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The Genus *Lactobacillus*: A Taxonomic Update

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Abstract Lactic Acid Bacteria (LAB) are a functional group of microorganisms comprising Gram-positive, catalase negative bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Among LAB, *Lactobacillus* is the genus including a high number of GRAS species (Generally Recognized As Safe) and many strains are among the most important bacteria in food microbiology and human nutrition, due to their contribution to fermented food production or their use as probiotics. From a taxonomic point of view, the genus *Lactobacillus* includes at present (October 2012), 152 validly described species, and it belongs to the family *Lactobacillaceae* together with genus *Pediococcus*, with whom it is phylogenetically intermixed. The updated phylogenetic analysis based on 16S rRNA gene sequence revealed that the family is divided into 15 groups of three or more species, 4 couples and 10 single lines of descents. In addition, other taxonomically relevant information for *Lactobacillus* species was collected. This study aims at updating the taxonomy of the genus *Lactobacillus*, presenting the phylogenetic structure of the *Lactobacillaceae* and discussing the clusters as possible nuclei of genera to be described in the future. It is expected that scientists and producers in the field of probiotics could benefit from information reported here about the correct identification procedures and nomenclature of beneficial strains of lactobacilli.

Keywords *Lactobacillus* · Taxonomy · 16S rRNA · Phylogeny

Introduction

Species of genus *Lactobacillus* are some of the most important taxa involved in food microbiology and human nutrition: several *Lactobacillus* species are remarkably essential in fermented food production and are used as starter cultures or food preservatives. Moreover, certain strains of human origins are being exploited as probiotics or vaccine carriers [1]. This genus includes a high number of GRAS species (Generally Recognized As Safe) among Lactic Acid Bacteria (LAB), a functional group comprising Gram-positive, catalase negative bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation [2].

Members of genus *Lactobacillus* are non-sporeforming, catalase negative (even if some strains are able to produce pseudocatalase), obligate saccharolytic rods or coccobacilli generally characterized by a low GC (guanine and cytosine) content of the genome although the upper limit of GC content reaches 59.2 mol% [3]. They are usually considered to have a fermentative metabolism [4], although recent molecular evidences challenge this definition [5]. Besides lactic acid, other side products consist of acetate, ethanol, CO_2, formate and succinate [4, 6].

Lactobacilli are generally aero-tolerant or anaerobic, aciduric or acidophilic. In general, they do not synthesize porphyrinoids and are devoid of heme-dependent activities [4].

The number of species was updated on October 1st, 2012.

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The growth temperature ranges from 2 to 53 °C, and they are able to grow in a pH range between 3 and 8. Optimal growth temperature and pH are usually 30–40 °C and 5.5–6.2, respectively.

They have complex nutritional requirements in terms of amino acids, peptides, vitamins, salts, fatty acids or fatty acid esters, and they are found in rich habitats where carbohydrate-containing substrates are available such as food (dairy products, grain products, meat and fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage, and sourdough), water, soil and sewage; they are part of the normal flora in the mouth, GI, and genital tracts of humans and many animals [6].

The taxonomy of lactobacilli has been based for years on phenotypic properties as carbohydrate fermentation patterns, resistance to different NaCl concentrations, growth in different media at defined temperature or pH range and resistance against antibiotics, expanded to include the cell wall composition, cellular fatty acids, isoprenoid quinones and other characteristics of the cells [7].

Originally, lactobacilli were grouped based on their growth temperature and the fermentation of hexoses [8] and subsequently according to their homo/heterofermentative potential [9, 10]. The subdivision of the genus *Lactobacillus* was revisited by Pot and colleagues [11], but the accepted "modern" definition is the one given by Hammes and Vogel [4] and Hammes and Hertel [6] which divides lactobacilli as obligate homofermentative, facultative heterofermentative and obligate heterofermentative, based on the type of fermented sugars and fermentation products. The homofermentative lactobacilli (commonly referred as metabolic group A) ferment hexoses almost exclusively (>85 %) to lactic acid via the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis; pentoses and gluconate are not fermented. Facultative heterofermentative species (metabolic group B) ferment hexoses to lactic acid via EMP and are able to degrade pentoses and gluconate via an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, with a resulting production of acetic acid, ethanol and formic acid under glucose limitation. Finally, the obligate heterofermentative lactobacilli (metabolic group C) possess a FDB aldolase, but not phosphoketolase, and they metabolize pentoses and hexoses exclusively via phosphogluconate pathway (corresponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO_2 [6].

Exceptions to this characteristics are known [6] and suggest that classification and identification based on metabolic characteristics could be misleading.

The availability of 16S rRNA gene sequence allowed the first phylogenetic analysis of the genus, performed by Collins et al. [12]. Based on the number of species described until then, lactobacilli were split into 3 phylogenetic clusters: *Lactobacillus delbrueckii* group, *Lactobacillus casei-Pediococcus* group, then further divided into 4 subgroups, and *Leuconostoc* group, which also contained some species of *Lactobacillus* [12, 13].

The massive description of novel species over the past 20 years has led to a progressive revision of the genus with the recognition of a growing number of variable phylogenetic groups [14–16]. Although the 16S rRNA gene sequence analysis contributed to the development of a more exhaustive taxonomy for lactobacilli, it has become evident that only little correlation exists between the traditional classification based on metabolic properties and the phylogenetic relatedness [6, 16].

Genome data represent the ultimate frontier from which a more reliable insight into the evolutionary relationships of *Lactobacillus* species must still be obtained. The recent explosion of genomic information allowed a better comprehension of lactobacilli features such as their physiology, metabolic capabilities, probiotic potential, key gene features and niche adaptation. Furthermore, the availability of genome sequences provided a good opportunity to reconstruct events of genome evolution revealing the natural relationship between some of *Lactobacillus* species [17–25].

The aim of the present study is to give the taxonomic update of genus *Lactobacillus*, review its phylogenetic structure after 5 years from the last survey and analyze the subgeneric groups which arise from 16S rRNA-based phylogeny in terms of species composition and relevant phenotypic data. As reported in the FAO/WHO Guidelines for the Evaluation of Probiotics in Food, identification procedures should be applied to each strain candidate as a probiotic. Identification precedes the safety evaluation, and the most updated methodology for identification should be applied combining phenotypic and genetic tests [26]. Identification of strains is a comparative procedure, therefore, when considering lactobacilli (among the most important probiotics), it must be noted that their biodiversity is high and appropriate references should be chosen. Vankerckhoven et al. [27] have also underlined the difficulties of LAB taxonomy, the unreliability of biochemical identifications and the importance of 16S rRNA gene sequence analysis as the election method for the taxonomic placement of probiotic cultures, although cases of insufficient discriminative power for closely related species are known. This implies that additional techniques, such as sequencing of more divergent protein-coding genes and/or fingerprinting techniques should be applied to differentiate strains and allot them to the correct species after 16S rRNA gene–based clustering. Above 98.7–99 % 16S rRNA gene sequence identity between two novel strains, DNA–DNA reassociation tests would be mandatory to identify the species [28].

The taxonomic analysis of genus *Lactobacillus* reported here is expected to provide up-to-date names and tools helpful for applications and scientific communication related to lactobacilli. Moreover, the revised phylogenetic framework together with the high heterogeneity of the genus revealed by phenotypic data furnish the basis for the correct genome data integration in the taxonomic analysis of lactobacilli. The combination of different data could lead to the revaluation of the taxonomic scheme of genus *Lactobacillus* and its feasibility to be split in more homogeneous genera.

Materials and Methods

16S rRNA gene sequences of the type strain of each species belonging to *Lactobacillus* and *Pediococcus* genera were obtained from Nucleotide database in NCBI (http://www.ncbi.nlm.nih.gov/nuccore); sequence alignments were performed with CLUSTALW 2.0 [29] and adjusted manually with Jalview v2.7 [30]. Unknown bases were disregarded, and 813 positions were included in the phylogenetic analysis. Phylogenetic trees were constructed using Jukes and Cantor [31] and Tamura Three Parameters [32] as distance matrix calculation methods, and neighbor-joining [33] and minimum evolution [34] as tree inference models as implemented in MEGA v5.0 software package [35]. The statistical reliability of the topology of the phylogenetic trees was evaluated using bootstrapping with 1,000 replicates [36].

16S rRNA gene sequences of *Eggerthia catenaformis* and *Kandleria vitulina* were included in the analysis as outgroups [37].

SplitsTree v4 [38] was applied to aligned 16S rRNA gene sequences to detect conflicting signals along the sequences which are then displayed as networks instead of bifurcating trees.

Results

The Phylogenetic Reconstruction of Genus *Lactobacillus*

From a taxonomic viewpoint, the genus *Lactobacillus* belongs to phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. At the time of writing, (October, 2012) the genus *Lactobacillus* is composed by 152 species, and it is the most numerous genus of the family and of the order. Its closest relative is genus *Pediococcus*; in 2000, Leisner and colleagues proposed a novel genus, *Paralactobacillus* [39], but the individual species (*P. selangorensis*) was recently reclassified as part of genus *Lactobacillus* [40]. Currently, seven species comprise subspecies: *Lactobacillus aviarius* (subsp. *aviarius*, and subsp. *araffinosus*), *Lactobacillus coryniformis* (subsp. *coryniformis*, and subsp. *torquens*), *Lactobacillus delbrueckii* (subsp. *delbrueckii*, subsp. *bulgaricus*, subsp. *indicus*, subsp. *lactis*, and subsp. *sunkii*), *Lactobacillus kefiranofaciens* (subsp. *kefiranofaciens*, and subsp. *kefirgranum*), *Lactobacillus paracasei* (subsp. *paracasei*, and subsp. *tolerans*), *Lactobacillus plantarum* (subsp. *plantarum*, and subsp. *argentoratensis*) and *Lactobacillus sakei* (subsp. *sakei*, and subsp. *carnosus*; http://www.bacterio.cict.fr/).

With respect to the last survey of *Lactobacillus* genus [16], 44 novel species were described and added to the genus; furthermore, other detectable changes regarded the reclassification of seven species as described in detail in the following list:

- reclassification of *Lactobacillus sobrius* Kostantinov et al., 2006 as *Lactobacillus amylovorus* Nakamura 1981 [41];
- reclassification of *Lactobacillus zeae* Dicks et al., 1996 as *Lactobacillus casei* (Orla-Jensen 1916) Hansen and Lessel 1971 [42];
- reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 as *Lactobacillus dextrinicus* [43];
- reclassification of *Paralactobacillus selangorensis* Leisner et al., 2000 as *Lactobacillus selangorensis* [40];
- reclassification of *Lactobacillus catenaformis* and *Lactobacillus vitulinus* as *Eggerthia catenaformis* and *Kandleria vitulina*, respectively [37];
- reclassification of *Lactobacillus kimchii* Yoon et al. 2000 as *Lactobacillus paralimentarius* Cai et al., 1999 [44].

The phylogenetic structure based on 16S rRNA gene sequence showing the phylogenetic relationships between members of genus *Lactobacillus* and *Pediococcus* is reported in Fig. 1. The combination of different models and methods allowed the recognition of 15 *Lactobacillus* robust groups (a group is defined when including three or more species) whose composition is reported in Table 1. The major part of the newly described species branched within the groups previously defined by Felis and Dellaglio [16], while *Lactobacillus manihotivorans*, *Lactobacillus vaccinostercus* and *Lactobacillus collinoides* groups were defined for the first time in this analysis. Interestingly, *L. collinoides* was previously associated with *L. manihotivorans* [16], but the recent description of five novel species (*Lactobacillus odoratitofui*, *Lactobacillus similis*, *Lactobacillus kimchicus*, *Lactobacillus nasuensis* and *Lactobacillus porcinae*) contributed to the generation of the two groups.

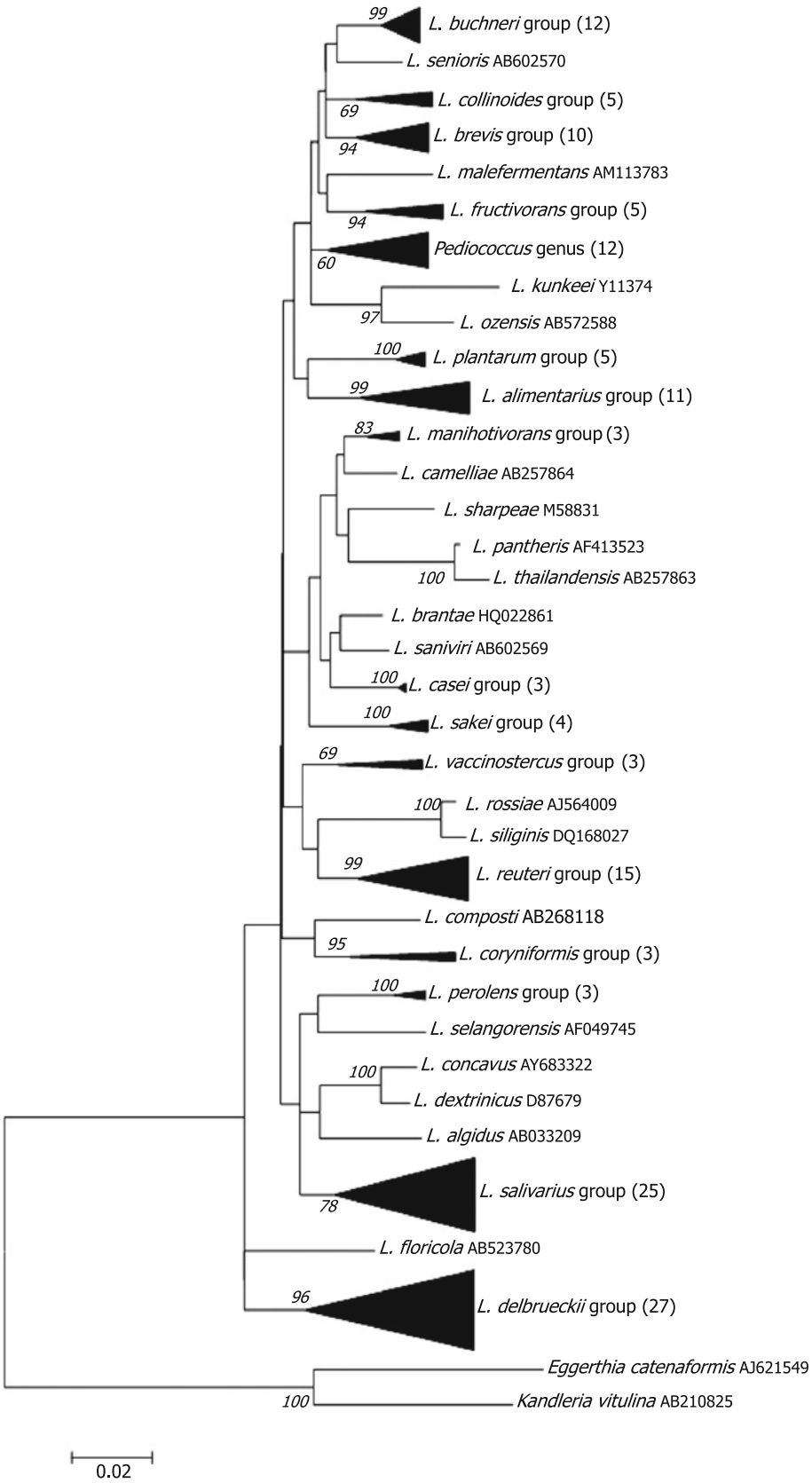

Fig. 1 Phylogenetic tree depicting the relationship between *Lactobacillus* and *Pediococcus* species based on 16S rRNA gene sequence. Tree was calculated using Tamura Three Parameters as distance matrix formula and minimum evolution as tree reconstruction method. Bootstrap values (1,000 replicates) are reported in percentage at nodes (values below 60 % are not shown). The *scale bar* represents the number of substitutions per site. Clusters containing more than three species were condensed and given the name of the first species described. Number of species for each group is indicated in *brackets*. Accession numbers are reported for species outside groups

Table 1 Phylogenetic group composition according to Felis and Dellaglio [16] and to the present study

Groups	Felis and Dellaglio [16]	This study
L. delbrueckii	*L. acetotolerans*, *L. acidophilus*, *L. amylolyticus*, *L. amylophilus*, *L. amylotrophicus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii*, *L. fornicalis*, *L. gallinarum*, *L. gasseri*, *L. hamsteri*, *L. helveticus*, *L. iners*, *L. intestinalis*, *L. jensenii*, *L. johnsonii*, *L. kalixensis*, *L. kefiranofaciens*, *L. kitasatonis*, *L. psittaci*, *"L. sobrius"*, *L. ultunensis.*	*L. acetotolerans*, *L. acidophilus*, *L. amylolyticus*, *L. amylophilus*, *L. amylotrophicus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii*, *L. equicursoris*, *L. fornicalis*, *L. gallinarum*, *L. gasseri*, *L. gigeriorum*, *L. hamsteri*, *L. helveticus*, *L. hominis*, *L. iners*, *L. intestinalis*, *L. jensenii*, *L. johnsonii*, *L. kalixensis*, *L. kefiranofaciens*, *L. kitasatonis*, *L. pasteurii*, *L. psittaci*, *L. taiwanensis*, *L. ultunensis*
L. salivarius	*L. acidipiscis*, *L. agilis*, *L. algidus*, *L. animalis*, *L. apodemi*, *L. aviarius*, *L. equi*, *L. mali*, *L. murinus*, *L. nagelii*, *L. ruminis*, *L. saerimneri*, *L. salivarius*, *L. satsumensis*, *L. vini*	*L. acidipiscis*, *L. agilis*, *L. animalis*, *L. apodemi*, *L. aquaticus*, *L. aviarius*, *L. cacaonum*, *L. capillatus*, *L. ceti*, *L. equi*, *L. ghanensis*, *L. hayakitensis*, *L. hordei*, *L. mali*, *L. murinus*, *L. nagelii*, *L. oeni*, *L. pobuzihi*, *L. ruminis*, *L. saerimneri*, *L. salivarius*, *L. sucicola*, *L. satsumensis*, *L. uvarum*, *L. vini*
L. reuteri	*L. antri*, *L. coleohominis*, *L. fermentum*, *L. frumenti*, *L. gastricus*, *L. ingluviei*, *L. mucosae*, *L. oris*, *L. panis*, *L. pontis*, *L. reuteri*, *L. secaliphilus*,*L. vaginalis*	*L. alvi*, *L. antri*, *L. coleohominis*, *L. fermentum*, *L. frumenti*, *L. equigenerosi*, *L. gastricus*, *L. ingluviei*, *L. mucosae*, *L. oris*, *L. panis*, *L. pontis*, *L. reuteri*, *L. secaliphilus*, *L. vaginalis*
L. buchneri	*L. buchneri*, *L. diolivorans*, *L. farraginis*, *L. hilgardii*, *L. kefiri*, *L. parabuchneri*, *L. parafarraginis*, *L. parakefiri*, associated with *L. acidifarinae*, *L. namurensis*, *L. spicheri*, *L. zymae*	*L. buchneri*, *L. dioliovorans*, *L. farraginis*, *L. hilgardii*, *L. kefiri*, *L. kisonensis*, *L. otakiensis*, *L. parabuchneri* *L. parafarraginis*, *L. parakefiri*, *L. rapi*, *L. sunkii*
L. alimentarius	*L. alimentarius*, *L. farciminis*, *"L. kimchii"*, *L. mindensis*, *L. nantensis*, *L. paralimentarius*, *L. tucceti*, *L. versmoldensis*	*L. alimentarius*, *L. crustorum*, *L. farciminis*, *L. futsaii*, *L. kimchiensis*, *L. mindensis*, *L. nantensis*, *L. nodensis*, *L. paralimentarius*, *L. tucceti*, *L. versmoldensis*
L. brevis	*L. brevis*, *L. hammesii*, *L. parabrevis*	*L. acidifarinae*, *L. brevis*, *L. hammesii*, *L. koreensis*, *L. namurensis*, *L. parabrevis*, *L. paucivorans* *L. senmaizukei*, *L. spicheri*, *L. zymae.*
L. collinoides	Associated with *L. manihotivorans*	*L. collinoides*, *L. kimchicus*, *L. odoratitofui*, *L. paracollinoides*, *L. similis*
L. fructivorans	*L. fructivorans*, *L. homohiochii*, *L. lindneri*, *L. sanfranciscensis*	*L. florum*, *L. fructivorans*, *L. homohiochii*, *L. lindneri*, *L. sanfranciscensis*
L. plantarum	*L. plantarum*, *L. paraplantarum*, *L. pentosus*	*L. fabifermentans*, *L. paraplantarum*, *L. pentosus*, *L. plantarum*, *L. xiangfangensis*
L. sakei	*L. curvatus*, *L. fuchuensis*, *L. graminis*, *L. sakei*	*L. curvatus*, *L. fuchuensis*, *L. graminis*, *L. sakei*
L. casei	*L. casei*, *L. paracasei*, *L. rhamnosus*, *"L. zeae"*	*L. casei*, *L. paracasei*, *L. rhamnosus*
L. coryniformis	*L. bifermentans*, *L. coryniformis*, *L. rennini*, associated with *L. composti*	*L. bifermentans*, *L. coryniformis*, *L. rennini*
L. manihotivorans	–	*L. manihotivorans*, *L. nasuensis*, *L. porcinae*
L. perolens	*L. harbinensis*, *L. paracollinoides*, *L. perolens*,	*L. harbinensis*, *L. perolens*, *L. shenzhenensis*
L. vaccinostercus	–	*L. oligofermentans*, *L. suebicus*, *L. vaccinostercus*
Pediococcus	2 clusters, not associated: the first comprises *P. cellicola*, *P. damnosus P. parvulus*, *P. inopinatus*, while the second includes *P. acidilactici*, *P. claussenii*, *P. pentosaceus*, *P. stilesii*	*P. acidilactici*, *P. argentinicus*, *P. cellicola*, *P. claussenii*, *P. damnosus*, *P. ethanolidurans*, *P. inopinatus*, *P. lolii*, *P. parvulus*, *P. pentosaceus*, *P. siamensis*, *P. stilesii*
Couples	*L. rossiae-L. siliginis*	*L. kunkeei-L. ozensis*
	L. vaccinostercus-L. suebicus	*L. rossiae-L. siliginis*
	L. manihotivorans-L. collinoides	*L. concavus-L. dextrinicus*
		L. pantheris-L. thailandensis
Single species	*L. kunkeei*, *L. malefermentans*, *L. pantheris*, *L. harpeae*, *"Paralactobacillus selangorensis"*	*L. algidus*, *L. brantae*, *L. camelliae*, *L. composti*, *L. floricola*, *L. malefermentans*, *L. saniviri*, *L. selangorensis*, *L. senioris*, *L. sharpeae*

Groups are ordered following the number of species included. Novel species with respect to Felis and Dellaglio [16] are underlined. Reclassified species are reported with quotation marks

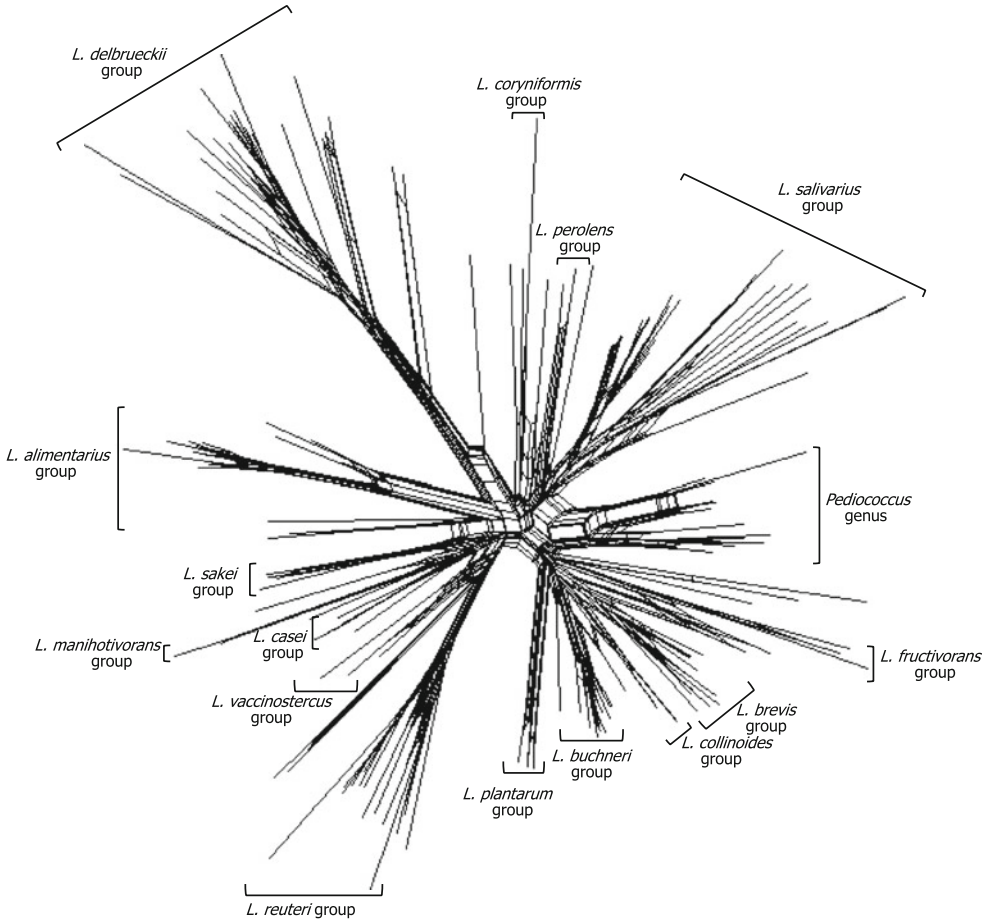

Fig. 2 Split graph resulting from split decomposition analysis of 16S rRNA gene sequences of *Lactobacillus* and *Pediococcus* species. Only group names are indicated

As for the species outside groups, *Lactobacillus rossiae* was always associated with *Lactobacillus siliginis,* while the position of the other species, for example *Lactobacillus algidus*, was variable, depending on the methods used for tree reconstruction (data not shown) and they constituted single lines of descent.

As depicted by the tree, genus *Lactobacillus* is paraphyletic and intermixed with *Pediococcus* species. The present phylogenetic structure confirmed the one inferred by Felis and Dellaglio [16], and it gave evidence that the description of 44 novel species (together with two novel *Pediococcus*) did not lead to a more homogeneous scenario of the genus.

Furthermore, the application of the split decomposition [38] employed to check the consistency of 16S rRNA gene–based tree and refine the phylogenetic analysis revealed the presence of interconnecting networks in the origin of the graph (Fig. 2). This may indicate a complex evolutionary history of genus *Lactobacillus* and, in general, of family *Lactobacillaceae* at 16S rRNA gene sequence level, characterized by more complicated events other than speciation, such as recombination or horizontal gene transfer, occurrence of which was not formally detectable with standard phylogenetic methods. As depicted in Fig. 2, the same phylogenetic groups were still observed, despite the unclear evolution of these bacteria.

The Taxonomic Description of Phylogenetic Groups

For each phylogenetic group, relevant taxonomic data were collected including type of glucose fermentation (which represents the historical subdivision of lactobacilli), GC content, aerobic/anaerobic patterns, cell wall composition, the lactic acid isomer produced and the motility, which are available for the most of the species. Production of ammonia from arginine and acetoin from glucose fermentation were involved together with NaCl tolerance even if for many species these data were not available. Other phenotypic data regarded temperature and pH growth range as well as isolation source.

Lactobacillus delbrueckii Group

Lactobacillus delbrueckii group is composed of 27 species, 5 of them, *L. equicursoris*, *L. gigeriorum*, *L. hominis*, *L. pasteurii* and *L. taiwanensis*, were added with respect to Felis and Dellaglio [16] (Fig. 3.1S). Taxonomically it is the most important phylogenetic group due to the presence of

L. delbrueckii, the type species of the genus, which name *Lactobacillus* is permanently linked to. It contains mainly the obligate homofermenters (20 out of 27), but also some facultative heterofermenters (6 species) and only one obligate heterofermenter. The GC content ranges between 33 and 51 mol%, which may be explained by changes in the codon usages originating from the degeneracy of the genetic code [45]. The majority of the species are characterized by Lys-D-Asp peptidoglycan type. This group is very heterogeneous in terms of lactic acid isomer produced and isolation source (Table 2.1S).

Lactobacillus salivarius Group

Lactobacillus salivarius group comprises 25 species, 11 of them were described in the last few years (*L. aquaticus*, *L. cacaonum*, *L. capillatus*, *L. ceti*, *L. ghanensis*, *L. hayakitensis*, *L. hordei*, *L. oeni*, *L. pobuzihi*, *L. sucicola* and *L. uvarum*) (Fig. 3.2S). Similar to *L. delbrueckii* group, it contains mainly homofermenters (16 out of 25 species) and also facultative heterofermenters (9 species). GC content ranges between 32 and 44 mol%. Each member produces L(+)-lactic acid or both L(+)- and D(−)-lactic acids, but no one produces exclusively the D(−)-isomer. The occurring peptidoglycan types are Lys-D-Asp and meso-Dpm-direct. Interestingly, this group clusters the majority of the motile species of *Lactobacillus* genus (*L. agilis*, *L. aquaticus*, *L. capillatus*, *L. ghanensis*, *L. mali*, *L. nagelii*, *L. oeni*, *L. ruminis*, *L. sucicola*, *L. satsumensis*, *L. uvarum* and *L. vini*) (Table 2.2S).

Lactobacillus reuteri Group

Lactobacillus reuteri group is composed by 15 species, and it is consistent with the previous description with the exception of the addition of two species, *L. alvi* and *L. equigenerosi* (Fig. 3.3S). It is mainly characterized by obligate heterofermentative lactobacilli, except *L. coleohominis* and *L. secaliphilus*, which are facultative heterofermentatives. Its members show a wide range of GC content (38–56 mol%). The peptidoglycan types are Lys-D-Asp, Orn-D-Asp and meso-Dpm-direct. Differently from the majority of the members, *L. frumenti* produces L(+)-lactic acid (Table 2.3S).

Lactobacillus buchneri Group

Lactobacillus buchneri group is composed by 12 species, 4 species were added in the last 5 years (*L. kisonensis*, *L. otakiensis*, *L. rapi*, and *L. sunkii*) (Fig. 3.4S). Similarly to *L. reuteri* group, it mainly contains obligate heterofermenters, and also facultative heterofermentative lactobacilli. GC content is between 38.8 and 42 mol%, and the most occurring peptidoglycan type is Lys-D-Asp. All the members of this group produce both L(+)- and D(−)-lactic acids, with the exception of *L. parakefiri*, which produces only L(+)-lactic acid (Table 2.4S).

Lactobacillus alimentarius Group

Lactobacillus alimentarius group comprises 11 species, 4 species were recently described (*L. crustorum*, *L. futsaii*, *L. kimchiensis* and *L. nodensis*) (Fig. 3.5S). It contains obligate homofermentative and facultative heterofermentative members, and the GC content ranges between 35 and 40 mol%. The peptidoglycan type is mainly Lys-D-Asp, except for *L. tucceti* which contains Lys-Gly-D-Asp type. Interestingly, the majority of the species were isolated from traditional and commercial sourdough (Table 2.5S).

Lactobacillus brevis Group

Lactobacillus brevis group is constituted of 10 species. In Felis and Dellaglio [16], this group comprised only 3 species. According to the phylogenetic analysis conducted in the present study, 4 species (*L. acidifarinae*, *L. namurensis*, *L. spicheri*, and *L. zymae*) were transferred from *L. buchneri* group, while other three species were recently described (*L. koreensis*, *L. paucivorans* and *L. senmaizukei*) (Fig. 3.6S). It contains both facultative and obligate heterofermentative species, and GC content ranges between 46 and 55 mol%. The peptidoglycan type is Lys-D-Asp. *L. koreensis* is the only motile species member of the group. All the species are able to grow between 5 and 10 % of NaCl concentration (Table 2.6S).

Lactobacillus collinoides Group

Lactobacillus collinoides group, formed by 5 species, was defined in the present study thanks to the description of *L. kimchicus*, *L. odoratitofui* and *L. similis* (Fig. 3.7S). Its members are heterofermentative, and their GC content is between 39.7 and 48.5 mol%. All of them are able to form D(−)-lactic acid and 3 species produce also the L(+) isomer (Table 2.7S).

Lactobacillus fructivorans Group

Lactobacillus fructivorans group is composed of 5 species, and it is consistent with its former definition, with the only exception of the description of *L. florum* (Fig. 3.8S). It contains mainly obligate heterofermenters (only *L. homohiochii* is facultative heterofermentative). GC content is between 35 and 42 mol% and peptidoglycan types are Lys-D-Asp and Lys-Ala (Table 2.8S).

Lactobacillus plantarum Group

Lactobacillus plantarum group comprises 5 species, of which 2 species, *L. fabifermentans* and *L. xiangfangensis*, were described in 2009 and 2011, respectively (Fig. 3.9S). This group is very homogeneous in terms of metabolic features, since members are facultative heterofermentative, and GC content, which ranges between 44 and 47 mol%. The peptidoglycan type is meso-Dpm-direct (Table 2.9S).

Lactobacillus sakei Group

Lactobacillus sakei group comprises 4 species, and it is consistent with its former description (Fig. 3.10S). All species are facultative heterofermentative, and their GC content is between 41 and 44 mol%. They produce both D(−)- and L(+)-lactic acids with the exception of *L. fuchuensis*, which forms only L(+)-lactic acid. This group is heterogeneous in terms of production of ammonia from arginine and acetoin (Table 2.10S).

Lactobacillus casei Group

Lactobacillus casei group consists of 3 species (Fig. 3.11S). This group is homogenous since its members are facultative heterofermentative, their GC content ranges between 45 and 47 mol% and the occurring peptidoglycan type is Lys-D-Asp. Furthermore, they are able to produce acetoin and all of them form L(+)-lactic acid (Table 2.11S).

Lactobacillus coryniformis Group

Lactobacillus coryniformis group is composed by 3 members (Fig. 3.12S). In the previous survey, this group comprised also *L. composti*, which now constitutes a single line of descent, according to the updated phylogenetic analysis. This group is homogeneous since its members are facultative heterofermentative, the GC content is 45 mol%, the occurring peptidoglycan type is Lys-D-Asp and both D(−)- and L(+)-lactic acid isomers are produced. None of them produce ammonia from arginine (Table 2.12S).

Lactobacillus manihotivorans Group

Lactobacillus manihotivorans group was defined for the first time in the present study thanks to the description of *L. nasuensis* and *L. porcinae* (Fig. 3.13S). They are homofermentative and characterized by a GC content between 47.6 and 59.2 mol%. Differently from *L. manihotivorans* and *L. nasuensis*, *L. porcinae* produces only L(+)-lactic acid. Interestingly, *L. nasuensis* is the only species able to grow at pH 8 (Table 2.13S).

Lactobacillus perolens Group

Lactobacillus perolens group is composed by *L. harbinensis*, *L. perolens* and the novel species *L. shenzhenensis* (Fig. 3.14S). *L. harbinensis* and *L. perolens* are facultative heterofermentative and produce only L(+)-lactic acid, while *L. shenzhenensis* is obligate heterofermentative. The GC content ranges from 45 up to 56 mol% (Table 2.14S).

Lactobacillus vaccinostercus Group

Lactobacillus vaccinostercus group is established according to the current phylogenetic analysis, and it is composed by 3 species (Fig. 3.15S). All of them are obligate heterofermentative, their GC content is between 35.3 and 41 mol% and the peptidoglycan type is meso-Dpm-direct (Table 2.15S).

Species Outside Groups

As for species outside the groups, they constitute 4 couples (Table 2.16S) and 10 single lines of descent (Table 2.17S). The more stable association is the one composed by *L. rossiae* and *L. siliginis*: both of them are obligate heterofermentative, the GC content is between 44.6 and 45.5 and they were isolated from wheat sourdough.

Discussion

Taxonomic analysis of probiotic strains is necessary for both basic research and in applied context since the reliable identification at the species level and correct naming are the basis for safety assessment, quality assurance and non-fraudulent labelling [7, 26, 27, 46, 47]. Taxonomic update of genera including probiotic species such as *Lactobacillus* is periodically required in order to avoid confusion due to nomenclatural modifications and renamings and to lead to an appropriate species designation of probiotic products [27, 46].

The taxonomic analysis of *Lactobacillus* genus and, in general, of the family *Lactobacillaceae*, based on the comparative analysis of 16 rRNA gene sequence, confirmed the heterogeneity of the genus, which was intermixed with *Pediococcus* members, and characterized by a complex evolutionary history. The paraphyletic structure of genus *Lactobacillus* shown by 16S rRNA gene–based phylogeny and the evolutionary analysis conducted in this study gave evidence that the taxonomy of lactobacilli is still far from being adequate and well established.

The majority of *Lactobacillus* species was found to be included in 15 robust subgeneric groups, 3 more than those

detected in the last survey of *Lactobacillus* genus. Notably, species belonging to the same cluster did not always share the same phenotypic properties. In fact, the collection of relevant taxonomic data revealed that the previously as well as the newly defined groups are phenotypically heterogeneous regarding the GC content, the isomer of lactic acid produced, the type of peptidoglycan in the cell wall and, most importantly, the metabolic profile, defined in terms of types of fermented sugars and fermentation end-products, upon which traditional taxonomic analysis is based.

The inconsistency between phylogenetic and phenotypic data is the key point that promotes the integration of genome data in the taxonomic analysis of *Lactobacillus* genus in order to find determinable characters which correlate with the phylogenetically based grouping (our unpublished results). Data derived from entire genome sequences are assumed to solve the inconsistency for each group and lead to a phylogenetic consolidation of lactobacilli with the potential emergence of new and more homogenous genera.

As for routine analysis and molecular identification of lactobacilli, in the genus *Lactobacillus* many species share more than 97 % and even 98.8 % [28] 16S rRNA gene sequence identity: for example, many species of *L. delbrueckii* group as well as *L. casei* and *L. plantarum* groups share a 16S rRNA gene sequence identity higher that those conventional cutoffs. Therefore, as previously indicated [27], scientists, probiotic producers and regulatory bodies should carefully evaluate each case and refer to the appropriate literature to determine species identity and strain differentiation within each species group.

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