii* ssp. *bulgaricus*, and *Lb. delbrueckii* ssp. *lactis* can be easily distinguished. Cheeses with similar production procedures (e.g. Brie and Camembert cheeses, or Emmental and Comté…) produced common TGGE bands. TGGE provides a description of the dominant bacterial species in a complex ecosystem, but bacterial minority (less than 1%) cannot be detected. So, TGGE seems to be an excellent molecular tool to analyse diversity within complex bacterial communities, but screening in new primers in a more discriminating area than V3 16S rDNA is necessary for lactobacilli species.

Randazzo et al. [150] obtained DGGE profiles derived from PCR and RT-PCR (reverse transcriptase-PCR) of DNA and RNA, and compared them in order to determine the expression level of the 16S rRNA genes of the most predominant bacteria during the manufacturing of Ragusano cheese. The evolution of the *Lactobacillus* community during the manufacturing and ripening process was reflected in the unstable DGGE profiles generated by using *Lactobacillus*-specific primers, which target all members of the *Lactobacillus* group, but include *Leuconostoc* and *Pediococcus* spp. too. Concurrently microbial enumeration on different media was done, and after cultivation, isolates were identified by both classical phenotypic methods and 16S rDNA sequence analysis. *Lb. delbrueckii,* that was dominant as indicated by DGGE, was never isolated on the selective media.

Tannock et al. [178] assessed the impact of probiotic consumption on the intestinal microflora by monitoring the faecal community by FISH (fluorescence in situ hybridisation) and DGGE. Heilig et al. [93] used DGGE to study the stability of the bacterial community of the gastrointestinal tract in various age groups, over various time periods, and successive changes in the *Lactobacillus* community were observed. They also assessed the specificity of the PCR and DGGE approach for studying the retention in faecal samples of a *Lactobacillus* strain administered during a clinical trial.

Van Beek and Priest [186] adopted a polyphasic approach, using light and electron microscopy and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of 16S ribosomal DNA to monitor the development of the lactic acid bacterial community during malt whisky fermentation. Their results revealed that

culture-dependent methods underestimated bacterial diversity and demonstrated the presence of novel lactobacilli and other taxa in malt whisky during fermentation.

These new techniques have the advantage of facilitating the direct study and analysis of population dynamics within complex microbial systems. These methods are only just beginning to be applied to ecological studies of cheese microflora.

3.3. PCR-SSCP

SSCP (single strand conformation polymorphism) analysis for molecular identification in microbial ecosystems is based on 16S rDNA sequences. No culture is required. SSCP detects sequence variations between DNA fragments, usually amplified by PCR from variable regions of the 16S rRNA gene and uses neutral, non-denaturing polyacrylamide gels. Short double-stranded DNA fragments are first generated using standard PCR protocols. Single-stranded DNA is created by combining a small aliquot of PCR product with an equal volume of formamide (80–95%), and then it is heat denatured. The two complementary strands of DNA will migrate differently and will therefore separate during gel electrophoresis. This fingerprinting method characterises microbial diversity, by comparing closed microflora [84]. SSCP analysis has been adapted for the rapid identification of both Gram-positive and Gram-negative bacteria to genus and species level. Analysis of the microflora of AOC Salers cheese resulted in the co-elution of *Lc. lactis, Lb. plantarum, Lb. pentosus* and *St. thermophilus,* all of which were associated with the main peak [84].

4. CONCLUSION

It is widely recognised that the identification of lactobacilli to species or strain level on the basis of physiological and biochemical criteria is very ambiguous and complicated. Numerous taxonomic changes have been observed in the *Lactobacillus* genus as qualification of old species in new genera or description of new species. This leads to a problematic genus characterisation by phenotypic tests and to an increasing use of classical culture-based molecular methods. New molecular techniques for microbial community analysis that do not require isolation of the microorganisms are very promising. They provide a complementary picture of the population obtained using culture-based techniques when applied to the analysis of milks and dairy products. However, these molecular approaches have several limitations, including the design of adequate primers, and the possibility that DNA isolation, amplification and cloning might be biased by certain strains and sequences. There is also a dependence on the detection threshold and on the number of lactobacilli, unfortunately low in high-quality raw milks. Nevertheless, these methods provide an overview of the diversity of microorganisms present in a particular sample. They provide qualitative and possibly semi-quantitative information, which should be complemented by quantitative PCR to obtain results as close as possible to reality.

We intended to produce a guide for the reader, covering pertinent techniques used for a polyphasic analysis of lactobacilli. This guide was not produced, since proposing a technique for a specified use is not completely reliable. In fact, the choice of the technique to use is variable depending on:

- the level of discrimination required;
- the type of product and matrix, the nature of undesirable organisms and the diversity of the lactobacilli present;
- the amount of time available;
- the available staff and equipment;
- the number of strains and isolates to be studied.

Furthermore, numerous techniques, culture-dependent or culture-independent, are based on the use of probes and primers. For these techniques the discrimination level depends on the existence or not of the

probes and primers at the taxonomic level desired. To date we are very far from having specific primers and probes for the 88 lactobacilli species, and regarding those which have been designed, their specificity and validity should be checked one by one with closed genera, species or strains. Another problem results from the given list of 88 lactobacilli species since it is not an official list (it does not exist) and thus to bypass possible misidentification all probes and primers should be validated against the same reference strain at the beginning to ensure their common specificity. Moreover, all the techniques mentioned in this review have not been applied to the lactobacilli using the same objectives. The genus primer designed by Dubernet et al. [62], has been used for PCR and PCR-TGGE, but not for hybridisation, but it is clear that it could be used. The difficulty of choosing a technique that has good dicrimination power depends not only on the techniques but also on the species or strains. Results also depend on the quality and exhaustivity of a database. Very good results at genus, species and strain level could be obtained by using FTIR, but if the database is not complete (not enough strains of different species, or of different origin), results will not be as good as they could be. Finally, only a few limited techniques can be applied with a high degree of confidence although they are dependent on database robustness: sequencing to identify at genus and species level, and sequencing or pulsed field gel electrophoresis to discriminate strains.

In conclusion, analysis of lactobacilli in cheeses and other dairy products is very complicated and the use of different techniques, especially molecular-based phenotypic or genomic techniques, is recommended.

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