



Short communication

Evaluation of a probiotics, *Bifidobacterium breve* BBG-01, for enhancement of immunogenicity of an oral inactivated cholera vaccine and safety: A randomized, double-blind, placebo-controlled trial in Bangladeshi children under 5 years of age

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ABSTRACT

To evaluate the probiotic, *Bifidobacterium breve* strain Yakult (BBG-01), for safety and enhancement of immunogenicity in an oral inactivated cholera vaccine, a randomized double-blind placebo-controlled study was performed. Bangladeshi children under 5-year-old received BBG-01 or placebo for 4 weeks with two doses of oral cholera vaccine. Serum/fecal antibodies and fecal bacterial flora in the study participants were monitored. All adverse events were mild and transient and had no significant difference between the two groups. Immunological responses were similar comparing the two groups. A negative correlation between *Bifidobacterium* and *Enterobacteriaceae* in the probiotic group suggests a possible involvement of BBG-01 in alteration of the enteric bacterial flora. In conclusion, BBG-01 is well tolerated by Bangladeshi children although the post vaccinal immunostimulatory effect of BBG-01 was not evident.

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1. Introduction

Probiotics are defined as “live micro-organisms when administered in adequate amounts confer a health benefit on the host” [1]. Immunostimulation using probiotics is under investigation. Previous studies show immunostimulatory effect of probiotics in concomitant administration with vaccine, but consistent result has not been obtained so far as to their immunostimulatory effects and clinical benefits [2–8].

It is known that the oral vaccines, which are designed to stimulate intestinal mucosal immunity, are less immunogenic when given to children in developing countries compared to industrialized countries [9]. Therefore, probiotics is expected to enhance immunogenicity of the vaccine. *Bifidobacterium breve* strain Yakult (BBG-01) is a probiotic strain, which is highly resistant to gastric and bile acids, and can be a good can-

didate probiotics to examine its immunostimulation for oral vaccines.

The protective efficacy of Dukoral[®], a commercially available oral cholera vaccine, is less in children than in adults demonstrating there is a great need for an effective vaccine to protect the children from the life threatening consequences of cholera [10,11]. We therefore performed a randomized controlled study to investigate whether BBG-01 enhances the immunogenicity of oral cholera vaccine for the children in Bangladesh.

2. Materials and methods

2.1. Study design and participants

The study followed a double-blind, randomized, placebo-controlled design with two parallel branches involving 128 participants in Dhaka City, Bangladesh. The inclusion criteria were healthy Bangladeshi male and female children aged 2–5 years. Major exclusion criteria were: any chronic illness; any recent illness that may compromise the immune system; those with a history of diarrhea in the preceding 2 weeks; malnutrition with a weight/height score of $\leq -2SD$ according to the NCHS (National

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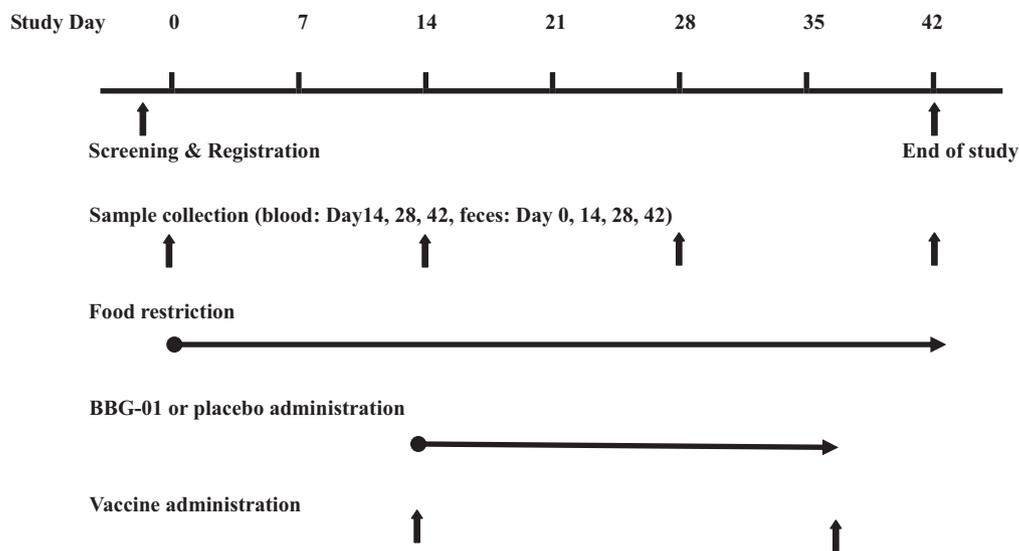


Fig. 1. Outline of the trial. Study days are shown at the top. The days for screening, registration, end of the study, sample (blood and feces) collection, and vaccine administration are indicated by short vertical arrows. Durations of food restriction and BBG-01 or placebo administration (daily) are indicated by horizontal arrows.

Center for Health Standard) with any symptoms; parasitic diseases; and pathogenic bacteria in the stools. Parents or guardians were provided their informed consent before enrollment. The trial was approved by the Institutional Review Board (IRB) of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B) and Kyoto University, Japan. The safety monitoring board at ICDDR, B supervised the safety of the participants enrolled in the trial. This study was registered with the Clinical Trials Data Bank (<http://clinicaltrials.gov/>), Identifier: NCT00464867.

2.2. Study procedures

The participants were randomly allocated to the BBG-01 group receiving BBG-01 and the oral cholera vaccine or to the placebo group receiving the placebo and the oral cholera vaccine. All the participants in both groups were requested to avoid fermented foods throughout the study period. The study agent (placebo or BBG-01) was administered once daily beginning on Day 14 through Day 42. The first dose of oral cholera vaccine was given on Day 21 and the second dose on Day 35. An overview of the study schedule is given in Fig. 1.

Probiotic preparation containing BBG-01 and placebo were provided by Yakult Honsha Co., Ltd., Japan. One gram of the probiotic preparation contained ca. 4×10^9 CFU of live BBG-01; whereas the placebo lacked BBG-01 and was prepared using one gram of cornstarch and hydroxycellulose L per sachet. The commercially available oral inactivated cholera vaccine Dukoral[®] (SBL vaccine AB, Stockholm, Sweden) consisting of whole killed cells and the cholera toxin B subunit was obtained from the manufacturer. Detailed information of the vaccine component and adverse events are described in product information [12]. Two doses of Dukoral[®] with a 14 day interval (at Day 21 and Day 35) were administered to all the participants in each group. Any adverse event that appeared during the course of the study period, were recorded and evaluated by the investigators.

2.3. Laboratory assays

Blood samples (3 ml on four occasions) and fecal specimens were collected on prescribed days (Fig. 1).

Serum vibriocidal antibody were monitored as described previously [13]. The blood samples were used to measure the antibodies in the lymphocyte supernatant (ALS) for IgA and IgG isotypes

specific for *Vibrio cholerae* O1 Ogawa lipopolysaccharide (LPS) and cholera toxin B subunit (CTB) [14–16]. Fecal and serum CTB- and LPS-specific antibodies were measured using the enzyme-linked immunosorbent assay (ELISA) [15]. A greater than 2-fold increase in serum/fecal anti-CTB and anti-LPS antibodies, a greater than 4-fold increase in vibriocidal antibody at Day 42 compared to Day 14 values were considered a significant response.

The fecal specimens collected on Days 0 and 42 were examined for bacterial flora. Frozen fecal specimens were processed and the extracted total RNA samples were analyzed using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method [17,18]. The RT-qPCR assay was performed using serial dilutions of the extracted total RNA sample with primer sets specific for the target bacterial groups. The specificity of the RT-qPCR assay using group-, genus- or species-specific primers was determined as described previously [17–19].

2.4. Statistical analysis

A power calculation was performed based on the published data [20,21]. Assuming 66% response to the intervention, 5% level of significance, power of 90% and the dropout rate to be 15%, a total of 128 children were required. The primary endpoint was serum vibriocidal antibody on Day 42 against Day 14.

Baseline continuous variables were compared in a 2-sample *t*-test. The proportions at the baseline, adverse events, and outcomes were compared using the Chi-square or Fisher's exact tests appropriately. Immunological data were displayed as geometric mean titer/concentration (GMT/GMC) with 95% confidence intervals, and significant differences were assessed by nonparametric methods, the Wilcoxon signed-rank test for paired samples and the Mann-Whitney *U* test for non-paired samples. Comparisons of fecal bacterial counts were assessed using the same non-parametric methods appropriately.

All analyses were two-sided, and a *P*-value of <0.05 was considered statistically significant. All statistical analyses were performed using Stata[™] V 9.2 (Stata Corp., USA), SigmaStat (SPSS, CA), or SPSS Ver. 11 (SPSS Japan Inc., Tokyo).

3. Results

Two participants in the placebo group were lost to follow up before the interventions (Supplementary Fig. 1) so that the data

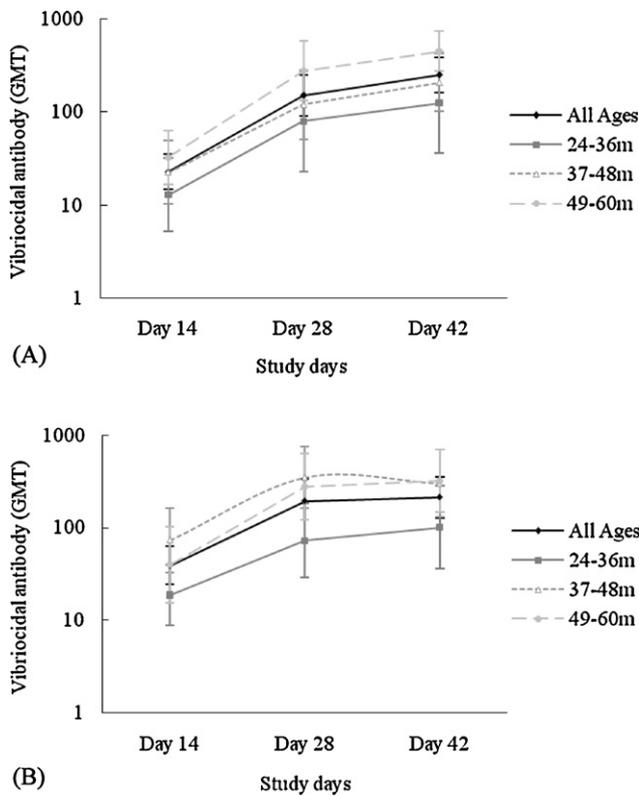


Fig. 2. Pre- and post-vaccinal geometric mean titer (GMT) of vibriocidal antibody in the placebo (A) and the BBG-01 (B) group. Blood samples (Days 14, 28 and 42) were examined for vibriocidal antibody. Results for all ages and three age subgroups (24–36, 37–48, and 49–60 months) are shown. 95% Confidence interval for GMTs are indicated by vertical bars.

from 64 participants in the BBG-01 group and 62 participants from the placebo group were used for the analysis. The baseline demographics and characteristics of the participants in the two groups were similar except for age distribution (Supplementary Table 1, $P=0.03$). The participants were divided into three subgroups by age (24–36, 37–48, and 49–60 months) for blood and fecal sample analysis.

There were no significant differences in the proportions of adverse events comparing the BBG-01 and the placebo group (Supplementary Table 2). The symptoms were not severe and the children recovered during the study period without any medical interventions.

The GMTs for the vibriocidal antibody are plotted in Fig. 2. The GMTs for vibriocidal antibody increased after the first administration of the vaccine with a reduced increase after the second vaccination in all age subgroups. Percent responders to immunization among total and three age subgroups of the participants were compared between placebo and BBG-01 group for various immunological parameters (Supplementary Table 3). The responders for vibriocidal antibody were 62.5% in the BBG-01 group and 75.8% in the placebo group (not significant). The placebo group exhibited a significantly higher proportion of responders than the BBG-01 group for serum CTB-specific IgA ($P=0.016$) in the all ages group, serum LPS- ($P=0.007$) and CTB-specific IgA ($P=0.027$) in the 24–36 months and fecal CTB-specific IgA in the 26–36 months age subgroup ($P=0.026$), while the BBG-01 group showed a significantly higher proportion of responders than in the placebo group for serum LPS-specific IgA in the 37–48 months ($P=0.042$) and fecal CTB-specific IgA in 49–60 months age subgroup ($P=0.027$).

Change in fecal bacterial flora before and after ingestion of BBG-01 or placebo was compared (Supplementary Table 4). The total

bacterial count in the BBG-01 group increased significantly after the intervention ($P=0.037$). *Bifidobacterium* count in the BBG-01 group was significantly higher than in the placebo group after intervention ($P=0.006$) while *Enterobacteriaceae* count was significantly lower in the BBG-01 group than in the placebo group ($P<0.001$). *Enterobacteriaceae* in the BBG group significantly decreased when compared before and after intervention ($P=0.028$). A negative correlation between the changes in *Bifidobacterium* and *Enterobacteriaceae* counts was detected ($R^2=0.4746$, $P<0.001$).

4. Discussion

Here we show BBG-01 administered for 4 weeks with two doses of an oral inactivated cholera vaccine was well tolerated by children under 5 years of age in a developing country. However, we did not observe a clear immunological evidence to support an immunostimulatory effect of BBG-01 in the children. The GMT ratio of vibriocidal antibody on Day 42 against Day 14 in all ages was 10.9 in the placebo group and 5.4 in the BBG-01 group (data not shown). As was shown in Fig. 2, the vibriocidal antibody elevated considerably after the first administration of the vaccine and less so after the second administration in both groups. This may indicate that the majority of the subjects may have been exposed to *V. cholerae* and even one dose of the inactivated oral cholera vaccine was immunogenic. Proportion of responders for CTB-specific IgA and IgG in ALS to detect vaccine specific antibodies produced during the short period after the vaccination were over 70% in all age subgroups indicating the oral cholera vaccine was highly immunogenic in the participants.

The result that the placebo group exhibited a significantly higher proportion of responders than the BBG-01 group for some IgA responses particularly in younger age groups makes us speculate BBG-01 may exert immunostimulatory effect through the IgA response in the older, but not the younger children. But this is not consistent with previously published data [22]. Whether BBG-01 actively suppresses the mucosal immune response in certain age groups is not clear and needs to be investigated further because BBG-01 is generally accepted as an immunostimulatory probiotics.

Similar to our study, Perez et al. reported that children in a low socio-economic status given the fermented milk with or without the probiotics were vaccinated and their immune response was followed, but additional probiotics did not affect antibody response to the vaccines [23]. Typical factors in developing countries, zinc deficiency [21,24] or probiotics deficiency in undernourished children [25–27] for example, may cause lesser immune responses. Other studies have suggested several factors that may explain the hyporesponsiveness of oral vaccines in developing countries including small bowel overgrowth (SBBO) and tropical enteropathy [9,28,29]. These factors can explain the hyporesponsiveness but the participants in the present study had good immunological response for oral cholera vaccine as was shown in Fig. 2, and were difficult to explain the suggested immunosuppression by BBG-01.

The result of fecal bacterial counts of the BBG-01 group and the placebo group suggests that BBG-01 affected intestinal bacterial flora by blocking the proliferation of the bacteria belonging to *Enterobacteriaceae*. Although the mechanism and the meaning of the negative correlation are unclear, a similar observation was described by Ishibashi and Yamazaki [30]; when *B. longum*-monoassociated mice were challenged with *Escherichia coli* O157, the intestinal *E. coli* O157 was reduced and the bacterial count remained low. Why BBG-01 suppresses *Enterobacteriaceae*, whose pathogenicity is not so strong as *E. coli* O157, is one of our interest. More investigations on BBG-01 including age-dependency and clinical significance of the negative correlation of fecal BBG-01 and *Enterobacteriaceae* counts will be needed to clarify possible useful-

ness of this probiotics. *Contributors*: F.M. and F.Q. were involved in the preparation of the protocol; F.M., M.N., and F.Q. obtained ethical approval; F.M. and M.N. obtained funding; and M.I.C., A.A.T., and T.A. recruited subjects, collected data, and managed the running of the trial. A.S. set up and undertook the microbiological investigations. K.N. and T.A. analyzed stool flora. F.M. undertook data cleaning and analysis with the assistance of M.I.C., A.A.T., T.A. and F.Q. The manuscript was written by all the authors, who vouch for the accuracy, completeness and impartiality. All authors approved the final version. A.C. was the guarantor. *Conflict of interest statement*: K. Nomoto and T. Asahara are staffs of Yakult Central Institute for Microbiological Research. The rest of the authors declare that they were free of conflicts of interest with Yakult Honsha Co., Ltd. and SBL vaccine AB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.12.133.

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