Capability of the Two Microorganisms *Bifidobacterium breve* B632 and *Bifidobacterium breve* BR03 to Colonize the Intestinal Microbiota of Children

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Background: The total number of bacteria present in the gut microbiota of a newborn is consistently lower than the average found in adults, with the extent of this difference being directly related to body weight and age. It could be assumed that a lower number of viable probiotic cells is necessary to achieve significant gut colonization in infants and children. This study assessed the capability of *Bifidobacterium breve* B632 (DSM 24706) and *Bifidobacterium breve* BR03 (DSM 16604), 2 strains able to significantly inhibit some gram-negative bacteria in vitro, to integrate into the intestinal microbiota of children.

Materials and Methods: Ten healthy children aged an average of 5.7 ± 2.6 were given an oily suspension containing *B. breve* B632 and *B. breve* BR03 for 21 consecutive days. The daily dose was 100 million live cells of each strain. Fecal specimens were collected and analyzed at the beginning (d_0) and at the end of the study (d_{21}). Total fecal bifidobacteria and coliforms have been quantified by microbiological plate counts.

Results: A significant increase in total fecal bifidobacteria (from 8.99 to 9.47 $\log_{10}$ CFU/g, $P = 0.042$) and a parallel decrease in total coliforms (from 8.60 to 7.93 $\log_{10}$ CFU/g, $P = 0.048$) was recorded after 21 days of supplementation.

Conclusions: An oily suspension has proved an effective way of providing probiotics to children. A lower viable cells concentration was sufficient to mediate this effect in the light of the fact that the intestinal microbiota of children harbors a considerably smaller amount of total bacteria compared with adults. In addition to gut colonization in healthy children, *B. breve* B632 and *B. breve* BR03 were able to decrease total fecal coliforms, therefore supporting their potential specific use in colicky infants.

Key Words: bifidobacteria, gut colonization, intestinal microbiota, coliforms, gaseous colic

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As part of the normal microbiota, the intestinal microbial community plays a very important role in terms of both quantity and influence exerted on the host's health, so it has been described as one of the most complex bacterial ecosystem known to date.[1] The processes leading to the development of a stable gut microbiota are complicated and involve a succession of different microbial types from infancy to adulthood.[2]

In the womb, the fetus is in a sterile environment, but, following birth, the newborn is exposed to microbes from the mother and the surrounding environment.[3] During lactation, the *Bifidobacterium* genus is predominant (10^{10} to 10^{11} CFU/g of feces), but *Bacteroides* and *Clostridium* are also well represented (10^8 to 10^9 CFU/g of feces). A less diverse gut microbiota with high counts of *Bacteroides*, *Clostridium*, *Enterobacteriaceae*, and *Staphylococcus* early in life has been associated with an increased risk for atopic disease.[4]

The gut microbiota is also a critical stimulus for the adequate maturation of the immune system, which contributes to reducing infections and aberrant immune responses.[5]

Recent studies have revealed diversity in the composition of the intestinal microbiota in infants with gaseous colic compared with healthy babies, with particular reference to the number of gas-producing coliforms that are present in higher concentrations in infants suffering from colic.[6] A study by de Weerth et al[7] showed that proteobacteria were significantly increased in infants with colic compared with control infants, with a relatively abundance >2-fold. In contrast, bifidobacteria and lactobacilli were significantly reduced in infants with colic. Moreover, the colic phenotype correlated positively with specific groups of proteobacteria, including bacteria related to *Escherichia*, *Klebsiella*, *Serratia*, *Vibrio*, *Yersinia*, and *Pseudomonas*, but negatively with bacteria belonging to the Bacteroidetes and Firmicutes phyla, the latter of which comprises some lactobacilli and canonical groups notorious for producing butyrate and lactate.[7] An alteration in the composition of the intestinal microbiota, therefore, as well as excessive production of gas from the microbiota itself, may favor the onset and maintenance of colic.

Colic is characterized by paroxysmal and inconsolable crying, accompanied by agitation, facial flushing, flexion of the lower limbs, and abdomen gas emissions, in an otherwise healthy infant.[8] Although colic is common in the first months of life, with an incidence ranging from 3% to 30%, the etiology is still unclear and is likely to be considered multifactorial.[9]

The therapeutic approach to dealing with colic is variable and poorly standardized. A recent approach for the alleviation of the most important symptoms associated with colic is the administration of probiotics to infants during the first months of life. As is widely recognized, a key factor for a probiotic to exert its beneficial action is the ability to integrate into the resident gut microbiota.[10]

In this regard, the total number of bacteria present in the intestinal microbiota of a newborn is consistently lower

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than the average found in adults, with the extent of this difference being directly related to body weight and age. It could be assumed that a lower number of viable probiotic cells is necessary to achieve significant gut colonization in infants and children.

This study assessed the capability of *B. breve* B632 (DSM 24706) and *B. breve* BR03 (DSM 16604), 2 strains able to significantly inhibit some gram-negative bacteria in vitro,[11] to integrate into the intestinal microbiota of children when supplemented at a dose lower than that normally taken by adults.

MATERIALS AND METHODS

Study Design

Ten healthy children aged an average of 5.7 ± 2.6 years were enrolled in this pilot study between September and October 2012 by their medical practitioner. The exclusion criteria were clinical evidence of chronic diseases or gastrointestinal disorders, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) or other similar drugs, the assumption of any probiotic product or antibiotics, treatment with acid-suppressant proton pump inhibitors or H2 receptor blockers, as well as participation in other clinical trials in the 2 weeks before enrollment.

Parents were properly instructed with regard to both the dose of the product to be administered to their children and the simple procedure for the collection of fecal samples. Each family was then given a vial filled with 9 mL of an oily suspension containing *B. breve* B632 and *B. breve* BR03 and instructed to give the child 5 drops a day in the morning on an empty stomach, at least 30 minutes before breakfast, for 21 consecutive days. The daily dose was 100 million live cells of each strain.

Quantification of Specific Microbial Groups in the Feces

Fecal specimens were collected and analyzed at the beginning (d_0) and at the end of the study (d_{21}). Samples for the enumeration of specific bacterial groups of gut microbiota (about 10 g) were collected in sterile plastic containers previously filled with 20 mL of Amies transport liquid (BD Italia, Milan, Italy), stored at 4°C in the parental home and delivered to the laboratory (Biolab Research Ltd., Novara, Italy) within 24 hours after collection. The weighed samples were transferred to a sterile container (Stobag), diluted with Amies liquid to achieve 1:10 wt/vol and homogenized in a Stomacher for 4 minutes at 230 rpm. Samples were then decimally diluted using a sterile saline and 0.1 mL of the appropriate dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} for total bifidobacteria; 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} for total coliforms) were plated on selective cultural agarised media. In particular, total coliforms were counted on Petrifilm CC (3M; Segrate, MI, Italy) and total bifidobacteria on TPY agar added to 12 μg/mL of nalidixic acid (Sigma, St Louis, MO).[12] Coliforms were incubated in aerobic conditions at 37°C for 24 hours, whereas bifidobacteria were incubated at 37°C for 48 hours under anaerobic conditions (GasPak system) with Anaerocult A kits (Merck, Darmstadt, Germany). Colonies were counted and results expressed as $\log_{10}$ of colony forming units (CFU) per gram of fresh feces.

Statistical Analysis

All values relating to the concentration of total bifidobacteria and total coliforms are expressed as mean number of viable cells/gram of feces ± SD (m ± SD). Paired *t* tests were used to weigh the results and compare them between d_0 and d_{21}. Differences were considered significant at $P \leq 0.05$.

RESULTS

Quantification of Specific Microbial Groups in Fecal Samples

All children completed the study protocol, therefore no dropouts were recorded. A significant increase in total fecal bifidobacteria (from 8.99 to 9.47 $\log_{10}$ CFU/g, $P = 0.042$) and a parallel decrease in total coliforms (from 8.60 to 7.93 $\log_{10}$ CFU/g, $P = 0.048$) was recorded after 21 days of supplementation (Table 1 and Fig. 1).

DISCUSSION

The development of the intestinal microbiota occurs primarily during infancy, and a distortion could potentially contribute to a wide range of diseases. Molecular techniques have improved our understanding of the infant gut ecosystem. Mode of delivery as well as type of nutrition, that is, breastfed versus formula fed, are considered to be key factors that provide differential colonization opportunities and thus composition of the neonatal gut microbiota.[13,14] High levels of bifidobacteria in the infant gut have been associated with the timely and appropriate development and maturation of the immune system.[15]

Bifidobacteria are gram-positive, non-spore forming, nonmotile, and anaerobes. The species belonging to the genus *Bifidobacterium* are generally considered safe to use in humans. *B. breve* is a species originally isolated from the feces of a newborn: it is typical of the baby, both breastfed and formula fed, and is the dominant species in breastfed infants.[16]

Several studies support the use of probiotics for the treatment of minor gastrointestinal problems in infants. Positive effects on newborn colic have been shown after administration of *Lactobacillus* strains, whereas no studies have been reported regarding the use of bifidobacteria for this purpose. A recent paper by Aloisio et al[17] was aimed at the characterization of *Bifidobacterium* strains capable of inhibiting the growth of pathogens typical of the infant gastrointestinal tract and of coliforms isolated from colicky newborns. Among the 46 *Bifidobacterium* strains considered, 16 showed high antimicrobial activity against potential pathogens. The examination of all different features made it possible to identify 3 *B. breve* strains and a *Bifidobacterium*

TABLE 1. Quantification of Total Bifidobacteria and Total Coliforms in Fecal Samples

Parameters	Baseline	Day 21	*P*
Total bifidobacteria	8.99 ± 1.08	9.47 ± 0.87	0.042
Total coliforms	8.60 ± 1.11	7.93 ± 1.23	0.048

The data are expressed as mean ± SD values ($\log_{10}$ CFU/g of feces) of 3 independent analysis. *P* values are calculated using Student *t* test and considered significant when $P < 0.05$.

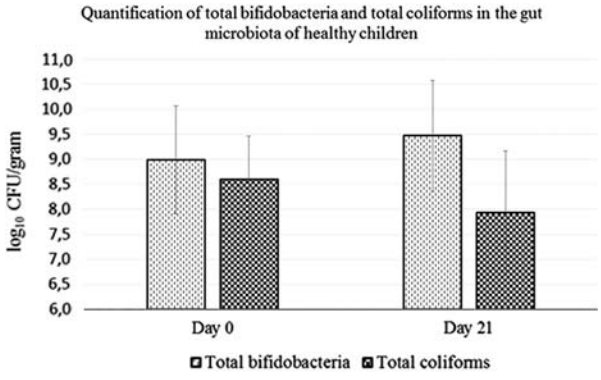

FIGURE 1. Concentration of total bifidobacteria and total coliforms in the feces of children at baseline and after 21 days of supplementation. Mean values ± SD are reported.

longum subsp. *longum* as potential probiotics for the treatment of enteric disorders in newborns such as infantile colic.

The mode of action of bifidobacteria and *B. breve* in particular is attributed, at least in part, to an ability to exert an inhibitory effect against harmful or even pathogenic microorganisms through a combination of different mechanisms, including the secretion of organic acids and the production of more specific antimicrobial substances, although no bacteriocin has so far been isolated from any *B. breve*. Bacteriocins are bacterially produced peptides that are active against other bacteria and against which the producer has a specific immunity mechanism.[18]

The 2 strains used in this study, which are *B. breve* B632 and *B. breve* BR03, showed the ability to integrate into the microbiota of healthy children, as suggested by the 0.48 $\log_{10}$ increase in total fecal bifidobacteria after 21 days of supplementation. An oily suspension has proved an effective way of providing probiotics to children. A lower viable cell concentration was sufficient to mediate this effect in the light of the fact that the intestinal microbiota of children harbors a considerably lesser amount of total bacteria compared with adults. In this way, 100 million viable cells of each strain, that is, a 10 times lower in amount than the minimum quantity generally considered as sufficient to achieve gut colonization in adults (1 billion), guaranteed a significant increase in fecal bifidobacteria.

In addition, they were able to decrease total fecal coliforms in parallel after a 3-week supplementation treatment, therefore confirming their prospective valuable use in colicky infants. In any case, further studies specifically involving newborns suffering from gaseous colic will be needed to confirm the positive evidence emerging from this pilot study. A validation clinical trial involving the selected strains is being planned.

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