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A Community-based Randomized Double Blind Controlled Trial of *Lactobacillus paracasei* and *Bifidobacterium lactis* on Reducing Risk for Diarrhea and Fever in Preschool Children in an Urban Slum in India

R. Hemalatha[1], Arthur C. Ouwehand[2*], Sofia D. Forssten[2], J. J. Babu Geddan[1], Raja Sriswan Mamidi[1], V. Bhaskar[3] and K. V. Radhakrishna[1]

[1]Clinical Division, Immunology and Microbiology, National Institute of Nutrition, Hyderabad, India.
[2]Active Nutrition, DuPont Nutrition and Health, Sokeritehtaantie 20, FIN-02460 Kantvik, Finland.
[3]Division of Biostatistics, National Institute of Nutrition, Hyderabad, India.

Author's contribution

This work was carried out in collaboration between all authors. Authors HR and ACO designed the study, author VB performed the statistical analysis, authors HR and ACO wrote the protocol, authors HR and KVR wrote the first draft of the manuscript. Authors SDF, JJB and RSM managed the analyses of the study. Authors HR, KVR and ACO managed the literature searches. All authors read, commented and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of the study was to determine the effect of probiotics on diarrhea and fever in preschool children in a community setting in a developing country.
Study Design: Double blind randomized controlled trial.
Place and Duration of Study: The study was performed in Addagutta; a slum of Hyderabad (India), from July 2010 to April 2011.
Methodology: Healthy preschool children (2-5 years, n=379) in an Urban Slum in India.

**Corresponding author: Email: arthur.ouwehand@dupont.com;*

Three randomly allocated groups of children received either of the two probiotics (*Lactobacillus paracasei* Lpc-37 *and Bifidobacterium lactis* HN019) or the placebo for a period of 9 months and were assessed for weight gain, linear growth and incidence of diarrhea and fever.

Results: Neither of the tested probiotics; *L. paracasei* Lpc-37 or *B. lactis* HN019 had any influence on weight gain or linear growth. There was no significant difference between the groups in incidence of diarrhea and fever when assessing the whole study period. However, during the wet season, in the months of August and September, incidence of diarrhea was significantly higher in placebo group (16.9%) compared to *L. paracasei* Lpc-37 (11.7 %) and *B. lactis* HN019 groups (8.4 %). Similarly, the incidence of fever was significantly higher in the month of August in the placebo group (11.5%) compared to the *L. paracasei* Lpc-37 group (7%) and *B. lactis* HN019 group (7.3%). Probiotic supplementation had no effect on fecal calprotectin, but fecal IgA and serum interleukin 8 were decreased significantly in the *B. lactis* HN019 group compared to placebo. Consumption of *L. paracasei* Lpc-37 lead to increased levels of fecal *L. paracasei*.

Conclusion: During the rainy season, when incidence of fever and diarrhea was highest, the administered probiotics reduced the incidence of these symptoms. Over the whole study period, the probiotics did, however, not influence incidence of diarrhea or fever.

Keywords: Probiotics; Lactobacillus paracasei; Bifidobacterium lactis; diarrhea; fever; children; developing country.

1. INTRODUCTION

Diarrhea is a major cause of mortality among under-five year old children and is an important public health problem in India. In 2010, diarrhea was responsible for 13% of child deaths in India and remains the second leading cause of death among children under five years globally [1]. Worldwide, nearly one in five child deaths or about 1.5 million each year is due to diarrhea [2]. Together, pneumonia and diarrhea account for 40 per cent of all child deaths around the world each year. Developing countries such as Africa (46%) and Asia (38%) contribute to 80% of all deaths of diarrhea across the globe, and India ranks number 1 among the developing countries in the total number of deaths due to diarrhea, with an estimated 386 600 children dying annually [3].

Children are more susceptible to diarrhea because of the immaturity of their immune system [4]. Children are also at greater risk than adults of life-threatening dehydration since water constitutes a greater proportion of children's bodyweight [2]. Prevention of acute diarrhea in children is an important public health challenge because a simple, safe, and cost-effective intervention to prevent acute diarrhea and its adverse health effects would have considerable public-health implications. Key measures that have been proposed to prevent diarrhea in young children include water sanitation, immunization, adequate nutrition, breast feeding and micronutrient supplementation (vitamin A and zinc) [2]. However, new interventions are needed to achieve substantial reduction in diarrheal morbidity and mortality in children under 5 years.

There is growing evidence that probiotic supplementation in both healthy and malnourished children reduces the duration of infectious and antibiotic associated diarrhea in young children [5,6]. Probiotics are live organisms that, applied to animals or human beings, beneficially affect the host by improving the properties of indigenous microbiota, hampering the growth of diarrheal pathogens, and boosting cellular and humoral immunity [7]. Although

there is evidence for the therapeutic benefits of probiotics in viral [8] or antibiotic associated diarrhea [9] among children, evidence for the role of probiotics in preventing acute diarrhea in young children in a community setting is less clear. There are very few studies that have assessed the effect of supplementation of probiotics in healthy preschool children in India [10]. Our objective was to study the effect of *Lactobacillus paracasei* Lpc-37 and *Bifidobacterium animalis* ssp. *lactis* HN019 supplementation on the incidence of diarrhea and fever in preschool children residing in an urban slum in India.

2. MATERIALS AND METHODS

2.1 Study Setting and Volunteers

The study was approved by the Scientific Advisory Committee (SAC) as well as the Institutional Review Board (IRB) of the National Institute of Nutrition (NIN, Hydrabad, India). The study has been registered in Clinical Trial Registry India; CTRI/2012/08/002942. Written informed consent was obtained from the parents or legal guardians of all the participating children. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki [11].

This study was carried out in the area of Addagutta, which is the largest slum in the capital city of state of Andhra pradesh, Hyderabad, India. Slum in the context of this article refers to a low income community. An initial screening of all households (750 households) with children aged 2-5 years was done for assessment of eligibility and enrollment (Fig. 1). All children of the same socioeconomic background were enrolled in the study. Enrolled children were those whose parents or legal guardians provided written informed consent and who did not meet any of the exclusion criteria. Excluded were children with major congenital birth deformities, acute illness at enrollment, chronic conditions affecting food intake or metabolism, participation in another clinical study and past history of surgical operation of gastrointestinal tracts.

2.2 Randomization

The study was a community based, prospective, cluster randomized, double blind, and placebo-controlled trial. Three clusters were randomized from the study area community and were coded. For easier management of the study and less manpower, cluster randomization was done. All the children from cluster A received product A and cluster B received B and cluster C received product C. The clusters were given an identification number and were assigned a treatment code by the senior scientist supervising the randomization. The randomization list and the supplements were given to the senior scientist at the institute who had no knowledge of the codes. All the investigators, including the medical doctor collecting clinical data and those collecting anthropometric measurement, as well as the statistician, were blind to the treatment. After completion of the data analysis, the groups were decoded. Thus, all the investigators involved in data collection, analysis and interpretation were blind to allocation. The parents or the caretaker of the children was unaware of the real nature of the product.

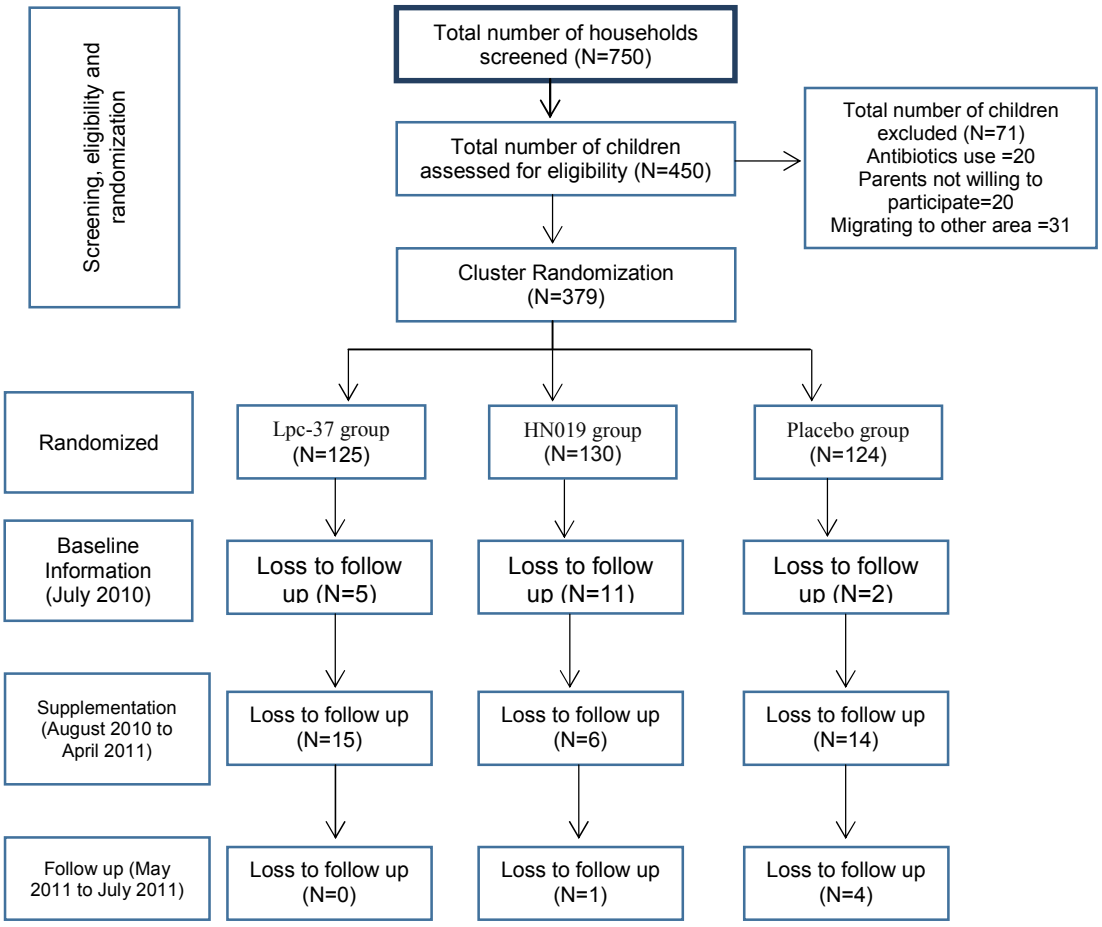

Fig. 1. Flow diagram of recruitment and allocation of volunteers through the phases of the trial. Lpc-37 group received *Lactobacillus paracasei* Lpc-37 and HN019 group received *B. animalis* ssp. *lactis* HN019. The children that were excluded prior to randomization never started the intervention and were hence not included in the analyses

2.3 Probiotic Supplementation

The probiotic bacteria, *Lactobacillus paracasei* Lpc-37 (ATCC SD5275) or *Bifidobacterium animalis* ssp. *lactis* HN019 (AGAL NM97/09513), each at a dose of 2-5x10^9 CFU/day in micro crystalline cellulose and placebo (micro crystalline cellulose) were prepared and were supplied in identical appearing capsules (packed in coded identical looking bottles) by Danisco USA (Madison, WI, USA). The probiotics and placebo were of the same color, weight, smell, and taste (normal taste of fermented milk product, without added flavor). The probiotic and placebo products were sent by the manufacturer to NIN in two batches at the beginning and middle of the study. The manufacturer performed tests at each shipment with the aim to assess stability and concentration of the probiotics. Probiotic supplementation to each and every child was directly supervised by the field investigators of the study who visited the study children every day to collect morbidity data. Contents from each capsule was suspended in 50ml milk and mixed thoroughly before giving it to the study children. All

children involved in the study received supplementation with one of the investigational products for a period of nine months (from August 2010 to April 2011).

2.4 Morbidity Data Collection

The primary outcome of the study was the incidence and duration of gastrointestinal complaints; in particular diarrhea, during the intervention period. Secondary outcomes were incidence and duration of fever and symptoms of respiratory tract infections (cough, runny nose) during the intervention period.

Data on morbidity of the children were collected for one complete year, that is one month prior to supplementation and were continued for one year to complete all seasons. The field investigators were trained and quality control testing for interrater reliability and reproducibility were done every three months. Diarrheal episodes separated by three symptom free days were considered as separate episodes. Diarrhea was defined as passing of more than three stools in 24 hours or stools with altered consistency, with or without mucus or blood or loose watery stools. A child was considered to have fever, if the caretaker reported fever (≥38ºC) during the previous day. Morbidity data on diarrhea and fever were collected on a daily basis. This was possible as the field investigator provided and supervised supplementation of probiotic daily. At baseline, morbidity data was collected for one month (July 2010) prior to the supplementation (intervention), during the intervention period for nine months (August 2010 to April 2011) and for a period of three months after the supplementation (May 2011 to July 2011).

2.5 Anthropometry

Weight (every month), height (once in every fourth month), Skin fold thickness at four sites were collected at baseline. Weight was measured to the nearest 100 grams using digital weighing scale (SECA, Hamburg, Germany) and height was measured to the nearest centimeter using measuring height rod (GPM anthropological instruments, Zurich, Switzerland).

2.6 Blood and Stool Collection

Stool (fecal) samples were collected from all children and blood samples were collected from a sub sample (N=63) of children. Blood sample was collected at the end of supplementation and was used to measure serum interleukins (IL-8, IL-10, IL-17) and macrophage inflammatory protein (MIP-1b) using ELISA kits (BIO-RAD, Hercules, CA, USA), according to the manufacturer's instructions. Stool samples were collected in sterile, DNase free plastic containers and were preserved at -80°C until analysis or transport to Finland on dry ice for further analysis. Stool samples were collected before, during (6 months after supplementation) and at the end of supplementation. The total quantity of faecal bacteria was determined by using a flow cytometric FACS Calibur-system (BD Biosciences, San Jose, CA, USA) as previously described [12]. Shortly, samples were fixed with 4% formaldehyde and stained with a fluorescent, nucleic acid binding dye, SYTO 24 (Molecular Probes, Leiden, the Netherlands).

Total *Lactobacillus* spp, *Bifidobacterium* spp, as well as the species of the administered probiotics, *Bifidobacterium lactis* and *Lactobacillus paracasei* were analyzed from the stool samples by qPCR. Bacterial DNA was extracted from the stool samples by an initial bead beating step of two 3x30s cycles at 6800rpm, thereafter a modified version of the Promega

Wizard genomic DNA purification kit (Promega, Madison, WI, USA) was used as described before [13]. The bacterial DNA concentrations were measured by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at -20ºC until further analyzed by qPCR.

One ng of extracted microbial DNA was analysed by qPCR with the ABI-PRISM 7500 sequencing detection system (Applied Biosystems Bridgewater, NJ, USA), using primers specific for *Lactobacillus* spp. [20], *Bifidobacterium* spp. [22], as well as the administered probiotics, *Bifidobacterium lactis* [32,21] and *Lactobacillus paracasei* [14]. Each sample was analyzed in triplicates and all samples from one subject were analyzed in the same run. To obtain standard curves, a 10-fold dilution series ranging from 10 pg to 10 ng of DNA from the bacterial standard cultures (*L. paracasei* Lpc-37 and *B. lactis* HN019) were included in the qPCR assays. For determination of DNA, triplicate samples were used, and the mean quantity per g wet weight was calculated.

Fecal concentrations of IgA and calprotectin were measured by using Human IgA ELISA Quantitation Kit E80-102 (Bethyl Laboratories Inc., Montgomery, TX, USA), and Phical ELISA test (Calpro AS, Oslo, Norway) respectively, before and at the end of the study, according to the manufacturers instructions.

2.7 Statistical Analysis

Data analysis was done using Statistical Package for Social Sciences (SPSS, IBM Chicago) Version 17 for Windows. For Dependent continuous variables, Mean and SD were calculated for continuous variables. For Normally distributed variables, One Way ANOVA with LSD *post hoc* test was done to determine significant differences between the groups. Where the distribution of variables was not normal, non-parametric tests was done. Kruskal-Wallis H test was done to see significant differences between more than two groups and when the difference between the groups was found to be significant, Mann-Whitney U test was performed to determine significant differences between two groups. For nominal variables, Proportions were calculated. Pearson Chi square test was done to determine significant differences. Fishers Exact T test was done for two by two tables and where the Proportion of cells was less than 20%. Repeated measures ANOVA was done to find any change in the incidence and duration of diarrhea before, during and after supplementation. Paired t test and Wilcoxon sign Rank test was done to determine differences within the group before and after supplementation. A test was considered to be significant when P value was less than 0.05.

3. RESULTS

NIN has been conducting active research and providing medical services in this slum over the past two decades. Majority of the households of the locality are “Pucca” Houses, and have safe drinking water facility and closed drainage system. A total of 379 children were enrolled for the study after initial screening and eligibility (Fig. 1). Mean age of the study children was, at enrollment, 38 months (range 24 – 48) with half of them being boys (Table 1). Both age and sex were not significantly different between the groups. The average height and weight of each group were not significantly different. However, at baseline, mean Weight for Age Z (WAZ) scores were significantly lower in the HN019 group compared to Lpc-37 group ($P < 0.01$), also mean Height for Age Z (HAZ) Scores were significantly lower in HN019 group compared to Lpc-37 group ($P < 0.001$) and placebo group ($P < 0.001$). All

children in the three groups showed consistent weight gain and linear growth. The increment of weight and height was comparable between the groups after supplementation (Table 1).

Table 1. Baseline characteristics (i.e. prior to the start of the intervention) and increments in height and weight of randomized children in all three groups

N	Lpc-37 group	HN019 group	Placebo group	All
	(125)	(130)	(124)	(379)
Age (months)	37.48 (8.21)†	38.93 (9.24)	37.41 (8.79)	37.94 (8.76)
Sex				
Male (%)	46	55	48	50
Female (%)	54	45	52	50
Weight (kg)	11.96 (1.8)	11.45 (1.86)	11.57 (1.74)	11.66 (1.81)
Height (cm)	90.67 (5.7)	88.50 (8.21)	89.28 (6.27)	89.47 (6.88)
WAZ[1]	-1.50 (1.07)	-1.98 (1.03)	-1.71 (1.36)	-1.73 (1.18)
HAZ[2]	-1.39 (1.23)	-2.23 (1.67)	-1.65 (1.93)	-1.76 (1.67)
WHZ[3]	-1.05 (1.20)	-1.09 (0.99)	-1.07 (1.06)	-1.07 (1.08)
Increment[4] in WAZ score	0.09 (0.85)	0.16 (0.62)	0.09 (0.80)	0.11 (0.76)
Increment[4] in HAZ score	-0.04 (0.44)	0.30 (0.35)	0.01 (0.95)	0.09 (0.65)
Increment[4] in WHZ score	0.19 (1.30)	-0.01 (0.92)	0.15 (0.89)	0.11 (1.05)

Lpc-37 group received Lactobacillus paracasei Lpc-37; HN019 group received Bifidobacterium lactis HN019; Placebo group received placebo; † Mean (SD); [1]Mean weight for age Z-score, WAZ, were significantly lower in HN019 group compared to Lpc-37 group (P <0.01); [2]Mean height for age Z-score, HAZ were significantly lower in HN019 group compared to Lpc-37 group and Placebo group (P <0.001); [3]Mean weight for height Z-score, WHZ; [4]Increment in WAZ, HAZ and WHZ from July 2010 to July 2011

The overall baseline incidence of diarrhea during the month of July (before Intervention) was 3.3% and was not significantly different between the groups. The number of episodes and total duration of diarrhea was also not significantly different between the groups. The overall incidence of fever was 10.7% and not significantly different between the groups, Table 2.

The overall incidence of diarrhea during the intervention period was 16.9%. The incidence of diarrhea was lowest in HN019 group (13.3%), followed by Placebo group (15.7%) and Lpc-37 group (21.9%), but were not significantly different between the groups (P = 0.22). The overall median number of episodes of diarrhea was 0 days (range 0-4) and so was the median duration of diarrhea (0 days, range 0- 9). There was no significant difference in the number of episodes and duration of diarrhea between the groups, Table 3.

The overall incidence of fever was 60.4% and was not significantly different between the groups. The overall median number of episodes of fever was one episode (range 0-5) and was not significantly different between the groups. The overall median duration of fever was two days (range 0-19) and was not significantly different between the groups, Table 3.

The overall incidence of diarrhea during the follow up period (May 2011 to July 2011) was 2.2% and was not significantly different between groups. The number of episodes and total duration of diarrhea was also not significantly different between groups. The incidence of fever was 13.4% and no significant difference was found between the groups. The number of episodes and total duration of fever was also not significantly different between the groups, Table 4.

Table 2. Morbidity data of the children during the run-in month of July in all three groups; without supplementation

		Incidence		Total no of episodes					Total duration (days)				
		Total number	Percentage (Number)	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD
Diarrhea	Lpc-37	120	4.2 (5)	0	0	1	0.04	0.20	0	0	5	0.15	0.76
	HN019	119	3.4 (4)	0	0	3	0.07	0.38	0	0	12	0.27	1.54
	Placebo	122	2.4 (3)	0	0	1	0.02	0.15	0	0	3	0.06	0.41
	ALL	361	3.3 (12)	0	0	3	0.04	0.26	0	0	12	0.16	1.01
Fever	Lpc-37	120	10.8 (13)	0	0	1	0.11	0.31	0	0	5	0.38	1.10
	HN019	119	10.1 (12)	0	0	2	0.11	0.34	0	0	9	0.58	1.82
	Placebo	122	11.0 (14)	0	0	2	0.12	0.35	0	0	6	0.36	1.12
	ALL	361	10.7 (39)	0	0	2	0.11	0.33	0	0	9	0.44	1.38

Lpc-37 group received Lactobacillus paracasei Lpc-37
HN019 group received Bifidobacterium lactis HN019
Placebo group received placebo

Table 3. Morbidity data of the children during the supplementation period (August – April)

		Incidence		Total no of episodes					Total duration (days)				
		Total number	Percentage (Number)	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD
Diarrhea	Lpc-37	105	21.9 (23)	0	0	4	0.32	0.73	0	0	7	0.69	1.48
	HN019	113	13.3 (15)	0	0	2	0.14	0.37	0	0	9	0.41	1.24
	Placebo	108	15.7 (17)	0	0	2	0.19	0.46	0	0	5	0.38	0.96
	ALL	326	16.9 (55)	0	0	4	0.21	0.54	0	0	9	0.49	1.25
Fever	Lpc-37	105	60.0 (63)	1	0	4	0.89	0.92	2	0	13	2.63	2.92
	HN019	113	60.2 (68)	1	0	5	1.01	1.13	2	0	19	2.52	3.55
	Placebo	108	61.1 (66)	1	0	5	0.99	1.05	3	0	19	3.19	3.74
	ALL	326	60.4 (197)	1	0	5	0.96	1.04	2	0	19	2.78	3.43

Lpc-37 group received Lactobacillus paracasei Lpc-37
HN019 group received Bifidobacterium lactis HN019
Placebo group received placebo

Table 4. Morbidity data of children after supplementation period (May – July)

		Incidence		Total no of episodes					Total duration (days)				
		Total number	Percentage (Number)	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD
Diarrhea	Lpc-37	105	0 (0)	0	0	0	0.00	0.00	0	0	0	0.00	0.00
	HN019	112	2.7 (3)	0	0	1	0.03	0.16	0	0	3	0.06	0.39
	Placebo	104	3.8 (4)	0	0	1	0.04	0.19	0	0	2	0.08	0.39
	ALL	321	2.2 (7)	0	0	1	0.02	0.15	0	0	3	0.05	0.32
Fever	Lpc-37	105	11.4 (12)	0	0	2	0.12	0.36	0	0	5	0.42	1.22
	HN019	112	13.4 (15)	0	0	1	0.13	0.34	0	0	7	0.44	1.26
	Placebo	104	15.4 (16)	0	0	1	0.15	0.36	0	0	5	0.50	1.22
	ALL	321	13.4 (43)	0	0	2	0.14	0.35	0	0	7	0.45	1.23

Lpc-37 group received Lactobacillus paracasei Lpc-37
HN019 group received Bifidobacterium lactis HN019
Placebo group received placebo

The overall incidence of diarrhea was highest during the wet (rainy) season in the months of August and September (9.8%) and was less than or about 1% during other months of the year, Fig. 2. The lowest incidence of diarrhea was in the month of June (0%). Overall there was no significant difference between the groups in incidence of diarrhea. However, further analysis revealed that there was a significant difference between the groups, in the month of September where the incidence was highest. The incidence of diarrhea was significantly higher in the placebo group (16.9%) compared to Lpc-37 group (4.8 %, P <0.05) and HN019 group (0.8 %, P <0.05) in the month of September. The overall incidence of fever was highest for the month of August (25.7%) followed by September (15%) and October (15%). The incidence of fever during the rest of the year was less than 10% with the lowest incidence in the month of May (7%). Here too, further analysis of the data indicated that the incidence of fever was significantly higher in the month of August in the placebo group (34.5%) compared to the Lpc-37 group (21.0%, P <0.05) and HN019 group (21.8%, P <0.05).

Few other infections then diarrhea were observed and these were not affected by the treatment.

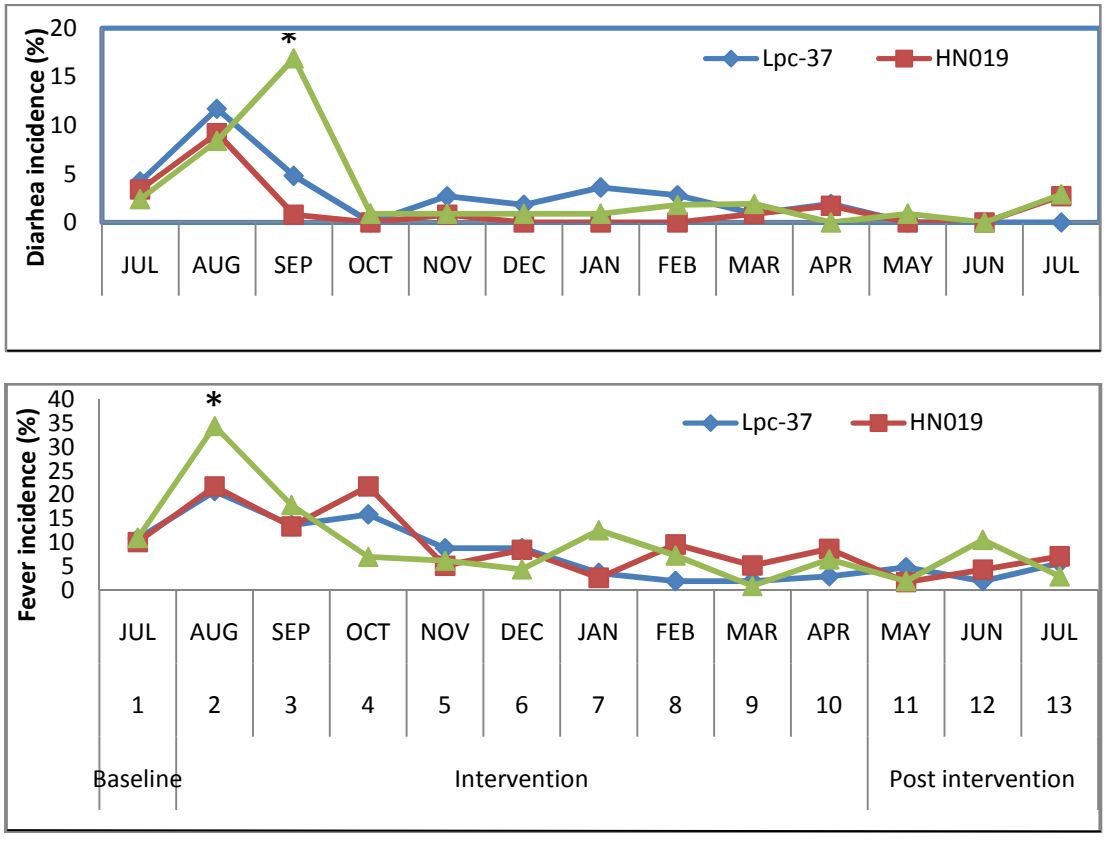

Fig. 2. Monthly Incidence of diarrhea (top graph) and fever (bottom graph) in children in all the Groups

**Placebo group (green) had significantly higher incidence of diarrhea and fever (P<0.05) than Lactobacillus paracasei Lpc-37 group (blue) and Bifidobacterium lactis HN019 group (red)*

Odds ratio was calculated for the incidence of diarrhea and fever with and without adjustment for confounding variables (Table 5). Compared to the placebo group, children in the Lpc-37 group had higher odds of diarrhea (1.52, 0.76 – 3.05) while HN019 group had lower odds of diarrhea (0.81, 0.37 – 1.73) but there was no significant differences between the groups with and without adjustment for confounding variables. The odds of having fever in children of Lpc-37 group and HN019 were slightly lower compared to placebo group but were not significantly different between the groups before and after adjustment for confounding variables.

Table 5. Binary logistic regression with diarrhea and fever as dependent variables, with and without adjustment of confounding variables in all the groups

		Odds ratio	(CI)	P value	Adjusted odds ratio[1]	(CI)	P value
Diarrhea	Lpc-37	1.52	0.76 – 3.05	0.24	1.73	0.84 – 3.55	0.13
	HN019	0.81	0.37 – 1.73	0.58	0.74	0.33 – 1.64	0.46
	Placebo	Reference			Reference		
Fever	Lpc-37	0.98	0.56 – 1.70	0.93	1.05	0.59 – 1.84	0.88
	HN019	0.95	0.55 – 1.64	0.84	0.93	0.53 – 1.63	0.81
	Placebo	Reference			Reference		

Lpc-37 group received Lactobacillus paracasei Lpc-37, HN019 group received Bifidobacterium lactis HN019 Placebo group received placebo, [1] Adjusted for age, sex, weight for age Z score (WAZ), height for age Z score (HAZ), and weight for height Z score (WHZ)

Stool bacterial counts and mucosal immune and inflammatory markers of children were determined on a randomly chosen subset of children, Table 6. The overall count of total bacteria, *Lactobacillus* and *Bifidobacterium* in the stool samples was similar in all three groups before and after supplementation of the probiotic. However, by the end of supplementation, *L. paracasei* was significantly higher (P <0.001) in those supplemented with this strain and, likewise, *B. lactis* was significantly higher in those supplemented with this strain compared to the other groups at the end of 6th month of supplementation (P <0.001) suggesting good colonization (not shown in the table). Nevertheless, by the end of supplementation *B. lactis* was found to be similar in all the three groups.

Calprotectin decreased significantly towards the end of study in all groups (P <0.001), but were not significantly different between the groups; neither before nor after supplementation. Stool IgA, however, was significantly lower (P <0.05) in children supplemented with *B. lactis* compared to other groups, Table 6.

Serum inflammatory markers (IL-8, IL-10, IL-17 and MIP-1b) were only available at the end of the supplementation and were analyzed in a randomly chosen subset of children, Table 7. IL8 was significantly lower (P <0.05) in children supplemented with *B. lactis* HN019 compared to other groups, while other measured cytokines were comparable in all the groups.

Table 6. Determination of bacterial count (log10/g) and immune markers in a random sub set of stool samples from children before and after supplementation in all groups

Stool	Group	Before		After	
		N	Mean (SD)	N	Mean (SD)
Total bacteria	Lpc-37	52	10.32 (0.4)	48	10.62 (0.3)
	HN019	44	10.23 (0.4)	42	10.55 (0.3)
	Placebo	44	10.33 (0.4)	43	10.51 (0.4)
	All	140		133	
Lactobacillus	Lpc-37	52	9.55 (1.4)	48	9.66 (1.1)
	HN019	44	10.06 (1.1)	41	9.76 (0.9)
	Placebo	44	9.99 (1.2)	43	9.66 (1.0)
	All	140	9.84 (1.2)	132	9.69 (1.0)
Lactobacillus paracasei††	Lpc-37	30	4.58 (0.5)	33	5.54 (1.0)[a]
	HN019	29	4.84 (0.8)	20	4.66 (0.4)[b]
	Placebo	23	4.66 (0.6)	28	4.68 (0.6)[b]
	All	82	4.69 (0.7)	81	5.02 (0.8)
Bifidobacterium	Lpc-37	52	8.83 (1.0)	48	9.09 (0.5)
	HN019	44	9.07 (0.6)	42	9.05 (0.5)
	Placebo	44	9.20 (0.4)	43	9.21 (0.4)
	All	140	9.02 (0.7)	133	9.12 (0.4)
Bifidobacterium lactis	Lpc-37	49	5.52 (0.6)	46	5.53 (0.6)
	HN019	44	5.50 (0.6)	42	5.69 (0.6)
	Placebo	43	5.75 (0.6)	43	5.69 (0.6)
	All	136	5.59 (0.6)	131	5.53 (0.6)
Calprotectin† (µg/g)	Lpc-37	117	440 (570)[1]	84	248 (410)[2]
	HN019	120	430 (447)[1]	91	352 (1056)[2]
	Placebo	117	476 (516)[1]	84	324 (742)[2]
	All	354	448 (512)	259	325 (742)
IgA†† (µg/g)	Lpc-37	117	3996 (3996)	64	4010 (5671)[a]
	HN019	120	3579 (4412)	66	2040 (4400)[b]
	Placebo	117	3395 (3546)	65	3199 (4295)[a]
	All	354	3656 (4001)	195	3073 (4865)

Lpc-37 group received Lactobacillus paracasei Lpc-37, HN019 group received Bifidobacterium lactis HN019, Placebo group received placebo
††Different alphabetical superscripts indicate significant differences between the groups
† Different numerical superscripts indicate significant differences ($p \leq 0.05$) within each group before and after supplementation

Table 7. Blood inflammatory markers in a randomly chosen sub set of samples from children after probiotic/placebo supplementation

After Supplementation	Lpc-37 group		HN019 group		Placebo group		All	
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)
IL-8†† (Pg/ml)	62	15.04 (27.3)[a]	60	6.80 (10.7)[b]	61	8.68 (9.9)[a]	183	10.22 (16.2)
IL-10 (Pg/ml)	63	38.02 (251.1)	60	3.89 (8.42)	60	8.34 (28.1)	183	17.10 (148.2)
IL-17 (Pg/ml)	48	18.27 (45.9)	51	41.87 (122.9)	49	81.52 (465.7)	148	47.34 (278.1)
MIP-1b (Pg/ml)	63	144.75 (121.3)	60	149.71 (119.8)	61	154.80 (96.3)	184	149.70 (8.3)

Lpc-37 group received Lactobacillus paracasei Lpc-37, HN019 group received Bifidobacterium lactis HN01, Placebo group received placebo, †† Different alphabetical superscripts indicate significant differences between the groups

4. DISCUSSION

Probiotics have consistently shown positive health benefits for the treatment of diarrhea in both young children and adults [14,15]. However, the role of probiotics for regular supplementation in prevention of diarrhea and other illness is less established. Most studies that have shown the efficacy of probiotics in reducing risk of various infectious diseases in children were in day care centers [16,17,18,19,20] and very few have been community based studies [21,22]. In a meta-analysis of 34 masked, randomized, placebo-controlled trials on efficacy of probiotics in prevention of acute diarrhea, probiotics reduced the associated risk of acute diarrhea among children by 57% (35-71%), and by 26% (7-49%) among adults [23]. They have further stated that there is a lack of data from community-based trials and studies from developing countries evaluating the effect of probiotics on acute diarrhea unrelated to antibiotic usage.

In our study, the overall incidence, total number of episodes or duration of diarrhea during the supplementation period was not significantly different for the groups supplemented with either of the two probiotics compared to placebo. Our results contrast those of a similar community-based double-blind, randomized, controlled trial on 3758 children aged 1-5 years in an urban slum in India. Children who were given a probiotic drink containing *Lactobacillus casei* strain Shirota for 12 weeks, had a 14% reduction of diarrhea [22]. Compared to the above study, our study had relatively small sample size; however, the duration of our study (nine months) was longer than the above study (three months) and our study involved all seasons of the year. This may also explain the difference between the two studies. Further analysis of the data indicated that during the rainy season; August-September, when incidence of diarrhea and fever is highest, both administered probiotics reduced the incidence in diarrhea; similar to the study with *L. casei* Shirota [22]. Outside the wet season, diarrhea incidence was low and the benefit of probiotics was limited; reducing the overall effect. When risk for disease is low, probiotics or any other protective measure for that matter, are unlikely to further reduce this risk. It may therefore not be beneficial (but also not detrimental) to consume probiotics during a low risk period. For seasonal diseases such as diarrhea in the present study or respiratory tract infections, it is more beneficial to focus on use during these higher risk periods.

L. paracasei Lpc-37 and *B. lactis* HN019 were chosen for our study, since they have been shown to be highly tolerant to acid and bile, have strong adhesion property to intestinal cell lines and thereby well suited for intestinal survival and function [24]. *L. paracasei* Lpc-37 has been shown to influence immune regulation, as demonstrated in a study with adult allergy patients [25]. *Bifidobacterium lactis* HN019 in combination with galacto-oligosaccharides has been shown to reduce incidence of dysentery, respiratory tract infection [26]. Consumption

of the probiotics was found to lead to an increase in fecal levels of the consumed species only for *L. paracasei*. This is in agreement with earlier observations [27]. Fecal *B. lactis* levels were high throughout the study; 10^9 bacteria/g regardless of the treatment. This may explain why no increase was observed upon consumption of *B. lactis* HN019. Similarly, the levels of fecal lactobacilli were found to be exceptionally high. *Lactobacillus* has earlier been reported to be only a minor component of the intestinal microbiota present at levels below 10^8 bacteria/g feces [27].

Studies on probiotics have documented antimicrobial effects, improvement in mucosal barrier function, and immunomodulation activity on both innate and adaptive immunity [28]. *In vivo* and *in vitro* studies have also shown that activation of macrophages, improvement in natural killer cell activity [29,30], increased numbers of IgA, IgM, and IgG secreting cells in the circulation, and increased fecal IgA concentrations provide beneficial effects on the balance of pro- and anti-inflammatory cytokines [31,32,33]. In our study, the total bacterial count and inflammatory and immune markers were higher than reported in the literature from the developed countries [34]. It is likely to be due to repeated infections in children of this age group, living in poor sanitary conditions typical of an urban slum in a developing country. Stool calprotectin and IgA concentrations were lower at the end of supplementation than baseline values, which was not entirely unexpected as the baseline stool samples were collected at the beginning of the rainy season (July), where both diarrhea and fever were more common than at the end of the study (April). The serum inflammatory marker, IL 8 was lower in HN019 group. Though the main function of IL-8 is clearance of pathogens during infections, it also contributes to promote inflammation. Findings in the current study indicate an immuno-modulatory function of HN019 and suggest studies to explore the potential of oral administration of HN019 in immune-mediated diseases.

The long term (nine months) supplementation of probiotics was not observed to have any influence on weight gain or growth. This observation therefore refutes the recently proposed theory that probiotics contribute to obesity [35].

4. CONCLUSION

Both probiotics supplemented for a period of 9 months did not have any influence on weight gain or linear growth in 2 to 5 year old children. Although, there was no overall significant effect on incidence, duration and episode of diarrhea and fever, during the wet season both probiotic strains, *Lactobacillus paracasei* Lpc-37 and *Bifidobacterium lactis* HN019, significantly reduced diarrhea in the month of September and fever in the month of August, in preschool children belonging to parents of low socioeconomic status in a developing country (India).

CONSENT

All authors declare that written informed consent was obtained from the children's parents of legal guardians before their participation in the study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committees; Scientific Advisory Committee (SAC) as well as the Institutional Review Board (IRB) of the National Institute of Nutrition (Hydrabad, India) and

have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki (2008 revision).

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COMPETING INTERESTS

ACO and SDF are employees of DuPont. DuPont manufactures and markets the investigated probiotics.

REFERENCES

1. WHO | Diarrhoeal disease [Internet]. WHO. [cited 2013 Feb 7]. Available from: http://www.who.int/mediacentre/factsheets/fs330/en/index.html
2. Wardlaw T, Salama P, Brocklehurst C, Chopra M, Mason E. Diarrhoea: why children are still dying and what can be done. Lancet. 2010;375(9718):870–2.
3. Black RE, Morris SS, Bryce J. Where and why are 10 million children dying every year? Lancet. 2003;361(9376):2226–34.
4. Cohen MB. Etiology and mechanisms of acute infectious diarrhea in infants in the United States. J. Pediatr. 1991;118(4 Pt 2):S34–39.
5. Huang JS, Bousvaros A, Lee JW, Diaz A, Davidson EJ. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. Dig. Dis. Sci. 2002;47(11):2625–34.
6. Van Niel CW, Feudtner C, Garrison MM, Christakis DA. Lactobacillus therapy for acute infectious diarrhea in children: a meta-analysis. Pediatrics. 2002;109(4):678–84.
7. Guarner F, Schaafsma GJ. Probiotics. Int. J. Food Microbiol. 1998;39(3):237–8.
8. Szajewska H, Skorka A, Ruszczynski, M, Gieruszczak-Bialek D. Meta-analysis: Lactobacillus GG for treating acute gastroenteritis in children--updated analysis of randomised controlled trials. Aliment. Pharmacol. Ther. 2013;38(5):467-76.
9. Szajewska H, Ruszczynski M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. J. Pediatr. 2006;149(3):367-72.
10. Sur D, Manna B, Niyogi SK, Ramamurthy T, Palit A, Nomoto K, et al. Role of probiotic in preventing acute diarrhoea in children: a community-based, randomized, double-blind placebo-controlled field trial in an urban slum. Epidemiol. Infect. 2011;139(6):919–26.
11. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects [Internet]; 2008. [cited 2013 Feb 7]. Available from: http://www.wma.net/en/30publications/10policies/b3/index.html

12. Apajalahti JHA, Kettunen H, Kettunen A, Holben WE, Nurminen PH, Rautonen N, et al. Culture-Independent Microbial Community Analysis Reveals that Inulin in the Diet Primarily Affects Previously Unknown Bacteria in the Mouse Cecum. Appl Environ Microbiol. 2002;68(10):4986–95.
13. Costabile A, Fava F, Röytiö H, Forssten SD, Olli K, Klievink J, et al. Impact of polydextrose on the faecal microbiota: a double-blind, crossover, placebo-controlled feeding study in healthy human subjects. Br. J. Nutr. 2012;108(3):471–81.
14. Eser A, Thalhammer F, Burghuber F, Högenauer C, Stockenhuber F, Wenisch C, et al. [Probiotics for the prevention of antibiotic-induced diarrhea]. Z Gastroenterol. 2012;50(10):1089–95.
15. Huang JS, Bousvaros A, Lee JW, Diaz A, Davidson EJ. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. Dig. Dis. Sci. 2002;47(11):2625–34.
16. Arvola T, Laiho K, Torkkeli S, Mykkänen H, Salminen S, Maunula L, et al. Prophylactic Lactobacillus GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. Pediatrics. 1999 Nov;104(5):e64.
17. Chouraqui J-P, Van Egroo L-D, Fichot M-C. Acidified milk formula supplemented with bifidobacterium lactis: impact on infant diarrhea in residential care settings. J. Pediatr. Gastroenterol. Nutr. 2004Mar;38(3):288–92.
18. Kotowska M, Albrecht P, Szajewska H. Saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea in children: a randomized double-blind placebo-controlled trial. Aliment. Pharmacol. Ther. 2005 Mar 1;21(5):583–90.
19. Vanderhoof JA, Whitney DB, Antonson DL, Hanner TL, Lupo JV, Young RJ. Lactobacillus GG in the prevention of antibiotic-associated diarrhea in children. J. Pediatr. 1999 Nov;135(5):564–8.
20. Weizman Z, Asli G, Alsheikh A. Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics. 2005 Jan;115(1):5–9.
21. Oberhelman RA, Gilman RH, Sheen P, Taylor DN, Black RE, Cabrera L, et al. A placebo-controlled trial of Lactobacillus GG to prevent diarrhea in undernourished Peruvian children. J. Pediatr. 1999;134(1):15–20.
22. Sur D, Manna B, Niyogi SK, Ramamurthy T, Palit A, Nomoto K, et al. Role of probiotic in preventing acute diarrhoea in children: a community-based, randomized, double-blind placebo-controlled field trial in an urban slum. Epidemiol. Infect. 2011;139(6):919–26.
23. Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect Dis. 2006;6(6):374–82.
24. Engelbrektson A, Korzenik JR, Pittler A, Sanders ME, Klaenhammer TR, Leyer G, et al. Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy. J. Med. Microbiol. 2009;58(Pt 5):663–70.
25. Roessler (nee Klein) A, Friedrich U, Vogelsang H, Bauer A, Kaatz M, Hipler UC, et al. The immune system in healthy adults and patients with atopic dermatitis seems to be affected differently by a probiotic intervention. Clin. Exp. Allergy. 2007;38:93-102.
26. Sazawal S, Dhingra U, Hiremath G, Sarkar A, Dhingra P, Dutta A, et al. Prebiotic and probiotic fortified milk in prevention of morbidities among children: community-based, randomized, double-blind, controlled trial. PLoS ONE. 2010;5(8):e12164.
27. Forssten SD, Salazar N, López P, Nikkilä J, Ouwehand AC, Patterson Á, et al. Influence of a probiotic milk drink, containing *Lactobacillus paracasei* Lpc-37, on immune function and gut microbiota in elderly subjects. Eur J Food Res Rev. 2011;1(3):159-172.
28. Saavedra JM. Use of probiotics in pediatrics: rationale, mechanisms of action, and practical aspects. Nutr Clin Pract. 2007;22(3):351–65.

29. Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (Bifidobacterium lactis HN019): optimization and definition of cellular immune responses. Eur J Clin Nutr. 2000;54(11):849–55.
30. Isolauri E, Sütas Y, Kankaanpää P, Arvilommi H, Salminen S. Probiotics: effects on immunity. Am. J. Clin. Nutr. 2001;73(2 Suppl):444S–450S.
31. Isolauri E, Joensuu J, Suomalainen H, Luomala M, Vesikari T. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by Lactobacillus casei GG. Vaccine. 1995;13(3):310–2.
32. Rinne M, Kalliomaki M, Arvilommi H, Salminen S, Isolauri E. Effect of probiotics and breastfeeding on the bifidobacterium and lactobacillus/enterococcus microbiota and humoral immune responses. J. Pediatr. 2005;147(2):186–91.
33. Viljanen M, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, Savilahti E. Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. Pediatr Allergy Immunol. 2005;16(1):65–71.
34. Berdat PA, Wehrle TJ, Küng A, Achermann F, Sutter M, Carrel TP, et al. Age-specific analysis of normal cytokine levels in healthy infants. Clin. Chem. Lab. Med. 2003;41(10):1335–9.
35. Million M, Maraninchi M, Henry M, Arougom F, Richet H, Carrieri P, et al. Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii*. Int J Obes. 2011;36(6):817-825.

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