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Taxonomic *Lactobacillus* Composition of Feces from Human Newborns during the First Few Days

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Abstract

Intestinal microbiota comprise a complex ecosystem whose equilibrium is crucial for the health of animal species. For humans, data exist on the microbiota composition in adult subjects, but few studies have addressed the microbiota composition in infants. In particular, data on the presence and species distribution of members of the genus *Lactobacillus* in newborns (less than one week old) are lacking. In the present work, the feces of healthy newborns were sampled to determine the taxonomic composition of *Lactobacillus* in the intestinal microbiota in a group of 16 neonates. In total, 1640 colony-forming units (CFU) were isolated, of which 420 grouped in the *Lactobacillus* genus by means of primary phenotypic characterization. The 420 isolates were further grouped into 125 strains on the basis of identical plasmid profiles. Of these 125 strains, 21 turned out to be permanent, *i.e.,* they were identified in the feces of the same subject on several consecutive days. Sugar fermentation, DNA/DNA hybridization, and S-layer protein determination enabled us to classify 52 of the 125 strains as follows: *L. paracasei* (40 strains), *L. delbrueckii* sp. (1 strain), and *L. acidophilus* (*sensu stricto*) (11 strains). Based on the same criteria, the remaining 73 strains were tentatively allotted to the Johnson subgroup B, although hybridization experiments with probes specific for *L. gasseri* and *L. johnsonii* species were not performed. The presence of new species among these 73 strains cannot be excluded. Surprisingly, the obligately heterofermentative lactobacilli, *L. reuteri* in particular, were entirely absent from the feces of healthy newborns.

Introduction

The human intestinal microbiota form a complex ecosystem in which more than 400 different bacterial species are currently known to coexist (for reviews, see [8, 10, 11, 12, 20, 21, 25]). Among intestinal bacterial species, lactobacilli are considered particularly important for the maintenance of good health, although they are not the predominant bacteria [20].

Lactobacilli of the human intestinal tract may be divided into three major groups, according to the metabolic end-products they produce. The first group is formed by obligately homofermentative lactobacilli. Previously, species of

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this group were mainly allotted to the *L. acidophilus* species. Now, however, they are classified into six distinct species and clustered according to Johnson [16] into two subgroups, namely Johnson subgroup A, composed of *L. acidophilus* (*sensu stricto*), *L. crispatus, L. amylovorus,* and *L. gallinarum;* and Johnson subgroup B, composed of *L. gasseri* [19] and *L. johnsonii* [13]. Strains of Johnson subgroup A are covered by S-layer proteins, and those of subgroup B lack this envelope [4, 5]. The second enteric *Lactobacillus* group frequently found among the enteric isolates is formed by facultatively heterofermentative strains. Because of their phenotypic resemblance to the *L. casei* type strain, they were all classified as such in the past. By using molecular biological techniques, these strains have now been classified into three species: *L. casei, L. paracasei* and *L. rhamnosus.* The third group of enteric lactobacilli is formed by obligately heterofermentative strains. These were all originally ascribed to the *L. fermentum* species, but now also comprise the *L. reuteri* species. The *L. paracasei* sbsp *L. pseudoplantarum, L. plantarum, L. salivarius,* and *L. delbrueckii* species have also occasionally been isolated [11].

The presence of lactobacilli in human babies is less documented than in adults. Conway [10] recently remarked that data to estimate the number of lactobacilli in week-old babies are insufficient. In addition, little is known about species distribution of the lactobacilli in neonates [2, 10]. The present work aimed to take an inventory and preliminary census of enteric *Lactobacillus* species in a group of healthy human neonates, born in the same hospital, over a 6-month period. To this end, a culture collection was obtained of lactobacilli isolated from the feces during the first days after birth. Strains were characterized by means of species-specific oligonucleotide probes and by checking for the presence of S-layer proteins, as well as by classical taxonomic tests.

Materials and Methods

Reference Strains

The following were used as reference type strains: *L. delbrueckii* sbsp *lactis* (DSM 20072), *L. casei* (ATCC 393), *L. paracasei* (ATCC 25302); Johnson subgroup A species: *L. acidophilus* (*sensu stricto,* ATCC 4356), *L. crispatus* (DSM 20584), *L. amylovorus* (DSM 20531), *L. gallinarum* (DSM 33199); and Johnson subgroup B species: *L. gasseri* (DSM 20243) and, *L. johnsonii* (ATCC 33200).

Isolation and Primary Characterization of Bacterial Isolates

Bacteria were isolated from fecal samples obtained from 16 neonates born at the Piacenza Hospital (Italy), over a 6-month period. Neonates were identified by a 4-letter code (Table 1). A two-capital-letter code, where the first letter indicates the sex (M/F), identifies each subject. The lower case letters signify the type of delivery (c = caesarean section, n = natural route) and of feeding (b = breast, f = formula), respectively. Specimens were taken, with parental consent, twice a day for the 1st 6 days of life, and kept in sterile swabs under anaerobic conditions. Selective primary isolation of lactobacilli was performed on LBS (*Lactobacillus* selection) agar (BBL, Cockeysville, MD, US). Plates were incubated under anaerobic conditions (Gas Pack system, BBL, Cockeysville, MD, US) at 37°C for 48 h. The resulting colony-forming units (CFU) were propagated in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, England), and then re-isolated by streaking a loopful of culture onto MRS agar plates.

Purification of the isolates by the above procedure was followed by primary phenotypic characterization, which enabled identification of those isolates belonging to the *Lactobacillus* genus. The following phenotypic characteristics were examined: morphology (optical examination by phase-contrast microscopy), reaction to Gram staining, catalase activity, and CO_2 production (evaluated by means of Durham bell [17]). The concentration of the two stereoisomers of lactic acid produced in MRS broth after 24 h of incubation, at 37°C, was determined by enzymatic assay (Kit No. 1,112,821, Boehringer Mannheim, Germany).

Grouping the Isolates

After characterization, the isolates were grouped according to their plasmid profile. Plasmid profiles were obtained from the isolates after alkaline extraction [30]. The conditions for agarose gel electrophoresis and plasmid DNA staining were the standard ones described by Sambrook et al. [24].

Taxonomic Identification of the Strains

Sugar fermentation patterns: Fermentation patterns of the strains were determined by using standard carbohydrate galleries (API 50 CH strip, Cat. No. 50300, Biomérieux SA, Lyon, France) in API 50 CHL medium (Cat. No. 50410, Biomérieux SA, Lyon, France), according to the manufacturer's instructions.

DNA/DNA probe hybridization: Total genomic DNA was isolated from the strains according to the method of Sambrook et al. [24], and deposited onto a positively charged nylon membrane (Boehringer Mannheim, Germany) [26]. Hybridization was carried out using DNA probes targeted to rRNA coding regions on bacterial DNA. The probes are species-specific for *L. casei* type strain (ATCC 393), *L. paracasei* type strain (ATCC 25302) [26], *L. acidophilus* (*sensu stricto*) type strain (ATCC 4356) [22]), and for the subspecies *L. delbrueckii* [15]. Hybridization and probe detection were achieved using digoxigenine-labeled oligonucleotides, according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Presence of S-layer proteins: S-layer proteins of obligately homo-

Table 1. Grouping of isolates on the basis of plasmid profile. Bold face numbers relate to permanent strains

	L(+)-lactic acid producers				DL-lactic acid producers			
	Group strains		Single strains[b]		Group strains		Single strains[b]	
Subject/ conditions[a]	No. of groups	Numerosity of groups	Plasmid present	Plasmid absent	No. of groups	Numerosity of groups	Plasmid present	Plasmid absent
FAcb[c]	0	0	3	0	1	2	2	1
MAnb	2	**7;** 3	2	0	3	**12;** 3; 2	0	1
FBnb	2	2; 2	1	0	5	**8;** 4; 3; 2; 2	1	0
MBnb	0	0	2	0	4	**6;** 4; 2; 2	1	0
FCcf[c]	0	0	1	0	1	2	1	0
MCcf[c]	2	2; 2	0	0	2	3; 2	0	1
FDcf	3	2; 2; 2	1	0	5	**11;** 4; 2; 2; 2	0	0
MDcf	3	2; 2; 2	1	0	5	**15;** 10; **8;** 2; 2	1	2
FEnb	2	**6;** 2	0	0	4	**25; 10;** 2; 2	0	1
MEcf	2	2; 2	1	0	7	**13;** 4; 4; 3; 3; 2; 2	0	2
MFcb	2	2; 2	0	0	2	**9;** 2	1	0
MGcf	1	**7**	1	0	5	**14; 12;** 2; 2; 2	0	0
MHcb[c]	1	2	1	0	2	2; 2	1	1
MInb	2	2; 2	1	0	7	**12; 10;** 3; 3; 2; 2; 2	1	1
MJnf	1	2	0	0	7	**11; 10; 8;** 2; 2; 2; 2	1	2
MKnb	2	2; 2	0	0	2	**10;** 2	0	0
Total	25	65	15	0	62	317	10	12

[a] Subjects were identified by a four-letter code. A two-capital-letter code, in which the first letter indicates sex (M/F), identifies each subject. The lower-case letters signify the type of delivery (c = caesarean section, n = natural route) and of feeding (b = breast, f = formula), respectively

[b] Strains that could not be grouped because of either a unique plasmid profile or plasmid absence

[c] Sampling was not completed in these subjects because of the onset of illnesses requiring antibiotic therapy

fermentative lactobacilli were extracted according to previously described procedures [6]. S-layer protein A (SlpA) extracted from *L. acidophilus* (ATCC 4356) [5] was used as a positive control. The Johnson subgroup B species *L. gasseri* (DSM 20243) and *L. johnsonii* (ATCC 33200) served as negative controls. Briefly, a bacterial pellet was obtained by centrifugation of 1 liter of an overnight culture of the *Lactobacillus* strain of interest. The pellet was resuspended in 50 ml of 6 M LiCl. The S-layer protein was extracted from the cell envelope by gentle agitation of the suspension for 1 h at 4°C. The suspension was centrifuged (10 min at 6000 × *g*). The supernatant was dialysed overnight, at 4°C, against at least three changes of sterile water. Particles formed during this step were centrifuged (15 min at 3000 × *g*). The pellet was then resuspended in 10 ml of 6 M LiCl, and, again, dialysed overnight. This procedure was repeated 4 times, until a single band of SlpA monomers was detected in the positive control by sodium dodecylsulphate polyacrylamide-gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis was performed according to Laemmli [18], using a stacking gel containing 3% acrylamide and a running gel containing 12% acrylamide. The percentage of bisacrylamide was 0.8%. Samples were resuspended in 100 µl of water and 20 µl of solubilizing solution (300 µl of β-mercaptoethanol, 4 ml of glycerol, 1 ml of 20% SDS, 1 ml of 0.1 M Tris/0.02 M EDTA, pH 8) at room temperature; 1 µl of 20% (w/v) bromophenol-blue in water was then added. SDS-PAGE gels were stained with Coomassie Brilliant Blue G-250.

Statistical Analysis

The 95% confidence interval for the proportion of L(+)-lactic acid producers over total number of isolates was calculated by proportionally weighing the number of isolates for each subject [9].

Results

Isolation and Primary Characterization of Bacterial Isolates

Bacterial isolates were obtained from fecal samples taken from 16 newborns (Table 1) during their first 6 days of life. Due to the development of illnesses that required antibiotic therapy, sampling from four infants was interrupted. In all, 205 fecal samples were taken, from which 1640 CFU were obtained on the LBS medium selective for lactobacilli. Fig. 1 illustrates the process by which the isolates were classified into strains.

Primary phenotypic characterization led to the assignment of 420 isolates to the *Lactobacillus* genus. The remaining 1220 CFU had coccic or bifidus-like morphology, produced catalase, or had some other characteristics not compatible with those of the *Lactobacillus* genus (*e.g.,* a marked motility) [17]. Under the adopted conditions, none of the

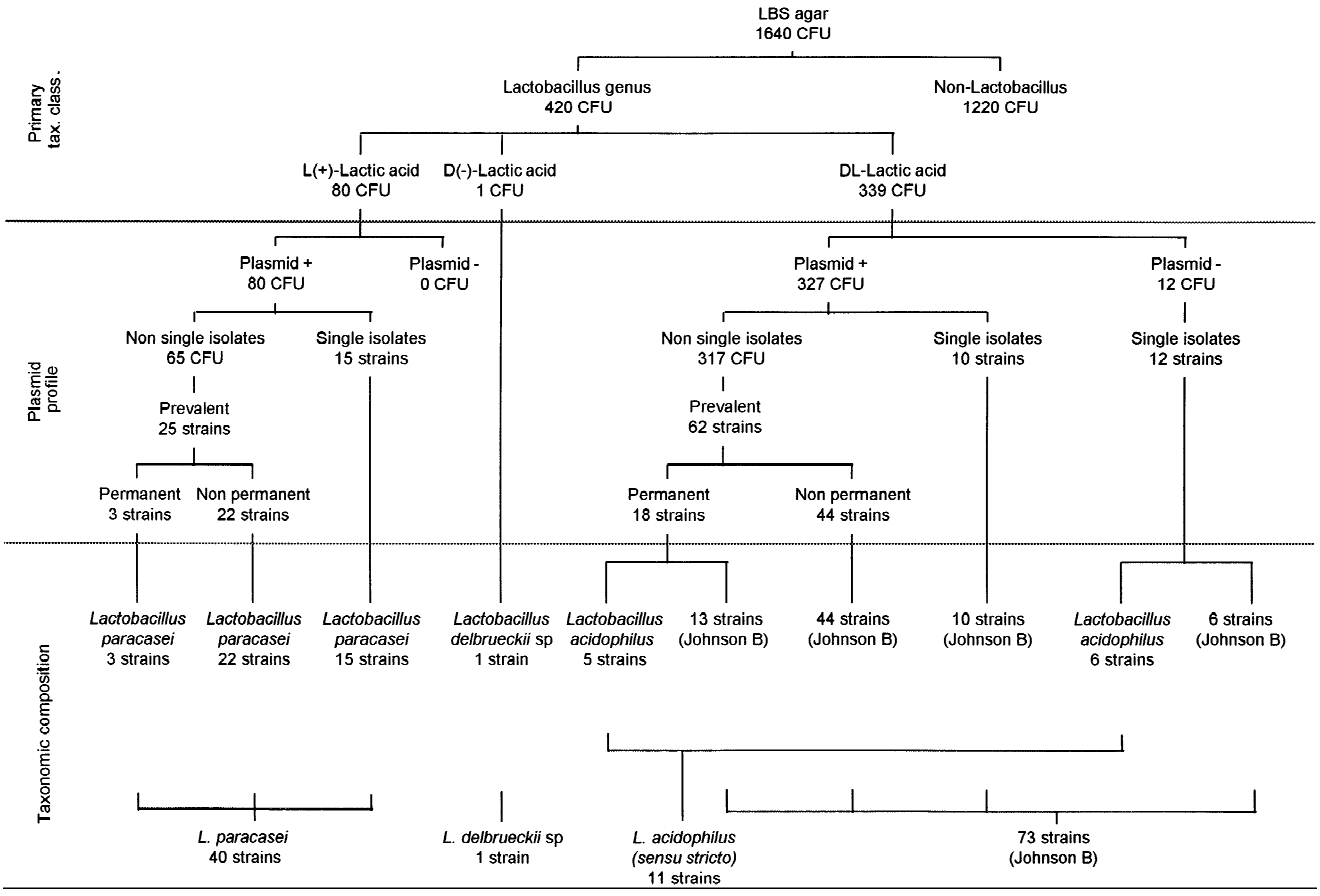

Fig. 1. Schematic representation of the process followed for classifying the isolates into strains, together with their final taxonomic location. The 73 strains labeled as Johnson B in parentheses were tentatively allotted to Johnson subgroup B, because they produced DL lactic acid, did not hybridize with *L. acidophilus* probes, and lacked S-layer proteins.

420 isolates was able to carry out heterofermentation leading to the production of carbon dioxide from glucose; *i.e.*, they were all facultatively heterofermentative or obligately homofermentative.

Of the 420 isolates, 80 isolates produced only the L(+)-lactic acid isomer, 1 isolate (from subject FDcf) produced only the D(−) form, and the remaining 339 isolates produced racemic lactic acid. The distribution of isolates producing different forms of lactic acid was essentially the same for the different subjects, as confirmed by the small amplitude (from 0.15 to 0.23) of the 95% confidence interval for the proportion of L(+)-lactic acid producers over total number of isolates.

Grouping the Isolates

Plasmid profiling was performed on all isolates phenotypically allotted to the *Lactobacillus* genus, with the exception of the D(−)-lactic acid-producing isolate. This isolate, found only once, was not subjected to the identification process adopted for the other isolates; rather, its taxonomic classification was determined by DNA/DNA hybridization (see below). Plasmid profile analysis was performed to identify those isolates lacking plasmids (Fig. 2, lanes 4 and 5), those having a unique plasmid pattern (Fig. 2, lane 7), and those showing identical plasmid patterns (Fig. 2, lanes 2, 3, and 6, 8). The number of plasmids harbored by each isolate ranged from zero to eight, with estimated molecular sizes ranging from 2 kb up to ~92 kb. Surprisingly, no plasmid-free isolates were present among the L(+)-lactic acid producers, and only 12 were present among the racemic (DL) producers.

Different isolates from the same donor, showing identical plasmid patterns, were recognized as belonging to the same strain. Such strains were defined as "prevalent strains." The plasmid patterns of isolates from different donors were

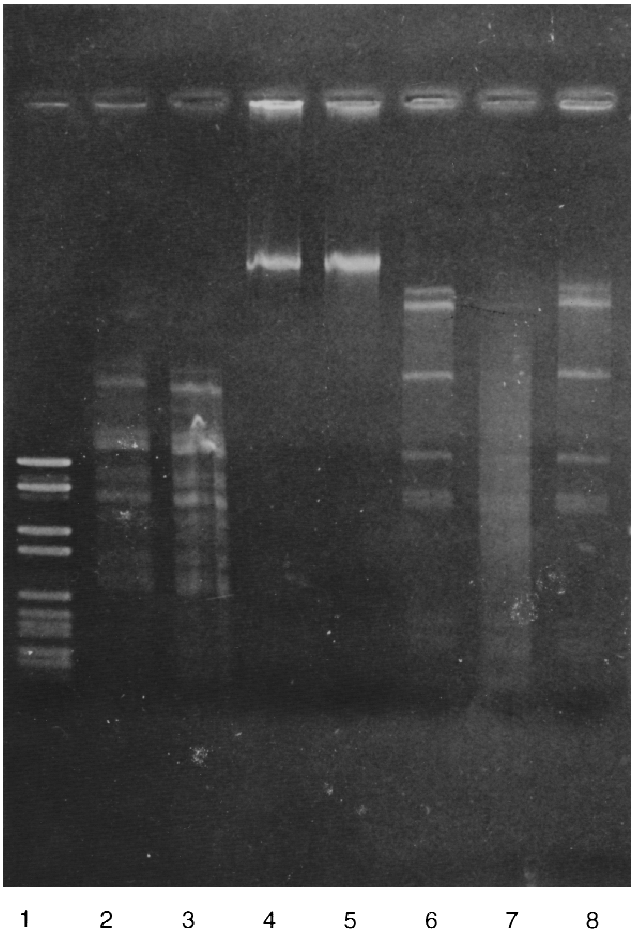

Fig. 2. Plasmid profile of 7 isolates from a single subject (MDcf). Lane 1, reference standards (Gibco Rrl Supercoiled DNA ladder, Cat. 15622-022). Lanes 2 and 3 show two isolates with identical plasmid profile, *i.e.,* belonging to the same strain. The same is true for lanes 6 and 8. In lanes 4 and 5 are two plasmid-free isolates. Lane 7 shows the plasmid profile of a different isolate.

never the same. Thus, plasmid-containing isolates occurring only once were termed ''single strains.'' The presence of plasmids allowed the grouping of the 80 isolates that produced L(+)-lactic acid in 25 prevalent strains and 15 single strains; the 327 DL producers were likewise grouped in 62 prevalent strains and 10 single strains.

Of the prevalent strains, those isolated from fecal samples taken on several consecutive days, from the same subject, were interpreted as being ''permanent strains,'' analogous to previous reports [23]. Three permanent strains were found among the 25 L(+) producers, and 18 among the 62 DL–lactic acid producing strains (Table 2, Fig. 1). By comparing these numbers with the corresponding ones for nonpermanent prevalent strains, (*i.e.* 22 and 44, respectively), and by considering, also, that all single strains are, by definition, nonpermanent, one must conclude that permanent strains (3 + 18 = 21) are clearly in the minority, compared to nonpermanent ones (22 + 44 + 15 + 10 = 91). There are no reasons to believe that plasmid-free strains would distribute differently, although evidence for this could not be obtained in this study.

Table 2. Number of isolates belonging to the same permanent strain in the various subjects[a]

Subject	L(+)	DL
FAcb	n.d.	n.d.
MAnb	**7**(3)	**12**(3)
FBnb	n.d.	**8**(2)
MBnb	n.d.	**6**(2)
FCcf	n.d.	n.d.
MCcf	n.d.	n.d.
FDcf	n.d.	**11**(2)
MDcf	n.d.	**15**(3)/**8**(2)
FEnb	**6**(2)	**25**(5)/**10**(2)
MEcf	n.d.	**13**(3)
MFcb	n.d.	**9**(2)
MGcf	**7**(2)	**14**(3)/**12**(3)
MHcb	n.d.	n.d.
MInb	n.d.	**12**(2)/**10**(3)
MJnf	n.d.	**11**(2)/**10**(2)/**8**(3)
MKnb	n.d.	**10**(4)

[a] The number of consecutive days in which it was possible to identify isolates with identical plasmid profiles from the same subject is indicated in parentheses
n.d., not detected

Nonpermanent prevalent strains were usually isolated two to four times, with the exception of a strain from subject MDcf, which occurred ten times. In contrast, permanent prevalent strains were isolated from 6 (minimum) to 25 (maximum) times.

Permanent strains were found in each of the 12 subjects in which it was possible to carry out the full sampling program, but not in the four subjects in which sampling had to be interrupted. The longest permanence of one of these strains was observed in subject FEnb. The strain was isolated on 5 consecutive days and belonged to the group of racemic lactic acid producers (Table 2). The simultaneous presence of up to 3 distinct permanent strains was observed in subject MJnf.

Taxonomic Identification of the Strains

One isolate of each strain, including those without plasmids, was examined for sugar fermentation pattern. All L(+)-lactic

acid producing strains were able to use pentoses as a source of carbon, suggesting that these strains belong to the *L. casei/paracasei/rhamnosus* group. Pentose fermentation enabled exclusion of the *L. salivarius* species. Since none of the L(+) producers was able to ferment rhamnose, the presence of the *L. rhamnosus* species also was excluded. In contrast, none of the DL-lactic acid producers was able to ferment pentoses, indicating, therefore, that the species *L. paracasei* sbsp. *pseudoplantarum* and *L. plantarum* were absent.

The strains were further characterized by DNA/DNA probe hybridization. All L(+)–lactic acid producers were found to belong to the *L. paracasei* species. Included in this group were the permanent strains isolated from subjects MAnb, MGcf, and FEnb.

The D(−)-lactic acid-producing strain was allotted to the *L. delbrueckii* group by means of a positive hybridization with a 23S rRNA gene targeted probe [15]. The strains that produced DL-lactic acid were targeted with the hybridization probe specific for *L. acidophilus* (*sensu stricto*) [22]. Of the 84 strains, only 11 could be classified as *L. acidophilus* (*sensu stricto*). Of these, five were permanent strains: they were isolated from subjects MAnb, MGcf (group of 12 isolates), MJnf (group of 8 isolates), FEnb (group of 10 isolates), and MKnb (Table 2). The remaining six *L. acidophilus* (*sensu stricto*) strains were all found among the plasmid-free strains. None of the DL-lactic acid-producing single strains was allotted to this species. It is noteworthy that no strains belonging to the *L. acidophilus* (*sensu stricto*) species were found among prevalent nonpermanent strains.

Detection of S-layer proteins by SDS-PAGE confirmed the presence of such proteins in all the 11 *L. acidophilus* (*sensu stricto*) strains (Fig. 3). In contrast, none of the remaining 73 strains, which produced DL-lactic acid, but were unable to ferment pentoses or to hybridize with the specific *L. acidophilus* (*sensu stricto*) probe, showed the presence of S-layer proteins. Thus, these strains were tentatively assigned to Johnson subgroup B.

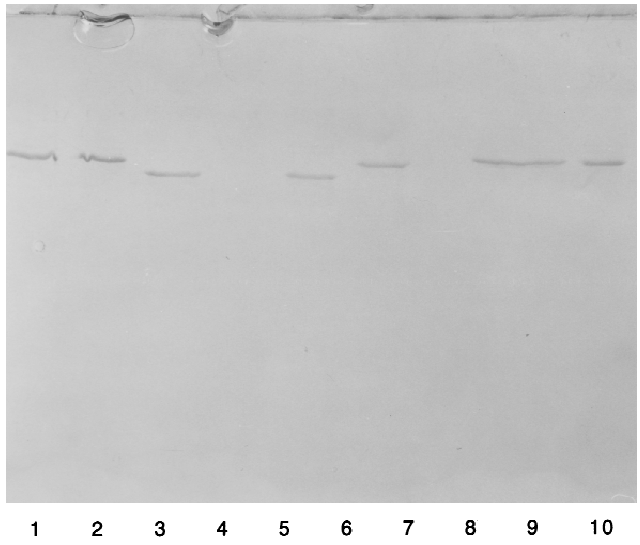

Fig. 3. SDS-PAGE analysis of LiCl extraction products from isolates producing racemic lactic acid. Lanes 4 and 7 were loaded with samples obtained from isolates lacking the S-layer protein and presumably belonging to the B group of Johnson. The other lanes were loaded with samples of isolates that hybridized with the oligonucleotide probe specific for *L. acidophilus* (*sensu stricto*). Lane 10 represents the positive control (*L. acidophilus* ATCC 4356).

Discussion

Very few data are available about the *Lactobacillus* species of the intestinal microbiota of newborns in their first week of life. Benno and Mitsuoka [1] reported that only *L. gasseri* was identified in 3 of 10 neonates during the first week of life. Consequently, a broad comparison was performed between our results and available data, including those regarding adult microbiota.

Among the *Lactobacillus* strains we have studied, no obligately heterofermentative lactobacilli were found. The absence of these species was surprising, because they have frequently been observed in fecal samples of both human and animal species [28]. In particular, *L. reuteri* has been indicated as a constant major component of animal (human included) intestine [21].

The facultative heterofermentative lactobacilli were numerically well-represented (40 strains); but no species variability was found, because they all belonged to the *L. paracasei* species. This group thus constituted one of the major components in the *Lactobacillus* population of our pool of subjects.

Of the homofermentative lactobacilli (84 strains), only 11 strains could be allotted to Johnson subgroup A and, in particular, to the *L. acidophilus* (*sensu stricto*) species. The *L. crispatus* species was absent. Although this species is considered by Mitsuoka [21] to be dominant in the human intestine, of the 13 *L. crispatus* strains identified by Johnson *et al.* [16] none was of human enteric origin. Results of other investigators [7, 14] seem to further contradict Mitsuoka's observation. Our data are consistent with the hypothesis that *L. crispatus* does not represent a major component of the human intestinal microbiota, at least in neonates. The remaining 73 strains probably belonged to Johnson subgroup

B, which comprises the two species now referred to as *L. gasseri* [19] and *L. johnsonii* [13]. Because *L. gasseri* is considered a dominant species in the human intestine [3, 21, 29], while *L. johnsonii* is only occasionally recovered [21], it is likely that many of these strains belong to the *L. gasseri* species. However, because further characterization of the strains to confirm this conclusion was not performed, it is also possible that they include unknown species.

In our screening process, plasmid profiling enabled us to exclude phenomena of cross contamination between the various subjects; in fact, in no subject was an isolate found that was identical to one identified in another of the subjects under examination. This also helped to rule out the environmental origin of the *Lactobacillus* population in our sample. We relied on a common method to group isolates belonging to the same strain, and to identify "permanent" strains—those that were present for several consecutive days in a particular subject. Twenty-one permanent strains were found. These strains should be considered able to colonize the intestinal environment, and, therefore, appear promising for the preparation of probiotic products.

Consistent with the hypothesis that the presence of permanent strains has a protective effect against pathogens, permanent strains were missing in all those subjects that developed illnesses. Conversely, permanent strains in newborns who remained healthy were identified from the earliest sampling. Of considerable interest is also the observation that the 4 subjects that developed illnesses were all born by means of caesarean section. From our limited set of data, however, neither the mode of delivery, nor the type of feeding, appear to determine the lactobacilli microbiota composition. This agrees with the findings of Simhon *et al.* [27].

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