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Intestinal Lactobacilli of Estonian and Swedish Children

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The prevalence of intestinal lactobacilli was compared for sets of Estonian (71) and Swedish (65) 1–2-year-old children. A total of 227 *Lactobacillus* isolates from 50 Estonian and 30 Swedish children were collected. The distribution of the three lactobacilli fermentation types, obligate homofermentative, facultative heterofermentative and obligate heterofermentative (OHOL, FHEL and OHEL) was assessed among 138 Estonian and 89 Swedish paediatric isolates of lactobacilli according to their gas production from glucose and also their growth at different temperatures. Furthermore, 76 selected OHOL, FHEL and OHEL isolates from 18 (36%) Estonian and 13 (43%) Swedish children were typed using gas chromatographic analysis. In addition, species-level identification was performed using an API 50 CHL kit (bioMérieux, Lyon, France) and internal-transcribed spacer PCR coupled with restriction analysis. The Swedish children examined were less frequently colonized with *Lactobacillus* sp. than the Estonian children (46% vs. 70% children; $p < 0.01$). The prevalence of the OHOL, OHEL and FHEL groups was found to be similar within both sets of children with FHEL present in 72% of Estonian and 80% of Swedish children. Utilizing both pheno- and genotyping systems seven species were found within the Estonian group of children versus three species within the Swedish group. The API 50CHL identified a further three *Lactobacillus* species in the Estonian group and one additional species in the Swedish group. Significantly, *Lactobacillus plantarum* strains were present in 33% of Estonian children tested but were not present in Swedish children ($p < 0.05$). Thus, among young children regional differences may occur in the number and species of intestinal lactobacilli. These differences between infants in the two countries with a low and a high prevalence of allergy are of interest in the suggested role of lactobacilli as immune modulators. *Keywords*: children, lactobacilli, obligate homofermentative, facultative heterofermentative, obligate heterofermentative species, prevalence.

INTRODUCTION

Lactobacilli are non-pathogenic inhabitants of the intestinal microflora in healthy humans. The most frequent species of lactobacilli in faecal samples include *Lactobacillus acidophilus*, *L. salivarius* and *L. fermentum* (1, 2). Recent results concerning lactobacilli from rectal biopsies have demonstrated that the most prevalent colonizing *Lactobacillus* sp. to be the *L. acidophilus* group, *L. salivarius*, *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. buchneri* and *L. reuteri* (3, 4). Other investigators (5–7) have documented significant differences between individuals in both the number and composition of species of intestinal lactobacilli. However, recent taxonomic changes to the genus *Lactobacillus* (8–10) have complicated the comparison of results from previous studies, performed using different methods.

Traditionally, the identification of lactobacilli has been based largely on morphology, Gram-staining, fermentation of glucose with lactic acid as the major product, and the ability to grow at various temperatures. Lactobacilli are divided into three groups based on their physiology; obligately homofermentative lactobacilli (OHOL) which convert glucose almost exclusively to lactic acid, and also facultatively and obligately heterofermentative lactobacilli (FHEL and OHEL) which convert hexoses/pentoses to lactic acid, acetic acid, ethanol and CO_2 in different proportions (1). Within the gut, the most prevalent species of the OHOL group are *L. acidophilus*, *L. gasseri* and *L. salivarius*, the dominant members of the FHEL group are *L. paracasei*, *L. rhamnosus and L. plantarum* whereas *L. fermentum*, *L. brevis*, *L. reuteri* and *L. buchneri* are the most common among OHEL group (1, 9).

It has been noted that there are large geographic variations in the composition of the human intestinal microflora. For example, lactobacilli are more frequently isolated in higher numbers from populations in Uganda, Japan and Southern India than in Western Europe (11, 12). Some studies have also indicated that the profiles of intestinal lactobacilli may also differ between the Swedish and Japanese populations (4, 13). However, these investi-

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gations did not employ identical methods. Bennet et al. (14) compared Ethiopian and Swedish neonates and found that faecal lactobacilli were more common among Ethiopian neonates. Similarly, we have previously demonstrated that lactobacilli are more common in Estonian than in Swedish neonates and infants (15, 16).

The composition of the intestinal microflora may be of importance for the development and priming of the immune system in early childhood (17, 18). The potential role of some lactic acid producing bacteria (enterococci, bifidobacteria) in the reduction of allergies has recently been postulated based on the analysis of intestinal microflora profiles in allergic Estonian and Swedish infants (19, 20). To better understand the role of *Lactobacillus* sp. for human health, studies on their physiological and biochemical properties, confirmed by molecular typing of intestinal lactobacilli, have to be conducted within different populations.

In this study we compared the predominant lactobacilli inhabiting the intestinal tract of 1–2-year-old children in Estonia and Sweden, with a low and high prevalence of allergy, respectively. The distribution of various fermentation types and representative *Lactobacillus* species was also examined.

MATERIAL AND METHODS

Subjects

The study groups were comprised of 71 Estonian (40 girls and 31 boys, mean age 18 months) and 65 Swedish (31 girls and 34 boys, mean age 16 months) children participating in a prospective study of allergic disease in relation to environmental factors. Faecal specimens were collected in Tartu, Estonia, from October 1995 to March 1997 and in Linköping, Sweden, from March 1995 to January 1997. The duration of exclusive and partial breast feeding and the number of atopic children and number of children treated with antibiotics according to questionnaire responses, were similar for both groups (16, 19).

Bacteriological analysis

Approximately 1 g of voided stool was collected in sterile plastic containers by the children's parents. In Estonia, the samples were kept in a domestic refrigerator at 4°C for no more than 2 h and then delivered to the laboratory, where these were frozen at −20°C. The Swedish samples were frozen at −20°C immediately at home, delivered to the laboratory within 1 month and then transported to Estonia on dry ice for analysis. All samples were maintained at −20°C.

Isolation and provisional identification of lactobacilli

Weighed samples of faeces were serially diluted (10^{-2} to 10^{-9}) under a stream of CO_2 in phosphate buffer with 0.04% thioglycolic acid (pH 7.2). For isolation of lactobacilli the dilutions were plated onto freshly prepared de Man-Rogosa-Sharpe (MRS) agar (Oxoid) and incubated in a microaerophilic atmosphere (CampyGen, Oxoid) for 72 h. Two to ten colonies, with different morphology, were picked from plates, with growth at the highest dilutions, into MRS broth and incubated in a 10% CO_2 environment for 24–48 h. Provisional identification was based on the ability of the isolate to grow in the MRS broth, and also on a Gram-positive rod-shaped non-sporing cell morphology and negative catalase reaction (1). A total of 227 isolates were provisionally identified as lactobacilli (138 isolates from 50 Estonian and 89 from 30 Swedish children) and were further analysed for fermentation type according to their physiological properties.

The ability of isolates to grow in MRS broth for 24 h in a 10% CO_2 environment at 15 and 37°C and to produce gas in MRS agar with 1% glucose was also assessed. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies OHOL; growth both at 15 and 37°C without gas production is characteristic of FHEL, whereas gas production at 37°C and variable growth at 15°C are characteristic of OHEL *Lactobacillus* species (1, 5, 21). The prevalence of OHOL, FHEL and OHEL strains among the predominant *Lactobacillus* isolates from Estonian and Swedish children were compared.

Thereafter, 10–15 isolates of the three fermentation types were randomly selected from the two groups of children: 76 isolates out of 227 from 18 (36%) Estonian and 13 (43%) Swedish infants colonized by lactobacilli. To verify the distribution of different fermentation types, estimated by physiological methods, the selected *Lactobacillus* isolates were subjected to gas chromatographic analysis of their metabolite profile. Furthermore identification to the species level was also carried out using the pheno- and genotypic methods outlined below.

Gas chromatographic analysis

The production of volatile and non-volatile fatty acids and ethanol (mg/ml) was estimated by gas chromatography, as described by Holdeman et al. (22). The gas chromatograph (Hewlett–Packard model 6890) was equipped with a hydrogen flame ionization detector and an autosampler (model 7683); the HP Chemical Station for GC System (A.06 revision) was used. Analyses were performed following cultivation of lactobacilli in MRS broth under microaerobic conditions (1). Reference strains from the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen (DSM) including *L. acidophilus* ATCC 4356, *L. salivarius* ssp. *salivarius* ATCC 11741, *L. delbrueckii* ssp. *delbrueckii* ATCC 9649, *L. plantarum* ATCC 14917, *L. paracasei* ssp. *paracasei* DSM 5622, *L. paracasei ssp. paracasei* ATCC 27216, *L. curvatus* ATCC 25601, *L. rhamnosus* ATCC 53103, *L. brevis* ATCC 14869, *L. fermentum* ATCC 14931, *L. buchneri* ATCC 4005 and *L. reuteri* ATCC 23272 were included as controls.

Lactobacillus sp. identification

The species of the lactobacilli were identified using an API 50 CHL kit (bioMérieux, France) which compares the fermentation patterns of an isolate with that of the type strain of the *Lactobacillus* species. According to APILAB Plus software database (API 50 CHL version 4.0), the identification levels of acceptable to excellent (ID% >80–99.9%, respectively) were deemed to be satisfactory. The API 50 CHL kit does not identify some human *Lactobacillus* strains, in particular the *L. reuteri* group. Reuterin production has been used as an identification marker for these strains (23). Therefore, OHEL *Lactobacillus* isolates were also tested for their ability to produce reuterin, using the biochemical plate assay technique carried out in BioGaia Biologies laboratory (Lund, Sweden).

Molecular typing

The 76 *Lactobacillus* isolates were subjected to internal transcribed spacer polymerase chain reaction (ITS-PCR) followed by restriction analysis. DNA extraction from *Lactobacillus* isolates was performed as described by Alander et al. (24) using lysozyme (Serva, Sweden; 20 mg/ml), mutanolysin (Sigma; 0.5 mg/ml) and proteinase K solution (Fermentas, Lithuania; 14.6 mg/ml). The DNA amplification was performed according to Jacobsen et al. (25) in a reaction volume of 50 (µl containing $1\times$ *Taq* polymerase buffer (Fermentas, Lithuania), 1.5 U *Taq* polymerase (Fermentas), 0.5 µM of each primer (16S-1500F and 23S-32R; DNA Technology AS), 200 µM deoxynucleoside triphosphates, 2 mM $MgCl_2$ and 2 µl of extracted DNA. Furthermore, the PCR product was restricted as described by Zhong et al. (26) using *Taq*I restriction enzyme (Fermentas). DNA fragments were separated by electrophoresis (1.5 h, 100 V) on an 2% agarose gel in $1\times$ TBE buffer, a size marker 100 bp DNA Ladder Plus (Fermentas) was also used. The banding pattern of the isolates was visually compared with that of the aforementioned *Lactobacillus* reference strains.

Statistical methods

The prevalence and proportions of different *Lactobacillus* sp. among the Estonian and Swedish children were compared, employing χ^2 or the Fisher exact test. The Student's t-test or Mann–Whitney rank sum test was applied to compare the amount of lactic acid produced by different isolates of *Lactobacillus* sp. The computer program 'STATGRAPHICS', Statistical Graphics Corp., USA, suggested the relevant statistical test according to the distribution of data.

RESULTS

Prevalence and distribution of different fermentation types of lactobacilli

Lactobacilli were isolated from 50/71 (70%) Estonian children and from 30/65 (46%) Swedish children ($p<0.01$). The distribution of different fermentation types (OHOL, FHEL and OHEL) of lactobacilli was similar in both the Estonian and Swedish toddlers (Table I) with FHEL prevailing in both groups (present in 72 and 80% of colonized children, respectively). Simultaneous colonization by two or more fermentation groups was established for 40% of the Estonian and 46% of the Swedish children colonized with lactobacilli (Table I).

The gas chromatographic analysis of metabolites from randomly selected isolates of OHOL, FHEL and OHEL confirmed the results of the physiological tests. Under microaerobic conditions, the isolates grouped as OHOL and FHEL produced abundant lactate (10.1 ± 2.5 and 14.6 ± 3.0 mg/ml, respectively) with small amounts of acetic acid. The production of lactic acid by OHEL strains was low (8.2 ± 1.3 mg/ml), differing significantly from the OHOL ($p<0.05$) and the FHEL ($p<0.01$) isolates. In contrast, OHEL strains produced ethanol in substantial amounts (11.4 ± 2.2 mg/ml), whereas only low amounts (up to 0.9 mg/ml) were detected in FHEL strains following

Table I

Distribution of different fermentation types (OHOL, FHEL and OHEL) among the predominant intestinal Lactobacillus sp. from Estonian and Swedish 1–2-year-old children

Fermentation type	Estonian children $n=50$		Swedish children $n=30$	
	Number	Prevalence (%)	Number	Prevalence (%)
OHOL	11	22	5	17
FHEL	36	72	24	80
OHEL	17	34	6	20
Presence of microbes of				
one	30	60	16	54
two	19	38	9	30
or three fermentation types	1	2	5	16

OHOL, obligate homofermentative lactobacilli; FHEL, facultative heterofermentative lactobacilli; OHEL, obligate heterofermentative lactobacilli.

Table II

Predominant faecal Lactobacillus sp. from healthy 1–2-year-old Estonian and Swedish children

Species	Estonian children		Swedish children	
	Number of children colonized[a] $n = 18$	Number of strains $n = 37$	Number of children colonized # $n = 13$	Number of strains $n = 39$
Identified both by API 50 CHL and ITS-PCR				
L. acidophilus	2	5	2	10
L. salivarius	1	1	0	0
L. paracasei	6	8	10	14
L. plantarum	6*	7	0*	0
L. brevis	3	3	0	0
L. fermentum	2	5	0	0
L. buchneri	1	1	4	9
Identified only by API 50 CHL				
L. acidophilus 3	2	2	0	0
L. delbrueckii	1	1	0	0
L. coprophilus	1	1	1	2
Unidentified	2	3	2	4

[a] either with 1 species or 2 different species.
* $p = 0.028$.

a 48 h of incubation. The OHOL strains did not produce ethanol. The metabolite profile of culture collection strains matched their fermentation type, demonstrating the validity of applied methods (data not shown).

Lactobacillus species identified among isolates of different fermentation types

The API computerized database identified 69 of the 76 (91%) strains as belonging to one of ten species including *L. acidophilus*, *L. delbrueckii*, *L. crispatus*, *L. salivarius*, *L. paracasei*, *L. plantarum*, *L. curvatus*, *L. brevis*, *L. fermentum*, *L. buchneri* and *L. coprophilus* and several subspecies (data not shown). Production of reuterin was not detected in any of the OHEL isolates and, therefore, no strain was identified as *L. reuteri.*

In total 63 strains were identified by molecular methods (Table II). Six strains identified by the API 50 CHL test as *L. acidophilus* (three Estonian isolates), *L. delbrueckii* ssp. *delbrueckii* (one Estonian isolate) and *L. coprophilus* (both an Estonian and Swedish isolates) could not be differentiated by molecular typing. Seven strains out of 63 (11%) were reassigned based on ITS-PCR results, these included one strain of *L. delbrueckii* ssp. *delbrueckii* to *L. acidophilus*, one strain of *L. crispatus* to *L. acidophilus*, one strain *of L. curvatus* to *L. paracasei* ssp. *paracasei*, one strain of *L. plantarum* to *L. paracasei* ssp. *paracasei*, and three strains of *L. brevis* to *L. buchneri*.

Within the Estonian group of children seven different species of *Lactobacillus* were identified using both pheno- and genotypic methods, as compared with three species within the Swedish group of children. The API 50 CHL identified three additional *Lactobacillus* sp. from Estonian children and one additional species from Swedish children. For seven strains, attempts at their identification failed using both methods for possible technical failures or lack of the particular reference strain (Table II). In addition, *L. plantarum* was identified as the prevailing species for six of the 18 Estonian children tested but none of the 13 Swedish children tested ($p < 0.05$). All children harboring *L. plantarum* were 2 years old.

DISCUSSION

Applying a three-step method in this study, we managed to compare the number and species composition of faecal lactobacilli in large groups of Estonian and Swedish 1–2-year-old children. Firstly, we estimated the prevalence of lactobacilli in 136 children. Secondly, the distribution of the three different fermentation types of *Lactobacillus* in 50 Estonian and 30 Swedish children colonized by lactobacilli was studied. Finally, we identified to the species level a number of randomly selected isolates from each fermentation group of 18 Estonian and 13 Swedish children.

We could confirm our previous observations that Swedish children are less frequently colonized with lactobacilli than Estonian children. However, no differences were observed in the prevalence of different fermentation types among the prevailing *Lactobacillus* isolates in colonised children from either group, indicating a gross uniformity of intestinal lactobacilli profiles between the two populations.

Using phenotypic methods (5, 21) and confirmed by gas chromatography (22), we found FHEL to be dominant in both countries. Furthermore, OHOL and OHEL were

equally common among the Estonian and Swedish children. To date little data has been acquired on the distribution of different fermentation groups of *Lactobacillus* sp. in children. Similar to our results, Reniero et al. (27) demonstrated that the *L. casei* ssp. *casei*, in principle belonging to FHEL group, continuously predominated in infants following weaning. However, only two infants were included in that study. A predominance of FHEL strains (*L. plantarum*, *L. rhamnosus*, *L. paracasei*) in rectal mucosa of Swedish adults (4) is also in agreement with our findings. In contrast, a study of 36 infants from the Japanese population demonstrated the predominance of OHOL strains including *L. gasseri*, *L. crispatus* and *L. salivarius* (13). However, these investigations did not employ identical methods for *Lactobacillus* sp. isolation and identification.

The phenotypic and gas chromatographic methods provided complementary tools for the assessment of the fermentation types of lactobacilli. For the identification of *Lactobacillus* sp. the API 50 CHL test kit (bioMérieux, Lyon, France) was applied. Identification failed in only 9% of the isolates examined using this method. In a similar recent study, only approximately half of the isolates could be identified by the API 50 CHL kit (28). The reason for this failure may have been the selection of unusual probiotic strains with modified properties. In the present study, comparative genotyping with the reference strains using ITS-PCR followed by restriction with *Taq*I restrictase reassigned a small (11%) percentage of the isolates tested.

All the ten species of lactobacilli encountered within the study are known to inhabit the human intestinal tract (1–3, 10). Whereas, *L. reuteri* and *L. gasseri* which have also been described in humans were not encountered. Within the Japanese population, *L. reuteri* was infrequently isolated (13). The prevalence of *L. gasseri* in the intestinal microflora is largely unknown, as the taxonomy of *Lactobacillus* sp. has been changed recently (8, 9). In our investigation, it is possible that API 50 CHL misclassified *L. gasseri* strains as *L. acidophilus* biotype 3. Only a few children were colonized with the latter species, however. The molecular identification of *L. gasseri* was complicated largely due to the absence of an appropriate reference strain.

In the present study, infants from both countries harboured one to three predominating strains of different fermentation types demonstrating a wide variety of intestinal *Lactobacillus* sp. among individuals. Within the Estonian group a total of ten species were identified, whereas only four species were found in Swedish children. The mean age of children was similar in both groups. Our study had limitations due to slight differences in the transportation and storage conditions between the Estonian and Swedish samples. Also, the random selection of predominant isolates for identification may have affected the results as in a wider sample there might always be the possibility for wider variety of species of lactobacilli. Further studies are needed to confirm our findings.

It has been suggested that there are geographical differences in the species composition of intestinal lactobacilli in adults due to dietary heterogenicity (3, 13). In adults, the prevalence of FHEL, particularly *L. plantarum*, has been clearly associated with a vegetarian as opposed to a typical Western type of diet (9). In our study, *L. plantarum* strains were encountered only from Estonian children. The infant diet and the duration of breast-feeding are quite similar in both countries. It is possible, however, that the differences in the time when mixed feeding is introduced in the two countries may influence the spectra of predominating *Lactobacillus* sp. as all *L. plantarum* strains were isolated from older children. The Estonian diet is still to a large extent based on locally produced foods and in addition, foodstuffs fermented by lactic acid bacteria are part of diet, even in children.

Thus, our data demonstrates that certain regional-specific differences in the prevalence of colonization with particular lactobacilli exist. Although the distribution of different fermentation types among predominant intestinal *Lactobacillus* sp. was similar in Estonian and Swedish 1–2-year-old children yet the species composition of prevailing lactobacilli differed. These differences between children in two countries with a low and a high prevalence of allergy are of interest in the light of the suggested role of lactobacilli as immune modulators.

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