

Detection of intestinal *bifidobacteria* and *lactobacilli* in patients with Hirschsprung's disease associated enterocolitis

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Background: The etiology of Hirschsprung's disease associated enterocolitis (HAEC) is unknown. Previous investigations have suggested that several factors such as dilation of proximal bowel, changes in colonic mucosal defence, and overgrowth of toxigenic bacteria may be related with it. This study was to quantify *bifidobacteria* and *lactobacilli* in the feces of Hirschsprung's disease (HD) patients with or without enterocolitis and those of normal children.

Methods: Fresh stool specimens were collected at the first three days of the admission from 30 HD patients (aged 2 weeks to 2 years) and 15 healthy age-matched non-HD patients in the morning once a day for at least three days. All of them have not been given probiotics or antibiotics at least 7 days before stool collection. Hematoxylin-eosin and acetylcholinesterase histochemical staining on rectal biopsies of patients with HD confirmed the diagnosis of HD in all 30 patients. The 30 HD patients were divided into two groups based on the clinical history of enterocolitis: the HAEC group ($n=10$) and HD group ($n=20$). Fecal *bifidobacteria* and *lactobacilli* were consecutively quantified by SYBR Green I-based real-time PCR assay. Data were analyzed using SAS v. 12.6 for Windows. All tests were two-tailed, and P values <0.05 were considered statistically significant.

Results: The mean levels of *bifidobacteria* were 7.35 ± 0.59 , 8.16 ± 1.17 , and 8.35 ± 0.74 in the HAEC, HD and control groups, respectively. The *bifidobacteria* colonization levels were lower in the HAEC group

than in the HD and control groups ($P<0.05$, $P<0.001$ respectively). The mean level of *lactobacilli* in the HAEC (5.51 ± 0.65) and HD groups (5.87 ± 0.78) was significantly lower than that in the control group (6.39 ± 0.56) ($P<0.05$). But there was no difference in log numbers of *lactobacilli* between HAEC and HD groups ($P>0.05$).

Conclusions: The scarcity of *bifidobacteria* and *lactobacilli* in HAEC patients may result in a decrease in epithelial barrier function and be a predisposing factor in the development of HAEC. This decline suggests that treatment with probiotics or prebiotics may be beneficial in these individuals. Further research will focus on whether probiotics can decrease the incidence of HAEC.

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Key words: *bifidobacteria*;
enterocolitis;
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lactobacilli

Introduction

In 1886 Harald Hirschsprung was the first to describe the disease that later bore his name. Hirschsprung's disease (HD), a major anomaly of the enteric nervous system (ENS), is characterized by the absence of intramural ganglion cells and the presence of hypertrophic nerve trunks in the distal intestine that results in functional intestinal obstruction.^[1] Hirschsprung's disease associated enterocolitis (HAEC) is a serious complication of HD with a variable incidence of 6%-58% and remains the major cause of morbidity and mortality in patients with HD.^[2,3] Despite multiple investigations and studies, a complete understanding of the etiology of HAEC is still unavailable.^[4,5] Numerous theories have been put forward to explain its occurrence including a physical dilation of the proximal bowel and fecal stasis, variations in the mucin components and production, rotavirus, mucosal immunity defects, and *Clostridium difficile*.^[4-6]

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Generally, the intestinal microbiota of infants is primarily composed of lactic acid bacteria, like *bifidobacteria* and *lactobacilli*. A mixture of *bifidobacteria* and *lactobacilli* producing lactic, acetic and other acids resulting in a lowering of pH in the colon are effective in enhancing motility of the colon so that these anaerobic microbial agents and their secreted products are intimately associated with the gastrointestinal mucosa and influence epithelial growth, differentiation, and immune activating properties.^[7,8]

However, there is little information on whether enterocolitis in Hirschsprung's disease patients will influence the colonization of *bifidobacteria* as well as *lactobacilli* in the gut of newborn infants. The objective of this study was to quantify *bifidobacteria* and *lactobacilli* in the feces of patients with Hirschsprung's disease associated with or without enterocolitis and age-matched control patients using a culture-independent method, SYBR Green I-based real-time PCR assay.

Methods

Subjects and specimens

This study was performed on patients treated at the Department of Pediatric Surgery of our institute between January 2006 and December 2006. The study was approved by the Joint Committee of Ethics of the School of Medicine, Shanghai Jiaotong University and Xin Hua Hospital. Fresh, discarded stool specimens were collected at the first three days of the admission from 30 HD patients (aged 2 weeks to 2 years) and 15 age-matched non-HD patients (mesenteric cysts, $n=4$;

cervical lymphangioma, $n=4$; cyst thyroglossal, $n=3$; facial hemangioma, $n=4$; detail in Table 1). All patients had not been given probiotics and antibiotics at least 7 days before stool collection. The rectal biopsy samples of the 30 HD patients revealed an absence of ganglion cells. Hematoxylin-eosin and acetylcholinesterase histochemical staining on rectal biopsies of patients with HD performed by pathologists confirmed the diagnosis of HD in all the 30 patients, who were divided into two groups based on the clinical history of enterocolitis: the HAEC group ($n=10$) and HD group ($n=20$). The clinical criteria for HAEC were diarrhea, abdominal distension, pyrexia, colicky abdominal pain, lethargy, and the passage of bloodstained stools.^[9]

Sample collection

The ward staff collected stool samples from each patient (after approval by the family) once a day for at least 3 days. Fecal specimens were cooled to 4°C immediately after collection, delivered to the laboratory within 6 hours, and frozen at -80°C directly on receipt until analysis.

DNA extraction

Bacterial DNA from fecal samples was extracted using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany), with some modifications. Finally, DNA samples were stored at -20°C

PCR primers

The genus-specific 16S rRNA-targeted primers sets used for quantitative real-time PCR in this study are listed in Table 2. *Bifidobacterium* genus-specific

Table 1. Clinical data of the 45 patients in this study

Group	Sex	Gestational age (wk)	Birth way	Birth weight (g)	Admission weight (g)	Feeding way	Age at time of stool collection (y)	Family history of HD	Extent of aganglionic segment		Associated congenital anomalies (Down's syndrome)
									Recto-sigmoid	Long segment or total colon	
HAEC	6M 4F	36.2±2.1	5VD 5CD	2452±206	11341.4±985.78	5BF 5FF	1.12±0.68	1	6	4	4 (2)
HD	11M 9F	37.5±2.3	12VD 8CD	2505±233	11978±1197.53	13BF 7FF	1.15±0.81	0	17	3	4 (0)
Control	8M 7F	37.8±1.5	8VD 7CD	2550±185	11983.8±1126.22	8BF 7FF	1.02±0.60	0	0	0	0 (0)

HAEC: Hirschsprung's disease associated enterocolitis; HD: Hirschsprung's disease; VD: vaginally delivered; CD: cesarean delivery; BF: breast feeding; FF: formula feeding

Table 2. Target groups and sequences of the PCR primers used in this study

Target groups	Primer	Primer sequence (30-30)	Reference
<i>Bifidobacteria</i>	Im26-f	GAT TCT GGC TCA GGA TGA ACG	10, 11
	Im3-r	CGG GTG CTI CCC ACT TTC ATG	
<i>Lactobacilli</i>	L159-f	GGA AAC AG(A/G) TGC TAA TAC CG	12
	L677-r	CAC CGC TAC ACA TGG AG	

primers Im26-f and Im3-r had been validated for *bifidobacterial* specificity by Kaufmann and Satokari, respectively.^[10,11] *Lactobacillus* genus-specific primers L159-f and L677-r had been validated for *lactobacillus* specificity by Heilig et al.^[12] Moreover, to check for specificity, the sequences of the selected PCR primers were compared to the sequences available at the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST).

Real-time PCR

PCRs were performed in 20 μ l final volumes in capillary tubes in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). The standard curve profiles of *bifidobacteria* and *lactobacilli* were generated by the LightCycler software. The resulting standard curve was shown as a graph of cycle number vs. log concentration. For each sample the crossing point was plotted against the known concentration of the standard.^[13] The crossing point was defined as the maximum of the second derivative from the fluorescence curve. The detection limit of the real-time PCR procedure with the DNA extract from feces and the PCR conditions described in this paper was found to be about 4×10^4 bacterial cells of *bifidobacteria* or *lactobacilli* per gram of feces (4.6 log units).

Statistical analysis

Data were analyzed using SAS v. 12.6 for Windows (SAS Institute Inc., Cary, NC 27513, USA). Copy numbers of 16S rRNA genes of *bifidobacteria* or *lactobacilli* per gram of sample were transformed into logarithms, and normal distributed data were subjected to statistical analysis. One way ANOVA was used for comparison of means. Student-Neuman-Keuls test was considered for the comparison of the groups. All tests

were two-tailed, and P values <0.05 were considered statistically significant.

Results

Quantitation of intestinal flora (*bifidobacteria* and *lactobacilli*) in the three groups is shown in Fig.

The mean levels of *bifidobacteria* were 7.35 ± 0.59 , 8.16 ± 1.17 , 8.35 ± 0.74 in the HAEC, HD and control groups, respectively. The *bifidobacteria* colonization level in the HAEC group was lower than in the HD and control groups ($P < 0.05$, $P < 0.001$ respectively).

The mean level of *lactobacilli* in the HAEC group was 5.51 ± 0.65 and in the HD group 5.87 ± 0.78 . Statistical analysis demonstrated a significant decrease ($P < 0.05$) in the log numbers of *lactobacilli* in the fecal samples of Hirschsprung's enterocolitis when compared with the control group (6.39 ± 0.56) (Fig.). There was no difference of log numbers of *lactobacilli* between the HD and HAEC groups ($P > 0.05$).

Discussion

HAEC is the most common cause of morbidity and death in HD patients.^[14] It is characterized by an acute inflammatory infiltrate into the crypts and mucosa of the intestinal epithelium in both aganglionic and ganglionic segments. Despite many investigations and studies, the etiology of this complication is poorly understood. Multiple theories have been put forward including a physical dilatation of proximal bowel, variations in mucin components and production, rotavirus and so on.^[15,16]

Studies^[17-22] have confirmed that luminal bacteria could play a major role in the initiation and perpetuation of HAEC. Thousands of endogenous bacteria live in the large intestine and may be an essential factor for certain pathological disorders. In 1986, Thomas et al.^[16] found cytopathic toxin neutralized by *clostridium sordellii* antiserum in the feces of 7 (54%) out of 13 children with HAEC. *Clostridium difficile* was isolated in 10 (77%) of these 13 children. In the control groups, *Clostridium difficile* was isolated in 18% of children with HD and 30% of those without HD. It is suggested that *Clostridium difficile* may be causally related to enterocolitis in HD.^[16] There is the possibility that HAEC could be prevented by the development of a "benign" colonic bacterial flora and aggressive treatment of *Clostridium difficile* could improve this "benign" colonic bacterial flora development. This conception has become a very exciting theory.^[17]

Bifidobacteria and *lactobacilli* are involved in a

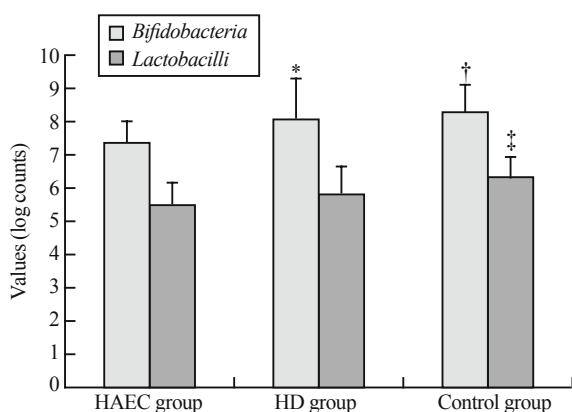


Fig. *Bifidobacteria* and *Lactobacilli* in fecal samples of the three groups. *: $P < 0.05$, compared with the HAEC group; †: $P < 0.001$, compared with the HAEC group. ‡: both $P < 0.05$, compared with the HAEC and HD groups respectively.

number of important probiotic microorganisms in the bowel. First, *bifidobacteria* and *lactobacilli* may take their action through a modulation of the intestinal bowel flora, which may result from competitive metabolic interactions with potential pathogens, production of anti-microbial peptides, or inhibition of epithelial adherence and translocation by pathogens.^[17] Second, *bifidobacteria* and *lactobacilli* have been proposed to modulate the host defenses by influencing the intestinal immune system.^[18] Third, these microorganisms have been reported to positively affect the intestinal barrier function.^[19]

It is important that reliable technologies are available for detection of *bifidobacteria* and *lactobacilli* in feces. Investigating the human colonic microbiota using culturing techniques has certain limitations, such as the requirement for samples to be processed quickly, the need for a specialized aerobic techniques, and low cell recoveries, while bacterial isolations and identifications are time-consuming and prohibitive for large scale studies. Gut colonization has been evaluated earlier by means of a platelet count analysis of fecal samples, but this is time-consuming and detects only cultivable bacteria which are estimated to account for only 10%-30% of all fecal bacteria.^[20] Recently, it has been shown that comparative analysis of 16S rRNA sequences can be used for culture-independent analyses of bacterial populations.^[21] Quantitative real-time PCR as an accurate, sensitive, specific, high throughput method, has been increasingly used for detecting and quantification of bacterial populations in many ecosystems. The PCR primers specifically detected and quantitated *bifidobacteria* and *lactobacilli* in feces. The method provided a highly sensitive means for identifying and quantitating these floras, and facilitated a high throughput bacterial analysis of biological samples.^[22]

In this study, the levels of *bifidobacteria* and *lactobacilli* decreased obviously in the feces of patients with HAEC. *Bifidobacteria* were at much higher mean levels than *lactobacilli* in the fecal samples over the entire time. This result is in accordance with others, showing that *bifidobacteria* dominate over *lactobacilli* in this age group.^[23] In our study, the *lactobacilli* colonization in the HAEC and HD groups was lower than that in the control group. The extent to which *lactobacilli* colonize the intestines of small children is controversial. Most studies have reported low *lactobacillus* colonization rates in Western countries,^[24] but some claimed that *lactobacilli* are present in substantial quantities (10^{7-9} cfu/g feces) in infant feces.^[25] In our study, *lactobacilli* in all fecal samples could be detected and quantified by real-time PCR. Variations in methodology may account

for the differences, since *lactobacilli* are difficult to identify by traditional biochemical methods. Now the development of genetic methods enables *lactobacilli* to be unequivocally identified and the question to be resolved. Another explanation may be that the expression of *bifidobacteria* or *lactobacilli* may be affected by gestational age, type of delivery, type of feeding, and antibiotic therapy. In this study, we took account of these factors.

The scarcity of *bifidobacteria* and *lactobacilli* in HAEC patients may result in a decrease in epithelial barrier function and be a predisposing factor in the development of HAEC. This decline suggests that treatment with probiotics or prebiotics may be beneficial in these individuals. Further research will focus on whether probiotics can decrease the incidence of HAEC.

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Competing interest: None declared.

Contributors: Shen DH and Chen JJ conducted the experiment, analyzed the data, and drafted the article. Shi CR contributed to conception and design, supervision, manuscript preparation. Yu SY contributed to acquisition of data and DNA extraction. All authors approved the final version for publication.

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