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Original article

Lactobacilli in the intestinal microbiota of Swedish infants

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Abstract

Lactobacillus colonisation was examined in 112 Swedish infants. Faecal samples obtained at 1, 2, 4 and 8 weeks and at 6, 12 and 18 months of age were cultivated quantitatively on Rogosa agar. Lactobacilli were speciated by PCR and typed to the strain level by randomly amplified polymorphic DNA (RAPD). Lactobacilli reached a peak at 6 months when 45% of the infants were colonised. *L. rhamnosus* and *L. gasseri* were the most common species in this period. Colonisation by lactobacilli in general ($P < 0.01$) and *L. rhamnosus* in particular ($P < 0.05$) was more common in breast-fed than in weaned infants at 6 months of age. *Lactobacillus* isolation reached a nadir of 17% by 12 months ($P < 0.0001$), but increased to 31% by 18 months of age $P < 0.05$). The food-related species *L. paracasei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* dominated in this second phase. A single strain persisted for at least 3 weeks in 17% of the infants during the first 6 months, most commonly *L. rhamnosus*. *Lactobacillus* population counts in colonised infants increased from $10^{6.4}$ cfu/g at 1 week to $10^{8.8}$ cfu/g at 6 months, and then dropped to $10^{5.4}$ cfu/g faeces at 12 months of age. *Lactobacillus* colonisation was not significantly related to delivery mode, or to presence of siblings or pets in the household. Our results suggest that certain *Lactobacillus* species, especially *L. rhamnosus*, thrive in the intestinal flora of breast-fed infants. After weaning they are replaced by other *Lactobacillus* species of types found in food.
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1. Introduction

Lactobacilli are a heterogeneous group of lactic acid producing anaerobic or microaerophilic bacteria. They are usually present in the intestinal microbiota of healthy adults, although in substantially lower population counts than several other bacteria. Lactobacilli are also found in a range of fermented foods [1]. They are commonly used as probiotics, with effects especially against acute diarrhoea in childhood [2,3] and have immune activating properties, most markedly on cell-mediated functions [4,5].

The extent to which lactobacilli colonise the intestines of newborn infants and small children is controversial. Most studies have reported low *Lactobacillus* colonisation rates in infants in Western countries [6–10], while some claim that lactobacilli are present in substantial quantities ($10^{7–9}$ cfu/g faeces) in infant stools [11,12]. Variations in methodology may account for the differences, since lactobacilli are notoriously difficult to identify by traditional biochemical methods. Another explanation may be that the *Lactobacillus* microbiota differ in different geographical areas. For example, lactobacilli are more frequently isolated from stools of Estonian as compared to Swedish infants [13]. The recent development of genetic methods enables lactobacilli to be unequivocally identified and the question to be resolved.

The isolation of lactobacilli from stools does not necessarily imply intestinal colonisation. Ingested lactobacilli that survive the gastro-intestinal passage may be cultivated from the stools [14–16]. Repeated isolation of a single strain from the same individual, however, suggests that the strain colonises and replicates in the gastro-intestinal tract.

Abbreviations: bp, base pair; RAPD, randomly amplified polymorphic DNA.

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The present study examines the *Lactobacillus* colonisation pattern of 112 Swedish infants. Stool samples were obtained at regular intervals over their first 12 or 18 months of life and cultured quantitatively for lactobacilli. Lactobacilli were speciated with specific PCR and individual strains were distinguished by randomly amplified polymorphic DNA (RAPD). Using this methodology, the population counts and time of persistence of each *Lactobacillus* strain in the infantile microflora could be determined.

2. Materials and methods

2.1. Infants

One hundred and twelve healthy Swedish infants born in 1998–2000 at the Sahlgrenska University Hospital (11,500 deliveries per year) were followed. The children were recruited to a prospective birth-cohort study aiming to investigate the relation between intestinal colonisation pattern and allergy development, the ALLERGYFLORA study. Parents-to-be were enrolled at the maternity clinic. Exclusion criteria were pregnancy complications and inability to comply with a quite demanding study protocol. Parents-to-be with atopic disease were targeted, in order to increase compliance and to obtain a study group with high incidence of allergy. In addition, parents-to-be without atopic history were included as controls.

Upon inclusion, information was obtained about atopic heredity, siblings and pets. During the infant's first year of life, the parents recorded their baby's feeding pattern and health status in a diary. A study nurse collected these records in telephone interviews at 6 and 12 months. Informed consent was obtained from the parents, and the Ethics Committee of the Medical Faculty of Göteborg approved the study.

2.2. Sampling and culture of stool samples

Quantitative cultures of the faecal microbiota were performed at 1, 2, 4 and 8 weeks, and at 6 and 12 months of age. From 65 infants, faecal cultures were also obtained at 18 months of age.

Freshly voided faeces were collected at home by the parents, and put in a plastic bag where an anaerobic atmosphere was generated (AnaeroGen Compact, Oxoid Ltd., Basingstoke, UK). The samples were kept refrigerated until transported to the laboratory, where they were processed within 24 h after collection. Using this procedure, bacterial counts were not altered as compared to immediate culture of faecal samples (data not shown).

The faecal samples were serially diluted and plated on Rogosa agar for selective outgrowth of lactobacilli [17]. The limit of detection was 330 ($10^{2.52}$) cfu/g faeces. The plates were incubated anaerobically at 37 °C for 3 days using the BBL GasPak anaerobic system (Becton Dickinson Microbiology Systems, Sparks, MD). From appropriate dilutions, one representative colony of each morphotype (differing in e.g. size, shape, colour or texture from other colonies) was separately enumerated, Gram-stained, examined in the microscope and subcultured to purity (0–6 per sample). Unbranched Gram-positive rods were regarded as tentative lactobacilli and analysed further (0–4 per sample).

2.3. Selection of lactobacilli using RAPD and a Bifidobacterium-specific PCR

It was soon evident that the Rogosa plates are not entirely selective for lactobacilli, but also permit growth of bifidobacteria. Moreover, bifidobacteria often appear as straight, non-bifid rods after culture on Rogosa, thus resembling lactobacilli microscopically. In order to select isolates for further speciation, a multiplex PCR was performed that allowed exclusion of all bifidobacteria [18]. In the same PCR, an RAPD analysis was performed simultaneously to distinguish different *Lactobacillus* strains. All isolates regarded as tentative lactobacilli were subjected to this combined analysis.

The isolates were grown overnight in *Lactobacillus* carrying medium (LCM) [19] containing 1% glucose (w/v), and a crude cell extract was prepared by bead beating as previously described [20]. One microlitre of supernatant was added to 50 µl of PCR buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics GmbH, Mannheim, Germany), 200 µM of each nucleotide (Roche), 0.2 µM of the primers PbiF1 (5′CCGGAATAGCT-CC-3′) and PbiR2 (5′-GACCATGCACCACCTGTGAA-3′), specific for bifidobacteria [18], as well as 15 µM of the RAPD-primer 73 (5′-ACGCGCCCT-3′) and 2.5 units of *Taq* RNA polymerase (Roche). The reaction mix was covered with mineral oil (Perkin–Elmer, CT) and amplification was conducted in a DNA thermal cycler (Perkin–Elmer) using the following temperature profile: 94 °C, 30 s; 50 °C 60 s, 72 °C, 90 s for 35 cycles, and a final extension step at 72 °C for 2 min. Gel electrophoresis was performed on submerged horizontal agarose gels (1.5% in TBE-buffer [21]) that were stained by ethidium bromide and photographed under UV-light. The amplicon specific for bifidobacteria had a size of 914 bp (base pairs) [18].

Isolates reacting with the primers specific for bifidobacteria were discarded. Other isolates were regarded as putative lactobacilli. *Lactobacillus* isolates from a single child showing the same band pattern on RAPD analysis were regarded as belonging to the same strain.

2.4. Identification of lactobacilli at the species level by multiplex PCR

At least one representative isolate of each different RAPD pattern from each child was speciated by multiplex PCR. In this multiplex PCR four different primer pairs were included, recognising the 16S-23S rRNA spacer region and its flanking 23S rRNA gene of the following groups of *Lactobacillus* species: group I, *L. delbrueckii*; group II, *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. johnsonii*, *L. helveticus* and

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