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Dominant cultivable *Lactobacillus* species from the feces of healthy adults in northern Spain

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Summary. The aim of this study was to identify numerically dominant cultivable lactobacilli species in the feces of healthy adults. Ten individuals from Asturias, northern Spain, were chosen. Bacterial colonies grown under anoxic conditions on MRS with cysteine were microscopically examined for lactobacilli. Isolates were subsequently grouped based on the analysis of their carbohydrate fermentation profiles and then identified by partial amplification, sequencing, and comparison of their 16S rRNA gene sequences. Lactobacilli varied from undetectable levels in three subjects ($<10^5$ CFU/g feces) to around 10^9 CFU/g feces. Among the 71 isolates obtained from seven individuals, 12 *Lactobacillus* species were identified. High inter-individual variation was observed in terms of total numbers, number of species, and dominant species. *Lactobacillus paracasei* was found in four of the seven individuals; *L. gasseri, L. delbrueckii*, and *L. plantarum* in three. Phenotyping showed that only one strain per species was in the majority in each individual. [**Int Microbiol** 2007; 10(2):141-145]

Key words: *Lactobacillus* spp. · *L. paracasei, L. gasseri, L. delbrueckii, L. plantarum* · gastrointestinal tract · intestinal microbiology · human microbiota · probiotics

Introduction

The *Lactobacillus* group represents only 1–6% of the intestinal microbiota of humans and they are not equally distributed along the gastrointestinal tract (GIT) [11,28]. However, these bacteria are essential to the maintenance and restoration of human health through their metabolic, trophic, and protective functions [5,19]. Owing to their ability to colonize the GIT and their long history of safe consumption, mostly in traditionally fermented dairy products, *Lactobacillus* species were among the very earliest probiotics [5,19,20]. The beneficial effects attributed to indigenous and probiotic lactobacilli arise from their colonization of the intestinal and genital mucosa [6,24], resulting in inhibition of pathogens [2,9], immunomodulation [15,17,25], and uptake and assimilation of cholesterol [18]. Nonetheless, despite their frequency and importance, detailed information on the species and strain compositions of lactobacillus populations from the human GIT is scarce, and our knowledge of the dominant species in most human communities is still vague.

In pioneer culturing studies, *Lactobacillus acidophilus, L. paracasei, L. crispatus, L. delbrueckii, L. gasseri, L. plantarum, L. reuteri, L. rhamnosus*, and *L. salivarius* were frequently reported as constituents of the intestinal microbiota [14,21]. The recent development of culture-independent molecular techniques has enabled more precise detection, identification, and quantification of fecal [8,12,26] and mucosa-associated [13] lactobacilli populations. These results have confirmed that, as in many other intestinal populations [4], there is wide inter-individual variability (both in culture and molecular studies). Species and strain compositions may vary along the GIT [11,28], over the human life cycle [8,22], and with the food habits of the community [7,14].

The aim of this work was to identify the numerically predominant cultivable lactobacilli species in the feces of healthy adults. Ten individuals from our region, Asturias,

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northern Spain, were selected for the sutdy. This work provides the first step in the design, use, and evaluation of probiotics. Furthermore, intestinal lactobacilli strains have been collected that will be assessed regarding their probiotic potential by subjecting them to a series of in vitro analyses.

Materials and methods

Sampling and processing of samples. Sample donors were selected and sampling was carried out as recommended by the Regional Ethics Committee (Principality of Asturias, Spain). The study subjects were four male and six female volunteers (mean age 45 years; range 34–66) who considered themselves healthy and who had no recent history of antibiotic treatment. Fecal samples were collected in sterile containers and transported to the laboratory in anoxic jars containing Anaerocult A (reducing agent) (Merck, Darmstadt, Germany). Samples were processed in an anoxic chamber (atmosphere 10% H_2, 10% CO_2 and 80% N_2) within 2 h of their arrival (Mac500, Down Whitley Scientific, West Yorkshire, UK).

Cultivable microbial counts. From each individual, between two and ten fecal samples were homogenized in a reducing medium containing brain-heart infusion (BHI) broth (Merck), 0.5% glucose, 0.5% yeast extract (Merck), 0.25% cysteine (Merck), 10 mg/l vitamin K_1 (Merck), and 0.02 g/l hemin (Sigma, St. Louis, MO, USA). Serial dilutions in the same medium were plated in duplicate on de Man, Rogosa, and Sharpe (MRS) agar (Merck) containing 0.25% (w/v) cysteine (MRSC) (Merck). Plates were then incubated in an anoxic chamber at 37ºC for 72 h. Random colonies were examined microscopically and the viable number of lactobacilli was calculatedas colony forming units (CFU).

Selection and preservation of isolates. After microscopy, between five and 22 colonies representative of all morphotypes were selected from one fecal sample of each individual (i.e., from the MRSC plates). Of the 78 colonies, 71 isolates with bacillary morphology were recovered, purified by sub-culturing in MRSC, and kept at –70°C in the same medium with 20% (v/v) glycerol (Merck) until use.

Phenotyping of the isolates. The isolates were phenotyped using a miniaturized carbohydrate-fermentation system (Phene-Plate system, Stockholm, Sweden) that assays the metabolism of 22 sugars and polyalcohols, namely, amygdalin, arabinose, arbutin, cellobiose, galactose, gluconate, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, palatinose, rhamnose, salicine, sorbitol, sorbose, sucrose, tagatose, trehalose, and xylose. A data matrix for the carbohydrate fermentation profiles was constructed and matches were sought using the Single Matching coefficient test (MVSP software; Kovach Computing Services, Anglesey, UK). Clustering was done using the unweighted pair group method with arithmetic averages (UPGMA).

Molecular identification: preparation of cell-free extracts, PCR amplification, and purification and sequencing of amplicons. Isolates were identified by partial amplification of their 16S rRNA genes, sequencing, and comparison of the sequences against those in public databases. Cells from isolated colonies were dispersed in TE buffer (10 mol/l Tris-HCl, 1 mol/l EDTA, pH 8.0) with 0.9% NaCl, boiled for 10 min, stored on ice for 5 min, and centrifuged at 16,000 ×*g* in a refrigerated (4ºC) centrifuge. The supernatants were then frozen at –70ºC until their use as template DNA in PCR amplifications.

Two PCR primers based on prokaryotic conserved regions encompassing the V1 and V2 hyper variable regions of the 16S rRNA gene were used: UPf (5′ TGG CTC AGG ACG AAC GCT GGC GGC 3′; positions 20–43 of 16S rDNA, *Escherichia coli* numbering), and UPr (5′ CCT ACT GCT GCC TCC CGT AGG AGT 3′; positions 361–338). Amplifications were done in a PCR Sprint thermocycler (Hybaid Thermoelectron, Waltham, MA, USA) under the following amplification conditions: initial denaturation at 95ºC for 5 min, 30 cycles at 94ºC for 45 s, 56ºC for 1 min, and 72ºC for 45 s, and a final extension step at 72ºC for 10 min.

Amplicons were purified using Microcon PCR filters (Millipore, Bedford, MA, USA) to remove unincorporated primers and nucleotides, and sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were then compared to those in GenBank [http://www.ncbi.nlm.nih.gov] and the Ribosomal Database Project (RDP) [http://rdp.cme.msu.edu/index.jsp] using programs available online. Sequences with a percentage identity of 97% or higher were considered to belong to the same species [16,23].

Nucleotide sequence accession numbers. The 16S rDNA sequences obtained in this work were deposited in the GenBank data library under accession nos. DQ644524–DQ644535.

Results

Lactobacilli counts ranged from around 1.0×10^5 CFU/g feces to 1.0×10^9 CFU/g feces. In three of the individuals, counts were below the detection limit (ca. 10^5 CFU/g), so that further analyses were not possible. One-way ANOVA showed the differences between the remaining seven individuals to be statistically significant ($P < 0.001$). In some individuals, especially individual E, there was a high degree of inter-sample variation. Between five and 22 colonies representative of all morphotypes were selected from one fecal sample of each of the seven subjects. This provided a total of 71 lactobacilli isolates (seven colonies were lost during subculturing), which were purified and subjected to phenotypic grouping and to assays with the commercial Phene-Plate fermentation system. All the sugars tested in this system were used by at least one strain but none was utilized by all strains—thus allowing maximum differentiation. High phenotypic variability was observed, with 24 different carbohydrate fermentation profiles obtained. The profiles were then examined for similarity using the Single Matching coefficient test and clustered by UPGMA.

Ten clusters were obtained at a similarity level of 82% (Fig. 1). The clusters branched into two unrelated groups, some of which consisted of single isolates. Strains in the first group, made up of clusters I, II, and III, used most of the carbohydrate sources. Those in the second group included the rest of the clusters; these were characterized by a more limited metabolism of sugars. The fermentation profiles of the ten clusters are summarized in Table 2 ONLINE, as supporting information [SI] digital material.

Cluster III, which included six different carbohydrate fermentation profiles (Fig. 1), consisted of three species: *L. paracasei* (14 isolates), *L. rhamnosus* (5 isolates), and *L. gasseri* (3

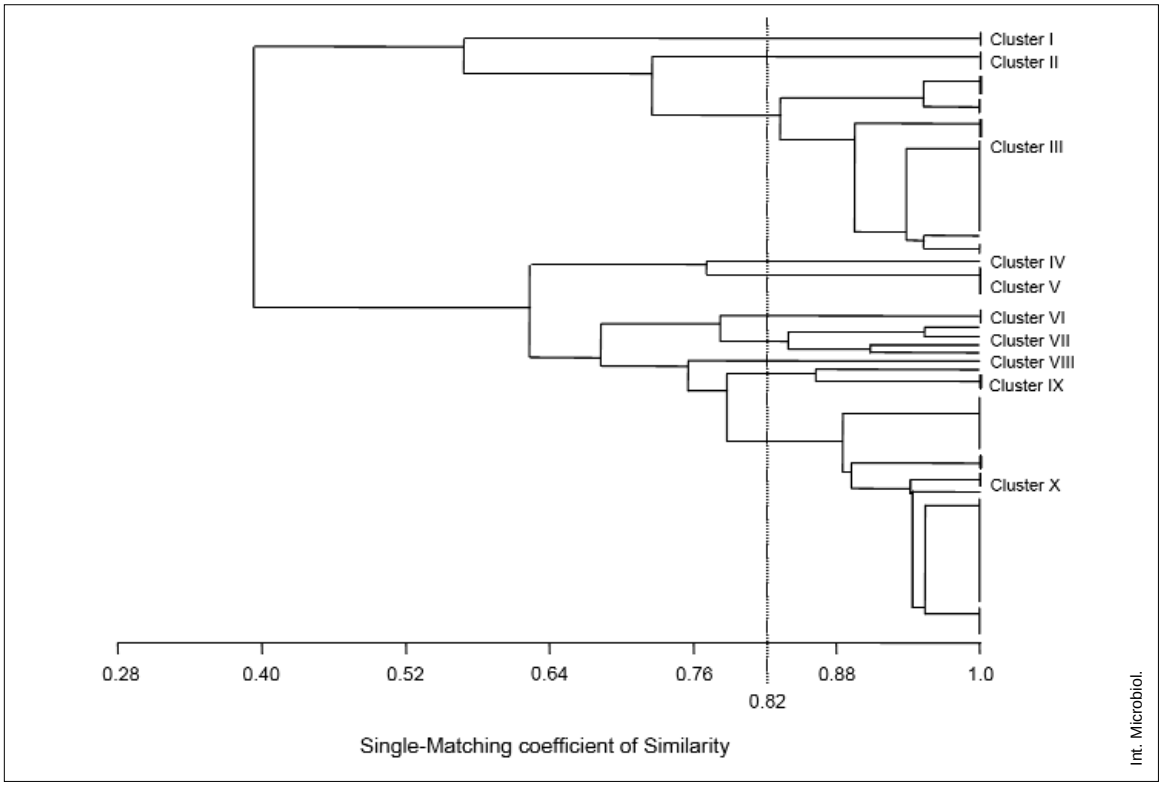

Fig. 1. Dendrogram showing the Single-Matching coefficient of similarity for the carbohydrate fermentation profiles of the isolates clustered by UPGMA. The dashed line indicates 82% similarity –the threshold for considering isolates as members of the same species. The species composition within clusters is summarized in Table 1S ONLINE.

isolates). Two profiles were obtained for each of these three species. As phenotyping was found to be of no use for identification purposes, all of the isolates were subjected to molecular identification. Comparison of the partial 16S rRNA sequences showed, in all cases, nucleotide identity with that of the described lactobacilli species of > 98% (data not shown). Moreover, strains belonging to the same species showed complete nucleotide identity within the sequenced stretch, for which a single sequence per species was submitted to GenBank. Consequently, all strains were assigned to one of the 12 species summarized in Table 1, according to the current genetic definition of bacterial species [16,23].

Two other phenotypic clusters (IX and X) contained more than one species. Additionally, although most *L. paracasei* strains were in cluster III, the single isolate of cluster IV was found to also belong to this species. Alternatively, this last strain might belong to the related species *L. casei*, which was indistinguishable from *L. paracasei* under the experimental conditions used in this study. The number of *Lactobacillus* species detected per individual ranged from one to seven, and none of the species was present in all individuals. All of the isolates of the same species from a single individual showed the same fermentation pattern, suggesting a single majority strain per species. The exceptions were the 16 *L. gasseri* isolates from individual E, which exhibited four different carbohydrate fermentation profiles. These results were further confirmed by random amplification of polymorphic DNA (RAPD) analysis (data not shown).

Discussion

In the present study, several media were initially assayed for their use in enumerating lactobacilli (including Rogosa acidified at pH 5.4, and MRS with and without cysteine), in an attempt to inhibit the growth of the dominant bifidobacteria. However, none of them yielded satisfactory results. Incubation of the plates in a CO_2-enriched atmosphere or under oxic conditions completely inhibited the growth of bifidobacteria, but also reduced counts of lactobacilli by up to three logarithmic units (data not shown). Our results agree with those of previous culture-based studies performed in other human communities, which showed that lactobacilli population sizes ranged widely between subjects [1,14].

Table 1. Distribution of dominant *Lactobacillus* species in the feces of seven healthy individuals from Asturias, northern Spain

Species	No. of species and isolates per individual[a]							Total
	A	B	C	D	E	F	G	
L. gasseri	5	2			16			23
L. paracasei		3		7	4	1		15
L. delbrueckii			2		2		4	8
L. rhamnosus				2		3		5
L. ruminis		5						5
L. brevis		1			3			4
L. plantarum				1		1	1	3
L. acidipiscis					2			2
L. johnsonii					2			2
L. parabuchneri					2			2
L. sakei							1	1
L. vaginalis			1					1
Total	5	11	3	10	22	14	6	71

[a]Individuals A–G. In three of the ten individuals examined, no lactobacilli could be recovered, indicating counts below the detection limit (ca. 1.0×10^5 CFU/g feces).

Furthermore, molecular studies have also shown that lactobacilli cannot be detected in variable percentages of individuals on a Western diet [8,13], as was the case for three subjects in this study.

The 71 isolates recovered were phenotypically characterized and identified at the species level by partial amplification, sequencing, and comparison of the sequences. Our strategy was based on the assumption that this number of isolates sufficiently covered the numerically predominant strains, as previously reported [12]. The phenotypic variation of *Lactobacillus* species is known to be large [10]. Moreover, the assay conditions and the involvement of plasmids in the fermentation of some sugars can further influence the results. Thus, the typing method did not allow the identification of the isolates with confidence, although it did provide an impression of the wide phenotypic diversity of fecal isolates.

The 12 lactobacilli species identified in this study were previously described as members of the fecal microbiota of humans [1,21]. While some studies reported *L. gasseri* and the species of the *L. paracasei* group as dominant in fecal and mucosal samples [12,26,28], in others, the dominance of species such as *L. plantarum* [1] and *L. fermentum* [221] was reported. Most of the dominant species are thought to originate from the oral cavity or from food [3,26]. This is supported by higher counts after the administration of exogenous probiotic lactobacilli strains [6,24,25]. The discrepancies reported by different authors regarding dominant species may in part be due to the isolation and identification methods used, but host-specific differences (age, sex, physiological state, host genotype, etc.) or dietary habits no doubt play a role as well [7,14,22,27]. High inter-individual variability in the number and species of lactobacilli [1,8,12] and other intestinal populations [4] has consistently been reported as well. Species and strains may also vary according to GIT location [11,28]. None of the strains recovered in the present study belonged to species *L. reuteri*. While Reuter observed the dominance of this species among the true intestinal lactobacilli [21], other authors reported it as only a minor component of the group [8,13,26].

In conclusion, most of the fecal lactobacilli populations detected in the ten healthy donors belonged to *L. gasseri*, *L. paracasei*, *L. delbruekii*, *L. rhamnosus*, and *L. ruminis* species. Even though only small numbers of individuals and isolates were analyzed here, our results are in agreement with those of previous studies and show high inter-individual variation in total numbers, the number of species present, and the dominant species. This work provides the first step in the selection of strains to be used as specific probiotics or, alternatively, to assess the effect of consuming commercial probiotics on the autochthonous populations of lactobacilli in the human adult-microbiota.

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