

Bacterial counts of intestinal *Lactobacillus* species in infants with colic

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Intestinal colonization by lactobacilli is suggested to be a prerequisite to normal mucosal immune functions. An inadequate level of lactobacilli may be involved in appearance of allergic disease of which, infantile colic, is often considered an early clinical manifestation. The aim of the study is to evaluate intestinal lactobacilli in breast-fed infants with infantile colic and healthy infants. Fifty-six breast-fed infants, aged 15–60 days were enrolled in the study and divided into two groups: colicky (30 cases) and healthy (26 cases) according to Wessel's criteria. Stool samples were collected, diluted and cultured on selective media. The colonies were counted, reported as colony forming unit (cfu) per gram of faeces and identified by biochemical methods. Different colonization patterns of lactobacilli were found among colicky and healthy infants. *Lactobacillus brevis* (4.34×10^8 cfu/g) and *L. lactis* (2.51×10^7 cfu/g) were found only in colicky infants while *L. acidophilus* (2.41×10^7 cfu/g) was found only in healthy infants. *Lactobacillus brevis* and *L. lactis* might be involved in the pathogenesis of infantile colic increasing meteorism and abdominal distension. Further studies are required to understand how the observed differences may be involved in the pathogenesis of this common disorder.

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Infantile colic is one of the most common problems in infancy with a frequency of 10–30% and can be described as follows: a healthy infant with paroxysms of excessive, high-pitched, inconsolable crying, frequently accompanied by flushing of the face, drawing up of the legs and passing of gas (1). Even if infantile colic is a common disorder, the aetiology is not completely understood. Abnormal gastrointestinal function and allergic problems such as food protein intolerance, seem to be related to this disorders (2). This syndrome begins before the sixth week of life in 90% of cases and generally resolves spontaneously by the fourth month, but sometimes might be considered the first clinical manifestation of atopic diseases (i.e. intolerance to milk cow's protein) (3).

It has been reported that intestinal microbiota plays an important role in the aetiopathogenesis

of allergic diseases. This is in accordance with Bjorkstein et al. who reported that allergic 2–3-yr-old children have an altered gut flora with a lower level of lactobacilli in comparison with healthy infants (4).

An aberrant gut microbial composition, such as inadequate lactobacillus level occurring in the first months of life, may affect the immune responses and could favour the development of different disorders (5, 6), such as infantile colic that could represent a precocious clinical manifestation of atopic diseases.

Lactobacilli could be considered one of the most attractive microbiota because of their role in the development of the immunophysiological regulatory mechanisms in the gut mucosa (6).

The aim of the present study was to establish if breast-fed infants with infantile colic have different patterns of intestinal lactobacilli from healthy breast-fed infants.

Materials and methods

Patient selection

The study recruited 56 colicky and non-colicky breast-fed infants, aged 15–60 days, seen in our Institution from May 2002 to April 2003. They were divided into two groups: colicky and controls. Colicky cry was defined as crying which cannot be consoled because of distinct pain which lasted for 3 h or more per day for 3 days or more in a week in an otherwise healthy infants, according to Wessel's criteria (1).

To prevent possible variability in the intestinal flora because of different kind of diet, all study-enrolled infants were exclusively breast-fed.

Infants were excluded if they received antibiotics and/or probiotics drugs and if they had clinical evidence of gastrointestinal disorders or other diseases that might mime colicky symptoms.

Parents gave written consent to the inclusion of their infants in the study. The study was approved by the local ethical committee.

Study design and sample collection

Before inclusion in the study, parents were given a structured questionnaire in order to obtain data concerning type of delivery, birthweight, crying time and family history of atopy. On entering the study, all infants were examined by a paediatrician. Sequential to recruitment, each breast-fed infant was assigned to one of the two groups, colicky or controls. One stool sample for each infant was collected. The faecal specimens were collected, transported to the Department of Public Health and Microbiology Laboratories and processed within 1 h after collection.

Bacteriological analysis

All samples were diluted 1:10 with normal saline. Further serial dilutions (10^{-2} to 10^{-9}) were made in normal saline and small volumes samples (100 μ l) were plated on Rogosa Bios agar (RG; Biolife, Milan, Italy).

All plates were incubated at 37°C for 3 days under microaerophilic and anaerobic conditions. The colonies count of different dilutions were recorded and all colonies of different morphology were cultured on De Man Rogosa Shape agar (MRS; BioMérieux, Rome, Italy) and incubated anaerobically for 24 h at 37°C. The microbial counts were reported as colony forming unit (cfu) per gram of faeces. The identification of lactobacilli species was achieved by biochemical methods using api 50CHL system

(BioMérieux) that allows the study of the carbohydrate metabolism of microorganisms. It consists of 50 microtubes each containing an anaerobic zone, for the study of fermentation and an aerobic zone for the study of oxidation or assimilation.

Statistical analysis

Statistic calculations were performed using commercially available software (Statsoft Windows, version Release 5, 1995; Statsoft Inc., Tulsa, OK, USA). Statistical significance was set at a $P < 0.05$.

Bacterial counts ($\log_{10}$ cfu/g) were calculated for each infant. Chi-squared test and, if significant, Fisher's exact test for two-by-two comparison was used to compare the distribution of categorical variables across groups.

Results

We studied 56 infants divided into two groups: colicky (30 cases) and non-colicky (26 cases). Infants characteristics are reported in Table 1.

Colicky infants had a higher rate of family history for atopic diseases (20/30) than controls (6/26) and a higher crying time (291 ± 35.9 min/day vs. 110 ± 40.1 min/day).

No significant differences were found between colicky and non-colicky infants in the rate of positiveness for lactobacilli (18 positives in controls and 20 positives in colicky infants) and in the amount of bacteria. The evaluation of the *Lactobacillus* species showed a different pattern of colonization in the two groups (Table 2).

Table 1. Characteristics (mean $\pm$ s.d.) of the study population

	Colicky	Non-colicky	Statistical analysis
Subjects (number of infants)	30	26	NS
Gender (M/F)	18/12	18/8	NS
Mean age $\pm$ s.d. (days)	32 ± 10	29 ± 13	NS
Type of delivery (Vaginal birth/ Caesarean section)	24/6	24/2	NS
Birthweight (g)	3464.14 ± 349.8	3508.56 ± 321.5	NS
Family history for atopy (i.e. almost one first degree parent with atopic diseases)	20	6	$P = 0.003$, CI 95% 0.168–0.692
Geographical (Italian/others)	30/0	26/0	NS
Crying time (min/day)	291 ± 35.9	110 ± 40.1	$P = 0.000$, CI 95% 125.73–172.27

Table 2. Positive infants for lactobacilli (positive cultures) and mean logarithms viable bacterial counts per gram of faeces calculated on the positive specimens of every species of *Lactobacillus* identified (mean counts and standard deviation)

	Controls (n = 26)	Colicky (n = 30)	Statistical analysis
<i>L. brevis</i>			
Positive cultures	0	9	$p = 0.007$
Mean (cfu/g)	0	4.34	CI 95%: 0.107–0.493
s.d.	0	1.58	
<i>L. lactis lactis</i>			
Positive cultures	0	7	$p = 0.028$
Mean (cfu/g)	0	2.51	CI 95%: 0.057–0.403
s.d.	0	9.71	
<i>L. acidophilus</i>			
Positive cultures	5	0	$p = 0.043$
Mean (cfu/g)	2.41	0	CI 95%: 0.041–0.339
s.d.	8.53	0	
<i>L. para-paracasei</i>			
Positive cultures	2	8	NS
Mean (cfu/g)	3.59	1.07	
s.d.	1.29	3.86	
<i>L. plantarum</i>			
Positive cultures	6	4	NS
Mean (cfu/g)	1.11	3.04	
s.d.	3.99	1.16	
<i>L. rhamnosus</i>			
Positive cultures	8	6	NS
Mean (cfu/g)	1.42	3.58	
s.d.	4.35	9.93	

Specifically, *L. brevis* (4.34 cfu/g; $P = 0.007$) and *L. lactis lactis* (2.51 cfu/g; $P = 0.028$) were found only in colicky infants while *L. acidophilus* (2.41 cfu/g; $P = 0.043$) was detected only in controls.

Discussion

Breast-feeding is a significant source of lactic acid bacteria probably because of combined action of breast milk components, such as antimicrobial compounds and prebiotic substances which may selectively stimulate the growth of lactic acid bacteria in the gut (7).

Lactobacilli consist of a diverse group of non-pathogenic, anaerobic, Gram-positive bacteria that produce lactic acid as a primary metabolic end product and are common constituent of human intestinal microbiota. This genus plays important physiological roles in the development of local and systemic immune responses (8). In particular, *Lactobacillus* spp., promote the immunological homeostasis of the host and oral tolerance induction (9).

Our data show different patterns of intestinal lactobacilli in colicky and non-colicky infants. The main difference found is the detection of *L. acidophilus* only in non-colicky infants. Some

authors reported that *L. acidophilus* plays a protective role by stimulating the immune system, the production of secretory IgA, enhancing macrophage activity and are also able to degrade food antigens (8–10). Literature data reported that lactobacilli can potentiate the production of cytokines (i.e. IFN- γ) that play an immunoregulatory role inducing good maturation and functionality of the gut-associated lymphoid tissue and promoting Th1–Th2 balance in building a correct intestinal barrier (9, 10). Close interaction between the intestinal immune system and gut microbiota is essential to maintain a disease-free state in the host, as the gut microbiota provide the immune system with stimuli resulting in immunological maturation and tolerance (11, 12). Moreover, it supports the view that non-pathogenic bacteria may directly alleviate intestinal inflammation through mechanisms altering signal transduction pathways of pro-inflammatory cytokines, in particular the NF- κ B transcription factor (13).

Our data show that infants with colic have a higher frequency of family history for atopy compared with control group indicating a further data in favour of the allergic hypothesis in the pathogenesis of colic (6, 14) even if some studies cannot support the relationship between infantile colic and allergic diseases (15).

It could be interesting to point out that we found *L. brevis* and *L. lactis lactis* only in colicky infants. These lactobacilli are both heterofermenting species producing, by glucose fermentation, ethyl alcohol and CO₂ as well as lactic acid and ATP. It is possible that alcohol influences intestinal motility (16) producing abdominal distension characteristic of colicky infants while CO₂ can lead to meteorism and flatulence also typical of this syndrome.

Our findings support the hypothesis that an inadequate balance of intestinal lactobacilli in infants with colic could lead to an immaturity of gut barrier and to aberrant antigen transfer and immune responses, thus explaining increased vulnerability to breaking down of oral tolerance, as reported by many authors (3, 8, 14).

In conclusion, culture-dependent mechanism may underestimate the actual numbers of the bacteria in faeces and the findings based on the study of faeces are indicative of the actual composition of microbiota in the gut but, even if preliminary in nature, our results suggest that differences in the composition of intestinal lactobacilli might influence the aetiopathogenesis of infantile colic, which is often considered an early clinical manifestation of atopy and confirm the crucial role of the balance of indigenous

intestinal bacteria for the maturation of human immunity to a non-atopic mode according to previous observations (4, 6, 17, 18).

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