# Evaluation of Combined Use of Probiotics, *Bifidobacterium breve,* on the Immunogenicity of the Oral, Whole Cell Killed Cholera Vaccine in Bangladeshi Children

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## Introduction

Cholera is a major health problem in many developing countries including Bangladesh and marked increase in the prevalence has been seen on all continents in the last decade. Case rates were highest among the age groups of 2-9 or 5-9 in previous epidemiologic studies carried out in Bangladesh (1, 2). So there is a great need for an appropriate vaccine to protect children from live threatening consequences of cholera. The B subunit whole cell killed vaccine is licensed in many countries of the world and recommended by WHO as a public health tool. Its protective efficacy has been demonstrated in adults in Bangladesh as well as in other countries, but less so in children. Thus there is an urgency for developing strategies to improve the immunogenicity of vaccine especially for protection of children in cholera endemic countries of the world. Different options for improved cholera vaccines are being considered including new and improved formulations of killed or live oral candidate vaccines as well as the use of micronutrient supplementation during the course of immunization.

Probiotics are defined as "live micro-organisms that when administered in adequate amounts confer a health benefit on the host". Studies have revealed the intestinal immunomodulatory effect of probiotics and that it can induce cytokines that may promote the differentiation of B cells into IgA-producing cells. Therefore, an option to increase the efficacy of the cholera vaccines that appears promising is the use of probiotics as adjunct to oral immunization based on the understanding that these agents could improve the mucosal immune responses, both innate and adaptive, and help reducing inflammation. The supplementation of probiotics to infants may also have a prophylactic effect against acute diarrheal diseases. In pediatric populations, effect of probiotic agents appears to be most significant against rotavirus diarrhea, suggesting that immunological mechanism is responsible for the beneficial effects. Bacterial species used as probiotics including *Bifidobacterium breve* are usually considered as nonpathogenic. *B. breve* strain Yakult is a strain of human intestinal origin having high resistance to gastric and bile acid. This strain was used as probotics to treat pediatric diseases including intractable diarrhea (3-5). In the present study we examined if supplementation with *B. breve* strain Yakult has a beneficial role in enhancing the immunogenicity of the oral, whole cell killed cholera vaccine in Bangladeshi children aged two to five years.

## Materials and Methods

**Study design:** This trial was a randomized placebo-controlled design with 2 parallel arms. Half of the total participants received *B. breve* (probiotic group) and half received placebo

(placebo group). A power calculation was performed, and we assumed a total of 128 children were needed to be enrolled, 64 in each group. Computer generated, blocked randomization list was prepared by an independent statistician outside the research group. A block size of eight was used.

**Participants:** The study participants were 2-5 year old children from an urban slum in the Mirpur area in Dhaka city in Bangladesh. This is a neighborhood situated around 10 km from the International Centre for Diarrheal Diseases, Bangladesh (ICDDR,B) hospital in Dhaka. Participants were recruited from an area of about 2 km around the ICDDR,B field office. Inclusion criteria included being healthy, aged between 2 to 5 years and consent was obtained from guardians or parents to allow their child to participate in the study. Exclusion criteria included any chronic disease, or any recent illness that may comprise the immune system as well as those with history of diarrheal illness (passage of loose or watery stool ≥3/24 h) in the past 2 weeks or under nutrition (≤−2 SD) were also excluded. Demographic and baseline data from study subjects were collected during the week prior to starting the study.

**Administration of probiotics and the vaccine:** The probiotic preparation containing *B. breve* strain Yakult and the placebo were provided by Yakult Co. Ltd., Japan. One gram of the probiotic preparation given to the study subject per day contained ca. 4 x $10^9$ CFU of *B. breve* and cornstarch whereas *B. breve* was lacking in the placebo. The B-subunit-whole cell killed cholera vaccine Dukoral® (SBL,Stockholm, Sweden) was used in the study. Each dose of the killed vaccine contained 1.0 X$10^{11}$ CFU of the killed whole cell vaccine plus 1 mg of cholera toxin B (CTB) subunit. The study schedule is schematically shown in Fig. 1. All children enrolled in the study were kept free from fermented food during the study period, from study day 1 through study day 42 after enrollment. According to the randomization list probiotics or the placebo was given to the study children from study day 14 up to day 45. Two doses of oral cholera vaccine were administered 14 days apart (on study day 21 and 35) among the study participants. Immunological responses were measured 7 days after intake of each vaccine dose.

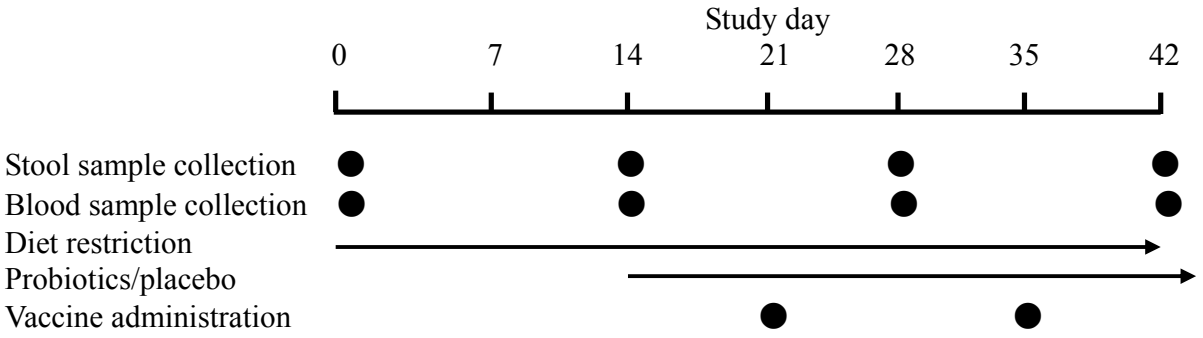


**Fig. 1. Study schedule**

**Collection and analysis of the samples:** Blood and stool specimens were obtained on study day 1, 14, 28 and 42. Peripheral blood mononuclear cells obtained from the blood sample were used to measure the antibody in lymphocyte supernatant (ALS): the IgA, IgM and IgG isotypes specific to *V. cholerae* O1 Ogawa LPS as well as to CTB (6). Plasma samples were used for determining vibriocidal antibodies using *V. cholerae* O1, Ogawa strain X25049 as target

bacteria (7). Those with ≥ 4 fold increase in response were considered responders. Plasma samples were also used to measure anti-CTB and anti-LPS antibody responses in the IgA, IgG and IgM isotypes using a kinetic ELISA procedure (6). A ≥2-fold increase was considered a significant response. Fecal samples were collected for evaluation of total IgA levels and determination of anti-CTB and anti-LPS-specific ELISA units per μg of fecal IgA. For IgA antibodies in faecal extracts, ≥2-fold increase was considered a significant response. Statistical analyses were carried out appropriately using nonparametric or parametric method by using Stata$^{TM}$ V 9.2 (Stata Corp., USA) as well as the SigmaStat (SPSS, CA). Paired samples were assessed by the Wilcoxon signed rank test, non-paired samples by Mann–Whitney *U* test. Proportions were compared using the $\chi^2$ or the Fisher's exact test as appropriate.

**Safety evaluation and ethics:** All adverse events occurring within the study period were recorded. Ethical approval for the study was granted by the IRB of ICDDR, B and Kyoto University, and all parents or guardians of each child participant gave written informed consent. This trial is registered with the ClinicalTrials.gov, number NCT00464867.

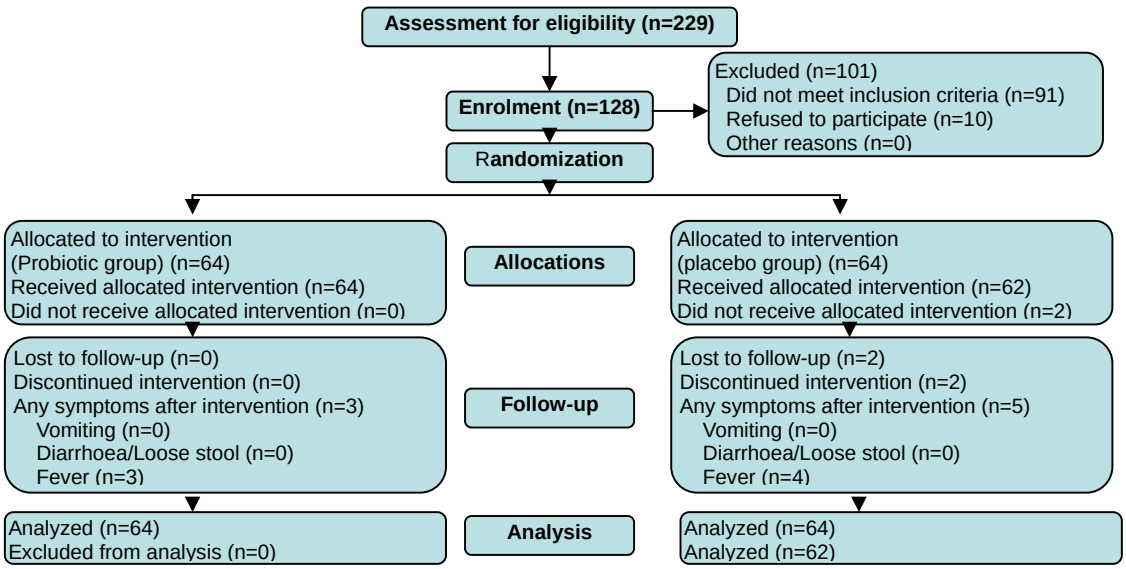


**Fig. 2. Flow of the enrolment and characteristics of the study subjects**

## Results

**Enrollment and study subjects:** Flow of the enrolment is summarized in Fig. 2. Of the 64 participants in the probiotic group, 37 were male and 27 female while in the placebo group 34 were male and 28 were female. The 128 subjects were randomized into one of the two groups (the probiotic group and the placebo group). Two subjects in the placebo group were lost to follow up before intervention was initiated. Therefore, the data from 64 subjects of the probiotic group and those from 62 subjects of the placebo group were analyzed.

**Safety of probiotics:** The surveillance for side-effects revealed only mild symptoms, and there were no significant differences in the frequency of adverse events in the probiotics

recipients (Fig. 2). Complaints of vomiting and abdominal cramp, fever and loose motion were observed in only a few children. There were no differences in the rates of any adverse events between children given probiotics or placebo recipients ($P = 0.74$).

**Effect of probiotics on vaccine efficacy:** The pattern of vibriocidal antibody elevation between the two groups was not significantly different between the groups (Fig. 3). The response rate was also comparable in the two groups of children (76-79% overall rates).

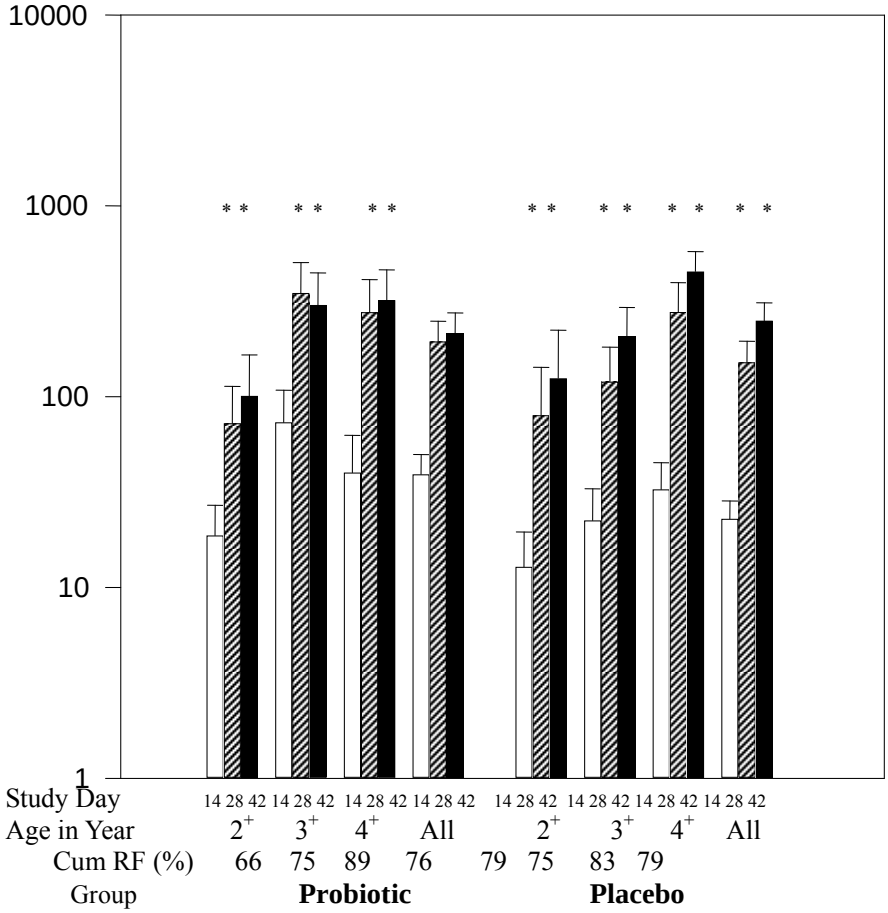


**Fig. 3. Vibriocidal antibody responses in groups receiving probiotics or the placebo. The bars indicate geometric mean titers and lines the standard errors of the mean. The asterisk indicates statistically significant difference in the response in vibriocidal antibody titers relative to that prior to immunization (day 14) and after immunization (day 21and day 35) ($P$ = 0.002 to < 0.001). Results are shown on the age distribution of the toddlers (2+, 3+, 4+) and for the group as a whole (All).**

About 86 to 97 percent of the children seroconverted with CTB-specific IgA antibody response in both serum and ALS specimens. No significant differences were seen in the magnitude of the CTB-specific IgA antibody titer between the probiotic and placebo group. No difference in the response to the LPS antigen was seen in the IgA isotype between the two groups (~56%). The

response to LPS in the IgG isotype was low (~19%). About 50% of children in both groups responded with LPS-IgA antibodies in feces while around 75% responded with CTB-IgA antibodies.

## Discussion

The results of this study have demonstrated the safety of *B.breve* in Bangladeshi toddlers. We, however, did not observe any changes in the immunological responses to the vaccine as the result of intervention with probiotics. The reason for this is not well understood. It could be due to the choice of the probiotic strain or due to the fact that the gut in children in Bangladesh are already colonized with a host of different bacteria and *B. breve* may not be able to exert its beneficial effect. This can also be an age related factor where younger or older children may show a more beneficial effect to the probiotics. Further studies and analyses are needed.

## References


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