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Lactobacilli and bifidobacteria in the feces of schoolchildren of two different socioeconomic groups: children from a favela and children from a private school

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Abstract

Objective: To determine the number of lactobacillus and bifidobacterium colonies in the feces of schoolchildren from two different socioeconomic levels.

Methods: We analyzed fecal samples of children aged 6 to 10 years without gastrointestinal symptoms or recent use of antimicrobials. The first group included 86 children living in a favela in the city of Osasco, state of São Paulo, southeastern Brazil. The second group included 36 children attending a private school in the same city. Body mass index (BMI) was used to assess nutritional status according to the reference values of the National Center for Health Statistics (NCHS). Specific anaerobic culture media were used for isolation of colonies for 48 and 72 hours at 37 °C. The number of colonies was determined using the plate-counting method.

Results: The mean lactobacillus (1.125×10^9 colony-forming units, CFU/g) and bifidobacterium (1.675×10^9 CFU/g) counts in the private school group were higher ($p < 0.001$) than those in the favela group: 0.250×10^9 and 0.350×10^9 CFU/g, respectively. In the favela group, children with BMI z score < -1.0 standard deviation (SD) ($n = 28$) showed lower mean ($p < 0.05$) lactobacillus (0.100×10^9 CFU/g) and bifidobacterium (0.095×10^9 CFU/g) counts than the children with BMI ≥ -1.0 SD ($n = 57$): 0.350×10^9 and 0.420×10^9 CFU/g, respectively.

Conclusion: The microbiota of schoolchildren living in unfavorable environmental conditions shows lower numbers of fecal lactobacillus and bifidobacterium colonies, especially in children with lower BMI values.

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Introduction

Lactobacilli and bifidobacteria are microorganisms of the intestinal microbiota, well-known for their beneficial effects for human health and therefore called probiotic bacteria. Probiotic bacteria predominantly colonize the colon, but might also have beneficial effects to the small intestine, as well as systemic effects via immune system.[1,2] A great number of these bacteria can be found in the colon of exclusively breastfed infants.[3-5] This is a very

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This study was conducted in the laboratory of the Discipline of Microbiology, Department of Microbiology, Immunology and Parasitology, and the Discipline of Pediatric Gastroenterology, Department of Pediatrics, Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil.

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important aspect since the composition of the colonic microbiota is established in the first months of life and tends to remain stable throughout life. Mode of delivery (vaginal or cesarean),[1-6] the first environmental influence on the intestinal microbiota, also plays an extremely important role. In later stages of life, exposure to inadequate environmental conditions may cause tropical (environmental) enteropathy. Tropical enteropathy can be defined as a diffuse subclinical atrophy of the villus architecture in the small intestine, associated with inflammatory T cell infiltration.[7-9] In tropical enteropathy, bacteria overgrowth in the proximal small intestine and alterations in indicator tests for enteral function may also occur, reflecting mucosal lesions.[10] It has been observed that, in areas at risk for environmental enteropathy, enteropathogens can be found even in the feces of children without diarrhea.[11] However, little research has been devoted to evaluate the participation of lactobacilli and bifidobacteria in the composition of the intestinal microbiota in areas that are most likely to develop environmental enteropathy. Studies[12,13] conducted in the 1970s, involving a small number of malnourished children, revealed changes in the colonic microbiota during nutritional therapy.

Therefore, the objective of this study was to evaluate the number of lactobacillus and bifidobacterium colonies in the feces of school-age children from two different socioeconomic levels. One of them consisted of children from a favela, an environment likely to expose them to the risk of environmental enteropathy. The number of lactobacillus and bifidobacterium colonies was also related to weight and height and the presence or not of diarrheagenic *Escherichia coli* in the feces.

Methods

Patients

This cross-sectional study, conducted between August 2006 and September 2007, analyzed fecal samples of 122 children: 86 children living in a favela and 36 children attending a private school. Our strategy intended to obtain two groups of two different socioeconomic levels. The project was approved by the Research Ethics Committee of Universidade Federal de São Paulo - Escola Paulista de Medicina (UNIFESP-EPM), and the children's parents or legal guardians signed a free informed consent form.

Eligibility criteria for both groups included the following: aged between 6 and 10 years and absence of diarrhea for over 30 days. Exclusion criteria were: use of antibiotics in the previous 15 days, clinical evidence of severe diseases, such as cardiopathy, nephropathy, chronic liver disease, immunodeficiencies, and chronic neuropathy, or hospitalization in the previous months.

The first group was formed in a favela popularly called "Morro do Socó", located in a neighborhood called Portal D'Oeste, on the outskirts of the city of Osasco, State of São Paulo, southeastern Brazil. Houses are sited on steep hillsides, in an area with difficult access, next to a sanitary landfill. Children were selected to compose a representative sample of this population. Residences in the area were selected by random numbers according to information obtained from Osasco Housing Department. A community leader identified the children in the randomly selected residences. Thus, a total of 100 children were selected for the study. Their parents or legal guardians were invited to participate in a meeting in which they were explained about all study procedures. Of the 100 children selected, 86 agreed to participate and performed complete laboratory examination. Therefore, this group represented a probability sample of children aged 6 to 10 years living in the area where the study was carried out.

The private school group comprised elementary students attending Oswaldo Cruz School in Osasco. The school committee of pedagogical coordination invited the students' parents or legal guardians to participate in the study. Of the 60 children whose parents or legal guardians showed interest in participating in the study, 43 actually agreed to participate. Of the 43 children seven (16.3%) did not perform all laboratory tests. Therefore, this group was composed of 36 children and is a convenience sample that includes all students who agreed to participate in the study.

Regarding sample size, we intended to have the same number of children in both groups, but this was not possible because private school students' parents showed little interest in participating in the study. Preliminary data from the pilot study allowed us to demonstrate that the number of children in the study was sufficient to identify a statistically significant difference for the main variable of the study, that is, the number of lactobacillus and bifidobacterium colonies in the feces of children from the two different socioeconomic groups.

To evaluate socioeconomic conditions, the children's mothers or legal guardians were interviewed. We used a structured questionnaire concerning demographic data, materials used in the construction of houses and availability of water supply, public sewage system and garbage collection, in addition to information necessary to the application of the Brazilian Economic Classification Criterion (Critério de Classificação Econômica Brasil).[14] This criterion classifies families in descending order, from A to E, according to possessions and schooling of the household head.

Methods

Nutritional status was assessed based on the children's weight and height (stature) measured according to the recommendation by Jelliffe.[15] Participants in underwear were weighted on a Filizola™ scale in a reserved place.

Standing height was measured without shoes using a portable wall-mounted stadiometer, and attention was given to this step to ensure that spine and feet were in a straight line. Weight-for-age, height-for-age and body mass index (BMI) z scores[16] were calculated using the Epi-Info software version 3.4.3,[17] according to the reference values of the National Center for Health Statistics (NCHS).[18]

The number of lactobacillus and bifidobacterium colony-forming units (CFU) in the feces was determined in a fecal sample collected by spontaneous evacuation. Within a 2-hour interval, feces were homogenized, and 1.0 g was extracted and diluted 1:5 in saline solution (1.0 g of feces + 4.0 mL 0.9% saline). The saline diluted sample was then homogenized and diluted again (1:100). From the 1:100 dilution, successive dilutions were performed (10^{-1} to 10^{-5}). For the cultivation 0.1 mL volume of each dilution was inoculated onto the surface of Rogosa SL (Difco) and Beerens agar plates for the cultivation of lactobacilli and bifidobacteria, respectively.[19] Plates were incubated under anaerobiosis at 37 ºC for 48 and 72 hours. After incubation, the number of CFU/g was determined and one colony of each selective medium was selected for morphologic analysis, after Gram staining.

Isolation and identification of diarrheagenic *Escherichia coli* in the feces was performed by biochemical and serologic methods, complemented by hybridization tests with genetic probes. Research was conducted by the staff of the laboratory of UNIFESP-EPM Discipline of Microbiology, using traditional methods.[20]

Statistical analysis was performed using the SigmaStat 3.5 software version 7.1 for Windows,[21] with a 5% significance level. Comparisons between groups were performed using the chi-square test or Fisher's exact test for the categorical variables and the Mann-Whitney test or Wilcoxon test for the continuous variables.

Results

Table 1 shows comparisons of age, sex, socioeconomic class, living conditions, and nutritional status. Both groups were matched for age. Regarding sex, there were more girls in the private school group (p = 0.029). The Brazilian Economic Classification Criterion showed that most families in the favela group belonged to classes C, D and E. In the private school group, families belonged to classes A and B, except for one single family that belonged to class C. In

Table 1 - Age, sex, socioeconomic class, public services at place of living, and weight-for-age, height-for-age and BMI z scores according to study group

Variables	Favela (n = 86)	Private school (n = 36)	p
Age (months)	100.0 (86.0; 111.0)	105.2 (88.8; 111.3)	0.495*
Sex			
Male	49 (57.0%)	12 (33.3%)	0.029†
Female	37 (43.0%)	24 (66.7%)	
Socioeconomic class			
A	0 (0.0%)	10 (27.8%)	
B	0 (0.0%)	25 (69.4%)	
C	27 (31.8%)	1 (2.8%)	< 0.0001†
D	41 (48.2%)	0 (0.0%)	
E	17 (20.0%)	0 (0.0%)	
Presence of illegal water supply connection	35 (40.7%)	0 (0.0%)	< 0.0001†
Garbage disposal in the public sewage system	8 (9.3%)	36 (100%)	< 0.0001†
Available garbage collection service	2 (2.3%)	36 (100.0%)	< 0.0001†
Weight-for-age z score	-0.77 (-1.31; -0.06)	+0.47 (-0.16; +1.58)	< 0.001*
Height-for-age z score	-0.25 (-0.86; +0.06)	+0.09 (-0.33; 0.85)	0.005
BMI z score	-0.58 (-1.35; -0.05)	+0.63 (-0.24; +1.67)	< 0.001*

BMI = body mass index.
* Median and percentiles (25th and 75th), Mann-Whitney test.
† Chi-square test with Yates' correction.
In 1/86 children of the favela group, weight, height, and data on the evaluation of socioeconomic class were not available.

the favela group, a large number of residences had illegal water supply connection, garbage disposal in the pit or open air and used open public garbage dumps. More than half (59.3%) of the houses in the favela were made of wood or wood and bricks. In contrast, all families in the private school group lived in brick-built houses, had legal water supply, garbage disposal in the public sewage system and garbage collection service available in the neighborhood.

The favela group had weight-for-age, height-for-age and BMI z scores lower ($p < 0.05$) than those observed in the private school group (Table 1).

Lactobacilli and bifidobacteria were not isolated in 8.1 (n = 7) and in 11.6% (n = 10), respectively, of the feces of the 86 children in the favela group, whereas lactobacilli and bifidobacteria were isolated in the samples of all children in the private school group. The proportion of children in the favela group with non-isolated fecal bifidobacteria (11.6%) was higher ($p = 0.032$, Fisher's exact test) than that of the private school group (0.0%). Lactobacillus and bifidobacterium colony counts (Table 2) were higher in the private school group in relation to the favela group ($p < 0.001$).

The Wilcoxon test showed that the number of bifidobacterium colonies was higher than that of lactobacillus colonies in both groups (favela, $p = 0.007$ and private school, $p = 0.002$). The Spearman coefficient showed a correlation between the number of lactobacillus and bifidobacterium colonies in each study group (favela, $r = +0.824$, $p < 0.000$ and private school, $r = +0.605$, $p < 0.000$).

To evaluate the relation between the number of lactobacillus and bifidobacterium colonies and nutritional status in the favela group, z scores were dichotomized at the cutoff point of -1.0 standard deviation (SD). As shown in Table 3, no statistically significant difference was observed in the number of lactobacillus and bifidobacterium colonies in children with weight-for-age and height-for-age z score < -1.0 SD, when comparing to those children with z score ≥ -1.0 SD. In the favela group, children with BMI z score < -1.0 SD showed lower lactobacillus and bifidobacterium counts than those with BMI z score ≥ -1.0 SD.

Table 4 shows the number of lactobacillus and bifidobacterium colonies according to the presence of diarrheagenic *Escherichia coli*, which were identified in 41 (51.9%) children in the favela group and in 6 (17.1%) children in the private school group. There was no statistically significant difference in the number of lactobacillus and bifidobacterium colonies, according to the presence or not of diarrheagenic *Escherichia coli*, between groups.

Discussion

In the city of Osasco, between 1980 and 2000, a fall was observed in infant mortality rates and in proportional mortality due to diarrhea.[22,23] However, groups living in unfavorable environmental conditions can still be detected in this city, including the region where the present study was carried out (Table 1).

The number of lactobacillus and bifidobacterium colonies in the feces of school-age children was lower in the children living in the favela than in those attending a private school. We could observe lower bacterial counts in the feces of children with BMI z score less than -1.0 SD.

Lactobacilli and bifidobacteria were isolated in all fecal samples collected in the private school. In the favela group, these bacteria were not isolated in the fecal samples of 10 (11.6%) of the 86 children. In Estonia and Sweden, lactobacilli and bifidobacteria were not found in the feces of 56.0 and 41.0%, respectively, of 27 allergic children at 2 years of age and in 40.0 and 29.0% of the 35 age-matched controls.[24]

These rates, both in the allergic and control groups, are higher than those observed in our study; however, age difference between the two samples should be taken into account.

Table 2 - Lactobacilli and bifidobacteria (CFU/g of feces) of children from both study groups

	Groups		
	Favela (n = 86)	**Private school (n = 36)**	**p**
Lactobacilli*	0.250×10^9 (0.070×10^9 – 0.750×10^9)	1.125×10^9 (0.500×10^9 – 1.950×10^9)	< 0.001
Bifidobacteria*	0.350×10^9 (0.050×10^9 – 0.900×10^9)	1.675×10^9 (0.900×10^9 – 2.650×10^9)	< 0.001

CFU/g = colony-forming units per gram.
* Median and percentiles (25th and 75th), Mann-Whitney test.

Although the proportion of individuals without lactobacilli and bifidobacteria in our study was lower than that found in the literature, repeated attempts to search for lactobacilli and bifidobacteria in initially negative individuals were performed to discard technical problems or issues related to the period of time between evacuation and beginning of sample processing. Such attempts, basically, confirmed our initial results.

Regarding the number of lactobacillus and bifidobacterium colonies, the counts obtained in the private school group were similar to those observed in the children attending Rubens Sverner Day Nursery of Hospital Israelita Albert Einstein (HIAE), in the city of São Paulo.[25] It is worth mentioning that only employees' children attended this nursery, i.e., this is not a charity day nursery, as other services offered by HIAE are, such

Table 3 - Lactobacilli and bifidobacteria (CFU/g of feces) according to nutritional indicators observed in the children in the favela group

	Z score		
	-1.0 SD	**≥ -1.0 SD**	**p***
Weight-for-age z score	(n = 32)	(n = 53)	
Lactobacilli	0.100×10^9 (0.035×10^9 – 0.670×10^9)	0.350×10^9 (0.097×10^9 – 0.762×10^9)	0.122
Bifidobacteria	0.130×10^9 (0.022×10^9 – 0.950×10^9)	0.400×10^9 (0.097×10^9 – 0.862×10^9)	0.270
Height-for-age z score	(n = 18)	(n = 67)	
Lactobacilli	0.150×10^9 (0.010×10^9 – 0.400×10^9)	0.290×10^9 (0.080×10^9 – 0.787×10^9)	0.210
Bifidobacteria	0.170×10^9 (0.050×10^9 – 1.050×10^9)	0.350×10^9 (0.076×10^9 – 0.847×10^9)	0.522
BMI z score	(n = 28)	(n = 57)	
Lactobacilli	0.100×10^9 (0.015×10^9 – 0.450×10^9)	0.350×10^9 (0.097×10^9 – 0.860×10^9)	0.015
Bifidobacteria	0.095×10^9 (0.020×10^9 – 0.625×10^9)	0.420×10^9 (0.100×10^9 – 1.062×10^9)	0.032

BMI = body mass index; CFU/g = colony-forming units per gram; SD = standard deviation.
* Median and percentiles (25th and 75th), Mann-Whitney test.
In 1/86 of the children studied, weight and height were not available.

Table 4 - Lactobacilli and bifidobacteria (CFU/g of feces) according to the presence of diarrheagenic *Escherichia coli* in the study groups

	Diarrheagenic *Escherichia coli*		
	Positive	**Negative**	**p***
Favela group	(n = 41)	(n = 38)	
Lactobacilli	0.210×10^9 (0.047×10^9 – 0.575×10^9)	0.195×10^9 (0.070×10^9 – 0.800×10^9)	0.772
Bifidobacteria	0.400×10^9 (0.047×10^9 – 0.775×10^9)	0.235×10^9 (0.080×10^9 – 0.900×10^9)	0.630
Private school group	(n = 6)	(n = 29)	
Lactobacilli	1.175×10^9 (0.060×10^9 – 1.700×10^9)	1.100×10^9 (0.500×10^9 – 2.012×10^9)	0.554
Bifidobacteria	1.150×10^9 (0.600×10^9 – 2.200×10^9)	1.800×10^9 (0.950×10^9 – 2.700×10^9)	0.405

CFU/g = colony-forming units per gram.
*Median and percentiles (25th and 75th), Mann-Whitney test.

as the outpatient clinic available to the population of the favela called "Paraisópolis". In this nursery, using a similar methodology for microbiological evaluation, the authors found between 0.86 and 1.30 x 10^9 CFU/g of lactobacilli and 1.20 and 1.30 x 10^9 CFU/g of bifidobacteria.[25] In the present study, the mean lactobacillus count in the private school was 1.125 x 10^9 CFU/g, higher than that observed in 2-year-old non-allergic Swedish and Estonian children[24] (median = 0.53 x 10^9 CFU/g), in 68 Japanese subjects[26] under 20 years of age (0.48 x 10^9 CFU/g), and in 5 English children[27] aged 16 months to 7 years. In addition, the mean number of bifidobacterium colonies (1.675 x 10^9 CFU/g) was also higher than that found in those studies: Swedish and Estonian[24] (median = 0.93 x 10^9 CFU/g), Japanese[26] (1.01 x 10^9 CFU/g) and English[27] (0.98 x 10^9 CFU/g). Such difference might result from differences in the culture techniques employed and from age differences, taking into account that the number of colonies may vary in children, adults and elderly people.[27] Other factors related to environment and life style may also affect the composition of the colonic microbiota. In the study conducted with Swedish and Estonian children, the (above mentioned) number of colonies in the controls was compared to that of allergic children, and no statistically significant difference was found.[24] On the other hand, in the study carried out in Japan,[26] children with atopic dermatitis showed less lactobacillus (0.527 x 10^9 and 0.489 x 10^9 CFU/g, respectively) and bifidobacterium (0.975 x 10^9 and 1.01 x 10^9 CFU/g) colonies than the controls, with statistically significant differences.

In this study, differences between mean lactobacillus and bifidobacterium counts were more significant than those observed in the Japanese children[26] with atopic dermatitis. Therefore, differences in colonic microbiota seem to be more intensely affected by the unfavorable favela environment than by influence associated with abnormalities in the immune system of individuals with atopic dermatitis. The present study was developed along with other projects[28,29] targeting the assessment of additional health and nutritional aspects of children living in the favela. We observed that favela children without diarrhea, in relation to private school children, showed increased frequency of bacterial overgrowth in the small intestine (28.2 and 3.0%, respectively)[28] and of diarrheagenic *Escherichia coli* (51.9 and 17.1%).[29] Thus, a small number of lactobacillus and bifidobacterium colonies might, hypothetically, be a part of the intestine global response to unfavorable environmental conditions. However, within this setting, no difference was observed in the number of lactobacillus and bifidobacterium colonies concerning the presence or not of diarrheagenic *Escherichia coli* in the feces of children living in the favela (Table 4). In such situation, probiotic bacteria would be expected to compete with diarrheagenic *Escherichia coli*. Evidence from the literature suggests that the increased number of lactobacillus and bifidobacterium colonies in the gastrointestinal tract is beneficial, since these colonies might inhibit or prevent adhesion[30-32] and provide a competition for receptors in the intestinal mucosa,[33] as well as protection against cell lesions caused by enteropathogenic bacteria.[34,35] Therefore, it was not possible to confirm such theory with the methods used in the present study, pointing out that the children were without diarrhea when the study was carried out. The questionnaire used in this project did not include specific questions about occurrence of atopic diseases. In this context, it is worth mentioning that there is no complete agreement among studies on the prevalence of allergic diseases in different socioeconomic groups.[36,37] We assume that low socioeconomic status and intestinal parasitosis are likely to be associated with a reduced risk of allergic diseases, in which, hypothetically, lower lactobacillus and bifidobacterium counts would be expected.[38,39] Therefore, the lower lactobacillus and bifidobacterium counts observed in the low-socioeconomic group in the present study seem not to confirm such hypothesis.[24,26]

Data shown in Table 3 demonstrate that children with BMI z score < -1.0 SD had less lactobacillus and bifidobacterium colonies, with a statistically significant difference. The choice of this cutoff point allowed a characterization of decreased counts of potentially probiotic bacteria in individuals with lower BMI values. This result could not be achieved if the traditional -2.0 SD cutoff point was adopted; taking into account the small number of children that would be included in this category (7 of 86: 8.2%, in the favela group, and 1 of 36: 2.8%, in the private school group – results not shown). In an analysis of the past 10-year literature, we could not find information on colonic microbiota in protein-calorie malnourished children. In the 1970s, studies[12,13] carried out in Guatemala evaluated the microbiota in the digestive tube of children with malnutrition associated or not with diarrhea. In one of these studies,[12] the colonic microbiota of four children with severe protein-calorie malnutrition was prospectively evaluated before nutritional treatment, during the stabilization phase and in two time points of the recuperation phase. An increase in the number of colonies of colonic anaerobes, which included lactobacilli and bifidobacteria, was detected, reverting the inversion of the ratio anaerobes/aerobes observed in malnutrition.[12] The other article,[13] which evaluated children with acute diarrhea secondary to *Shigella dysenteriae* infection, demonstrated in eight patients an increase in the sum of lactobacillus and bifidobacterium colonies between the third and the tenth day of antibiotic therapy.

Over recent years, probiotics have been used in the prevention of gastrointestinal abnormalities and in malnutrition induced in experimental animals.[40,41] On the other hand, findings from recent experimental studies in animals show that intestinal microbiota might interfere in the regulation of the energy balance.[42] In Finland, a group of 25 overweight children aged 7 years showed

a lower number of bifidobacterium colonies and a larger number of *Staphylococcus aureus* colonies in their colonic microbiota at the end of their first year of life, when compared to 24 normal-weight schoolchildren.[43] In the present study, overweight was observed in 5 (13.9%) of the 36 children attending private school, although the number of lactobacillus and bifidobacterium colonies in the feces of these children did not differ significantly from that observed in normal-weight children in the same group (results not shown). This information, however, should be analyzed with caution, considering the small number of children included in the private school group.

In conclusion, the microbiota of school-age children living in unfavorable environmental conditions shows reduced numbers of fecal lactobacillus and bifidobacterium colonies, especially of those children with lower BMI values.

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