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Role of Probiotics VSL#3 in prevention of suspected sepsis in low birth weight infants in India: a randomized controlled trial

Brief Title: Probiotics in prevention of suspected sepsis in LBW infants

Anju Sinha[1](PhD), Subodh S. Gupta[2](MD), Harish Chellani[3](MD), Chetna Maliye[2](MD), Vidya Kumari[3](PGDMCH), Sugandha Arya[3](MD), B.S Garg[2](PhD), Sunita Dixit[4](DNB), M.S. Prasad[3](MD), Rajni Gaind [3], Vijayshri Deotale[2](MD), Manish Taywade[2](MD), Vasantha Thavraj[1] (MD) Ajit Mukherjee[1](PhD), and Malabika Roy[1](MD)

1. Indian Council of Medical Research, 2. Mahatma Gandhi Institute of Medical Sciences, Sewagram, 3. Safdarjung Hospital, New Delhi. 4. District Hospital Wardha.

Corresponding author:

Anju Sinha, Scientist 'D', Indian Council of Medical Research, New Delhi 110029.Email: apradhandr@gmail.com

Telephone: +91 26589493

Mobile: +91 9811422241

Keywords: Neonatal sepsis, Low Birth weight, Prevention, Probiotics.

This trial is registered with Clinical Trial Registry of India (CTRI), number CTRI/2008/091/000049

Abstract

Objectives: To assess the effect of the probiotic VSL#3 in prevention of neonatal sepsis in low birth weight (LBW) infants. **Design**: Randomized, double-blind, placebo-controlled trial. **Setting**: Community setting in rural India. **Participants**: LBW infants aged 3-7 days. **Interventions**: Infants were randomized to receive probiotic (VSL#3, 10 billion cfu) or placebo for 30 days, and were followed up for two months. **Main outcome measure**: possible serious bacterial infection (PSBI) as per Integrated Management of Neonatal Childhood Illnesses algorithm, diagnosed by field workers/physicians. **Results**: 668 infants were randomized to VSL#3 and 672 to placebo. By intention-to-treat analysis, the risk of PSBI among infants 1.5-1.99 kg was significantly reduced (RR 0.29 [95% CI 0.10 to 0.84]) in the probiotics group. The reduction in the overall population did not reach statistical significance (RR 0.79 [95% CI 0.56 to 1.03]). Probiotics reduced mean days of hospitalization (4.6 ± 4.4 vs 6.9 ± 5.6 in the placebo arm [p=<0.0001]) but not the risk of hospitalization (RR 0.66 [95% CI 0.42 - 1.05]). The onset of PSBI in 10% of infants occurred on the 40th day in the probiotics arm versus 25th day in control arm (p=0.063). **Conclusions**: VSL#3 significantly reduces PSBI risk in infants weighing 1.5-1.99 kg. Confirmation of its effect on overall risk in LBWs is warranted in a larger study with a more specific primary outcome measure. **Trial registration:** The study is registered at the Clinical Trial Registry of India (Registration No. REF CTRI/2008/000049).

Article summary: "Strengths and limitations of this study

- Probiotics have been reported to be effective in preventing neonatal necrotising enterocolitis and nosocomial infections in preterm LBW babies.
- In our study, daily supplementation of LBW infants with probiotic VSL#3 (10 billion cfu) for 30 days led to a non-significant 21% reduction in risk of neonatal sepsis. A significant effect was observed among infants weighing 1.5-1.99 kg. Survival analysis showed 15 day delay in the onset of sepsis in the intervention arm.

- Our study used IMNCI algorithm for diagnosis of possible serious bacterial infection (PSBI-suspected sepsis) by field workers. A larger study with sufficient power and a more specific primary end point (such as Physician's diagnosis of neonatal sepsis) is warranted to confirm the preventative effect of VSL#3 on neonatal sepsis in LBW infants.
- Our study was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 days of life, therefore the role of probiotics on early onset sepsis could not be evaluated.

Introduction

Neonatal infections are responsible for more than a quarter of the 1 million neonatal deaths every year in India.[1]Low Birth Weight (LBW) is a very important indirect cause of death in neonates, accounting for40%to 80% of neonatal deaths.[2]Infections (sepsis, pneumonia and meningitis) are known to evolve more rapidly in LBW infants, leading to severely increased disease and higher rate of death. Prevention of infection in LBW babies would directly decrease neonatal morbidity and mortality. Management of neonatal sepsis with antibiotics faces the problem of drug resistance, attributed to availability over the counter, indiscriminate use and incomplete courses in India. Researchers are evaluating immunotherapy (with immune globulin, myeloid colony stimulating factors, probiotics, glutamine supplementation, recombinant human protein C and lactoferrin) as adjuvants for the prevention of neonatal sepsis.[3]

Probiotics have attracted much interest and debatein the neonatal literature during the last decade.[4]FAO/WHO defines probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[5]Probiotic microorganisms have particular characteristics: human origin, safety in human use, bile acid resistance, survival in the intestine, temporary colonization of the gut, adhesion to the mucosa, and bacteriocine production. The ingestion of probiotics is associated with modification in physiological homeostasis of the intestinal flora, which is important in preventing disease, especially infections.[6]The best evidence for efficacy of specific probiotics strains has been obtained with randomized controlled trials and meta-analysisis in the

prevention and treatment of antibiotic-associated diarrhoea,[7] gastroenteritis and acute diarrhoea,[8] and in alleviation of lactose intolerance.[9]

Clinical trials evaluating the role of probiotics (Infloran) in preterm very low birth weight infants[10-12] reported a reduction in incidence of necrotizing entero-colitis (NEC), overall mortality[10] and severity of NEC.[11] A meta-analysis[13] and systematic reviews[14,15] of randomized trial suggested a beneficial effect of probiotic treatment on reducing the incidence and all-cause mortality due to NEC. Following on from the evidence on VLBW and premature infants, we hypothesized that the probiotic preparation VSL#3 might reduce morbidity due to sepsis in LBW infants. We aimed to estimate reduction in the incidence of suspected sepsis in 0-2 month old low birth weight infants in the intervention arm with a daily supplementation of probiotic (VSL#3, 10 billion cfu) over a period of 30 days. If found efficacious, this could be an important public health intervention for prevention of neonatal infections.

Methods

Study design and participants

We undertook a randomized, double-blind, placebo-controlled (1:1) trial from January 2009 to November2011 at two tertiary care hospitals and the adjoining community areas (Safdarjung hospital in New Delhi and Mahatma Gandhi Institute of Medical Sciences Wardha, India). We screened newborn infants aged 3 days, born in the hospitals weighing 1500-2500 g, residing within 20-25 km of the hospital, and not planning to shift residence for at least the next two months. We excluded extremely premature infants (< 34 weeks), sick infants, those with congenital malformations incompatible with life and those with families not giving consent. Eligible babies, for whom parents/guardians gave informed consent, were enrolled on days 3-7 of life. Participants were enrolled by a physician in the hospital and followed up in the community for two months for occurrence of neonatal sepsis and other morbidities. Baseline information on demographic characteristics was obtained for assessment of Standard of Living Index.[16] Ethical clearance was obtained from the two

participating institutes. A Data Safety and Monitoring Committee (DSMC) met every six months and reviewed severe adverse events.

Study medication

Infants were randomly assigned to receive probiotic or placebo by the study physician. The intervention consisted of administration of the probiotic preparation VSL#3 at a dose of 10 billion colony-forming units (cfu) for 30 days, starting on third day of life. The content of the probiotic sachet was mixed in expressed breast milk in a plastic cup and fed to the infant. Sterilized plastic cup and stirrer were provided along with the sachets. A similar-looking maltodextrin preparation in the same outer packing was administered to the control group. The supplement was prepared by CD Pharma India Pvt. Ltd. The preparations withstood a temperature up to 28 degrees Celsius and were therefore kept in a cold chain (refrigerators/vaccine carriers) at the homes of enrolled infants.

Randomization and masking

A computer generated stratified block randomization with permuted block size of 4 was used. We stratified infants by birth weight (1500-2000g, 2001-2500g) and sex. A team of scientists at INCLEN Trust, New Delhi, used a computer-generated table for subject allocation. Allocation concealment was ensured by sequentially numbering the sachet packets containing VSL#3 or placebo after block randomization. Identical packaging of VSL#3 and a placebo with similar consistency and colour was provided. Parents of enrolled infants, investigators and field workers were masked to treatment allocation. Data analysis was performed in a blinded manner. The codes remained with the INCLEN Trust, and were disclosed to the DSMB and ICMR on completion of data analysis.

Follow-up and assessment

Follow-up visits were done by the field worker, for supervising supplementation over 30 days, and detection of morbidities over two months. Visitation was daily during the first week, biweekly in weeks 2-4 of life, and weekly in the second month. Detection of neonatal sepsis was performed during visits using the Integrated Management of Neonatal and Childhood Illnesses (IMNCI) algorithm {ref}

for detection of possible serious bacterial infection(see supplementary material*)*. Field workers referred and accompanied sick infants to the study hospital for treatment. At the hospital, the infants were examined by a physician, blood cultures were obtained, and treatment was carried out as per the protocol of the hospital.

Information on compliance and morbidities was recorded. An enrollment card was provided which parents were asked to carry whenever they sought treatment for the infant in between study visits. Effort was made to contact local practitioners visited independently by parents of infants and collect the details of treatments prescribed. Study staff were trained in the IMNCI algorithm and given practice on eliciting signs of neonatal sepsis. Study procedures were standardized and regular exercises were conducted so as to reduce inter- and intra-observer variability. Quality assurance measures included supervisory checks in the field work, data collection and data cleaning. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry (in EPI Info version 6.0) with built-in range and consistency checks.

The primary outcome was risk of possible serious bacterial infection (PSBI) as per the IMNCI algorithm, diagnosed by the field workers or physicians. Secondary outcomes were estimation of the effect of VSL#3 on overall morbidity pattern in 0-2 month-old LBW infants; stool colonization patterns in 10% of subjects (to be reported separately); and assessment of side effects due to the probiotic VSL#3, if any. On the recommendation of the DSMC, data on diagnosis of sepsis by a physician was also recorded as an amendment to the protocol.

Statistical analysis

Bang et al[2] reported a 17% incidence of neonatal sepsis in the community. Assuming a 10% loss to follow-up, 1340 infants were needed (670 in each group), to observe a 30% reduction in incidence of sepsis at 5% significance with 80% power. Analyses were done by intention to treat. 'R' software[17] (version 3.0.0) was used for calculation of PSBI risk, incidence rates, confidence intervals and

adjusted incidence rate ratios. We used Kaplan-Meier survival analysis curves with Herrington Flemming variation[18] of the log rank test to compare the event rates in the probiotic and placebo arms.

Role of the funding source

Funding source played no role in the study design, data collection, analysis and interpretation, writing of the report or decision to submit it for publication.

Results

Between January 2009 and November 2011, 5927 LBW newborn infants were screened and 1340 eligible LBW infants were enrolled (Figure1)**.**Of the 5927 screened, 4587 were excluded (reasons given in Fig 1). The probiotic and placebo groups were comparable with regard to baseline characteristics such as mode of delivery, mean birth weight, mother's schooling, religion of the family, standard of living index (SLI), and maternal morbidities during current pregnancy (Table 1).

The intervention and control groups were similar in mean number of field worker visits performed (20.8 ± 3.7 in probiotic versus 20.5 ± 4.0 in placebo groups; p= 0.154), mean number of doses of interventional product consumed (29.1 ± 4.4 in probiotics versus 28.7 ± 5.2 in placebo; p= 0.129), and mean number of days of follow-up visits(56.3± 2.2 in probiotics versus 56.1±3.8 in placebo; p=0.239).

Possible serious bacterial infection (PSBI)

Based on the intention-to-treat analysis there was a non-significant 21% reduction in the overall risk of PSBI in the probiotic group (84 cases in 688 infants in the probiotic arm vs.107 cases in 672 in the placebo arm; RR 0.79 [95% CI 0.56 to 1.03]; p = 0.080) (Table 2**).**In the probiotic group there wasa significant 71% reduction in the risk of infants with birth weights 1.5-1.99 kg (4 cases in 74 infants in probioticvs. 14 cases in 75 in the placebo arm; RR 0.29 [95% CI 0.10 to 0.84]; p = 0.014).A 32%

reduction in the risk of PSBI among female infants was observed (36 cases in 348 infants in probiotic vs. 53 cases in 349 in placebo group; RR 0.68 [95% CI 0.46 to 0.99]; p= 0.056).

A post-hoc analysis based on the ITT analysis showed a non-significant 29% reduction in the overall risk of physician-diagnosed sepsis in the probiotic group (38 cases in 688 infants in the probiotic vs.54 cases of 672 in the placebo group; RR 0.71 [95% CI 0.47 to 1.06], p = 0.091). There was no case of suspected sepsis diagnosed by physician in the group of 74 infants taking probiotics and weighing 1.50-1.99kg, as compared to 8 cases in 75 infants of this weight in the placebo group (RR 0.06 [95% CI 0.00 to 1.01], p = 0.007).

We also calculated the incidence rates of PSBI computed with the person-time data collected during home visits (**Table 3)**. The PSBI incidence rate in the probiotics arm was 2.61per 1000 days follow-up, versus 3.40 per 1000 days in the placebo arm (RR 0.77 [95% CI 0.59 to 0.99], p=0.0493). Among babies weighing 1.50-1.99kg, the incidence rate of PSBI per 1000 days was 1.67 and 4.57 in the probiotic and placebo groups, respectively (RR 0.36 [95% CI 0.15, 0.87; p=0.008).

In the post-hoc analysis of physician-diagnosed sepsis the incidence rate in the probiotic arm was 1.07per 1000 days, versus 1.59 per 1000 days with placebo(RR 0.67[95% CI 0.45 to 0.99], p =0.048). In the 1.5-1.99 kg weight stratum, there was no case of sepsis diagnosed by the physician, versus an incidence rate of 2.40 per 1000 follow-up days in the placebo arm (RR 0.00 [95% CI 0.0, 0.35]; p = 0.002).

Comparison of event rates

Kaplan Meier survival analysis curves were plotted to compare the event rates in the probiotic and placebo arms (Figure 2).This shows a divergence between the curves for probiotic and placebo, starting after a week of supplementation and remaining throughout the follow-up period. The onset of first episode of PSBI in 10% of infants occurred on the 41st day in the probiotic arm versus 24thday in

control arm (p=0.063), and onset of first episode of suspected sepsis diagnosed by physician in 5% of infants occurred on 53rd day in probiotic arm versus 26th day in control arm (p=0.071).

Other morbidities

There was no significant difference between the groups for proportion of babies who had local infection (3.0% in probiotic vs. 3.4% in placebo group, p=0.69), feeding problems (18.9% in probiotic vs. 16.4% in placebo group, p=0.21), or other morbidities (35.9% in probiotic vs. 34.2% in placebo group, p= 0.52).

Adverse events: hospitalizations and deaths

Hospitalization and death in enrolled infants were considered as moderate and severe adverse events respectively (Table 4). During the study 29 infants in the probiotic and 44 in the placebo arm needed to be hospitalized (p=0.038). Mean number of hospitalization days was 4.6± 4.4 in the probiotic versus 6.9± 5.6 in the placebo arm(p <0.0001). There were three deaths, one in the probiotic and two in the placebo arm. Verbal autopsy reports of deaths reviewed by the DSMB did not attribute them to the intervention. No side-effects of VSL#3 were reported.

Discussion

Overall, supplementation with the probiotic VSL#3 in LBW infants was associated with a 21% (non-significant) reduction in the risk of suspected sepsis (PSBI) diagnosed by the field worker. However, in the sub-group of infants weighing 1.5-1.99 kg, the reduction in risk of PSBI was statistically significant (reduction of 71%; p=0.014). The primary analysis in this study was based on PSBI classification by field worker as per the IMNCI algorithm as an indicator of neonatal sepsis. [19] The classification PSBI under IMNCI is described as sensitive but not specific for detection of neonatal sepsis.[20]Prior to closure of the study, the DSMC recommended conducting post-hoc analyses using physician's diagnosis of sepsis as the outcome measure. In this analysis there is a 33% overall reduction in risk of sepsis. Moreover, in the sub-group of infants weighing 1.5-1.99 kg, there is a

100% reduction, with no cases observed in the group receiving probiotic supplementation.. Probiotic intervention significantly reduced the mean number of hospitalization days. The Kaplan Meier survival analysis shows a 15-day delay in the onset of sepsis in the intervention arm; this translates to a disease-free window during the 28-day period, crucial for neonatal survival. Moreover, considering a higher case fatality in sepsis at early ages, this becomes even more important. Our results may not be definitive or robust enough; however, there is a consistency in them, and we do not consider this as a "negative trial". Although our study is not large enough, it may be misleading to interpret it as proving that there is no effect of the probiotic intervention or no difference between the study groups. More evidence needs to be generated, since interpretation of no effect might discourage further studies.[21]

Physician's diagnosis of sepsis is more meaningful than PSBI, owing to its specificity. The reported post-hoc analyses increase our confidence in the results. However, physicians used their clinical judgement for diagnosing sepsis; there was no standardized definition used, and this is a limitation of the study. Future trials should evaluate the role of VSL#3 on incidence of sepsis with a precise definition of the outcome measure. The incidence of sepsis observed in the study was lower than the expected effect size used in determining the sample size of the study. Home visits,[22,23] health education messages about exclusive breastfeeding and hygiene, and referral by field workers could improve care and care-seeking, resulting in lower morbidity and mortality and a type II error for the overall result of our study. Our study has several other limitations. It was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 day of life, so we cannot comment on the role of probiotics on early onset sepsis. We followed infants for a period of two months, and cannot comment on the long term effects of probiotic supplementation. There are concerns regarding heterogeneity in probiotic products.The literature suggests greater protection with double or triple probiotic strains.[13]Probiotic VSL#3 is a mix of 8 strains namely-*Streptococcus thermophilus, Bifidobacterium breve, B. Longum, B. Infantis, Lactobacillus acidophilus, L. Plantarum, L. Paracasei,* and *L. Delbrueckii spp bulgaricus.*In a randomized placebo-controlled

clinical trial in India, VSL#3 resulted in early recovery and reduced need for oral rehydration salts in rotavirus-affected children aged 6 months to 2 years.[24]

In previous studies, probiotics have been found to prevent necrotizing entero-colitis (NEC) by preventing colonization of the gut by pathogens, promoting colonization with beneficial organisms, improving maturity and function of gut mucosal barrier and modulating the immune system to the advantage of the host.[11,12]A Cochrane review showed a trend toward a non-significant benefit in reduction of sepsis[25]. The mechanism for efficacy of probiotics in reducing the incidence of sepsis in VLBW infants is probably similar to that for NEC.[26,11] However, in a further study by Lin et al[12] the effect of reduction in incidence of sepsis was not confirmed. This study was conducted among severely ill hospitalized VLBW infants with central line, total parenteral nutrition and prolonged use of mechanical ventilation. Probiotics exert their effects by positively influencing normal microbe–microbe and host–microbe interactions and may augment the protection afforded by commensal flora through competitive interactions, direct antagonism of pathogens, and/or production of antimicrobial factors. The preventive mechanisms could fail in the face of severe conditions as in case of the study by Lin.[12]Probiotics alone would not overcome the infection induced by invasive procedures. However, in the community setting such as in our study, among LBW predominantly breastfed infants, probiotics could be effective in preventing sepsis, since the primary effect of orally administered probiotics is in the gastrointestinal tract with prevention of bacterial translocation.

Neonatal infection is a high priority area of research. Research on immunotherapy[3]has provided very few leads. To our knowledge, at present there are no proven interventions beneficial in preventing sepsis in LBW infants[27,] apart from exclusive breastfeeding and practice of hygiene. This study provides an indication that microbial interference by beneficial bacteria is helpful in decreasing neonatal morbidity. Considering a 30% prevalence of LBW in India[28] and 30% mortality due to sepsis in newborns,[29] even a modest decline in the incidence of sepsis due to preventive intervention with probiotics could avert thousands of neonatal deaths. When produced at large scale it would be a cost-effective intervention for a major public health problem.

We observed a significant positive treatment effect in the subgroup of infants weighing 1.5-2.0 kg. This mandates conduct of a larger study with sufficient power to conclusively evaluate the role of probiotics among LBW infants in a population at high risk of mortality from sepsis. There is also a need to conduct this kind of study for all neonates to assess if probiotics could be beneficial even for children who are not LBW.

Contributorship statement

AS conceptualized the study, prepared the protocol and drafted the report. All the authors reviewed and approved it. AS, SSG, HC, BSG were responsible for the design of the trial; AS SSG MSP were responsible for preparing the standard operating procedures and data collection instruments; SSG, HC,CM, VK, SA, SD, VD and MT were responsible for implementation of the trial and clinical management of subjects; SSG designed the database and managed the data; SSG and AS were responsible for the analyses and interpretation. AS, SSG, HC and SA edited the draft manuscript. VT closely monitored implementation, AM, RG and MR and contributed at different stages of study implementation. All authors had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis, they approved the final version to be published, and agreed to be accountable for the accuracy and integrity of the manuscript.

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Competing Interest

"All authors have completed the Unified Competing Interest form available atwww.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that (4) [AS, SSG, HC, CM, VK, SA, BSG, SD, MSP, RG, VD, MT, VT, AM and MR] have no non-financial interests that may be relevant to the submitted work."

We declare that we do not have any conflict of interest.

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Data Sharing: "No additional data available".

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Table 1. Comparison of baseline characteristics, Intention-to-treat population

	Probiotics (N=668)		Placebo (N=672)		p-value
Mean (SD)birth weight	2261 ± 179		2263 ± 179		0.22
Female sex	349	52.2%	352	52.4%	0.99
Mother's schooling					
≤ 8 years	292	43.7%	285	42.4%	0.63
> 8 years	376	56.3%	387	57.6%	
Religion					
Hindu	489	73.2%	501	74.6%	
Muslim	46	6.9%	41	6.1%	0.80
Others	133	19.9%	130	19.3%	
Standard of Living Index					
Low	98	14.7%	85	12.6%	
Medium	348	52.1%	382	56.8%	0.20
High	222	33.2%	205	30.5%	
Mode of delivery					
Vaginal	633	94.8%	629	93.6%	0.36
LSCS + Others	35	5.2%	43	6.4%	
Morbidities during pregnancy					
Hypertension	23	3.4%	18	2.7%	0.42
Anaemia	55	8.2%	63	9.4%	0.46
PROM	22	3.3%	30	4.5%	027
None	568	85.0%	561	83.5%	
Mean SLI score	22.2 ± 7.9		22.3 ± 7.7		0.82

Table 2. Cumulative risk of possible serious bacterial infection/clinically suspected sepsis

	Probiotics				Placebo				Cumulative risk ratio		p-value **
	n	N	Cumulative risk		N	N	Cumulative risk				
			(%)	95% CI			(%)	95% CI	RR	95% CI	
Possible serious bacterial infection (PSBI, by field investigator)											
All strata	84	668	12.6%	10.3,15.3	107	672	15.9%	13.3,18.9	0.79	0.56,1.03	0.080
1.5-1.99 Kg	4	74	5.4%	1.7,13.49	14	75	18.7%	11.3,29.1	0.29	0.10,0.84	**0.014**
2.0 – 2.49 Kg	80	594	13.5%	11.0,16.5	93	597	15.6%	12.9,18.7	0.86	0.66,1.14	0.303
Male	48	320	15.0%	11.5,19.4	54	323	16.7%	13.0,21.2	0.90	0.63,1.28	0.553
Female	36	348	10.3%	7.5,14.0	53	349	15.2%	11.8,19.4	0.68	0.46,0.99	0.056
Suspected sepsis (by physician)											
All strata	38	668	5.7%	4.2,7.7	54	672	8.0%	6.2,7.7	0.71	0.47,1.06	0.091
1.5-1.99 kg*	0	74	0.0%	0,5.9	8	75	10.7%	5.2,19.9	0.06	0.00,1.01	**0.007**
2.0 – 2.49 kg	38	594	6.4%	4.7,8.7	46	597	7.7%	5.8,10.1	0.83	0.55,1.26	0.381
Male	21	320	6.6%	4.3,9.9	30	323	9.3%	6.6,13.0	0.71	0.41,1.21	0.205
Female	17	348	4.9%	3.0,7.7	24	349	6.9%	4.6,10.1	0.71	0.39,1.30	0.270

* As there was no case among the exposed, the risk ratio and its confidence interval were calculated by adding 0.5 to each cell. Fisher's exact p-value was calculated instead of chi-squared test.

** p-values less than 0.05 have been shown in bold.

Table 3. Incidence rate for PSBI clinically suspected sepsis per 1000 days of follow-up

	Probiotics				**Placebo**				Incidence rate ratio		p-value **
	n	Person-days	Incidence rate/ 1000 days		N	Person-days	Incidence rate/ 1000 days				
			Rate	95% CI			Rate	95% CI	RR	95% CI	
Possible serious bacterial infections (by field investigator) and sepsis by physician											
All strata	98	37532	2.61	2.12,3.18	128	37681	3.40	2.83,4.04	0.77	0.59, 0.99	**0.049**
1.5-1.99 Kg	6	4204	1.67	0.52,3.11	19	4159	4.57	2.75,7.13	0.36	0.15, 0.87	**0.008**
2.0 – 2.49 Kg	92	33328	2.19	2.23,3.39	109	33522	3.25	2.67,3.92	0.67	0.64, 1.12	0.248
Male	58	17946	3.23	2.45,4.18	69	18107	3.81	2.97,4.8	0.85	0.60, 1.20	0.357
Female	40	19586	2.04	1.46,2.78	59	19574	3.01	2.29,3.89	0.68	0.45, 1.01	0.056
Suspected sepsis by physician											
All strata	40	37532	1.07	0.76,1.45	60	37681	1.59	1.21,2.05	0.67	0.45, 0.99	**0.048**
1.5-1.99 Kg*	0	4204	0.00	0.00,1.11	10	4159	2.40	1.15,4.42	0.00	0.0, 0.35	**0.002**
2.0 – 2.49 Kg	40	33328	1.20	0.86,1.63	50	33522	1.49	1.11,1.97	0.80	0.53, 1.22	0.307
Male	23	17946	1.28	0.81,1.92	35	18107	1.93	1.35,2.69	0.66	0.39, 1.12	0.126
Female	17	19586	0.87	0.51, 1.39	25	19574	1.28	0.83,1.89	0.68	0.37, 1.26	0.221

* As there was no case among the exposed, the risk ratio and its confidence interval were calculated by adding 0.5 to each cell. Fisher's exact p-value was calculated instead of chi-squared test.

** p-values less than 0.05 have been shown in bold.

(Table 4) Comparison of Adverse events between probiotics and placebo arms

	Probiotics	Placebo	Total	p-value**
Hospitalization required	29	44	73	**0.038**
Mean days hospitalized	4.6±4.4 days	6.9±5.6 days		**<0.0001**
Median days hospitalized	3 days	6 days	-	-
Deaths	1	2	3	NS

** p-values less than 0.05 have been shown in bold.

Figure1 Participant flow through the trial

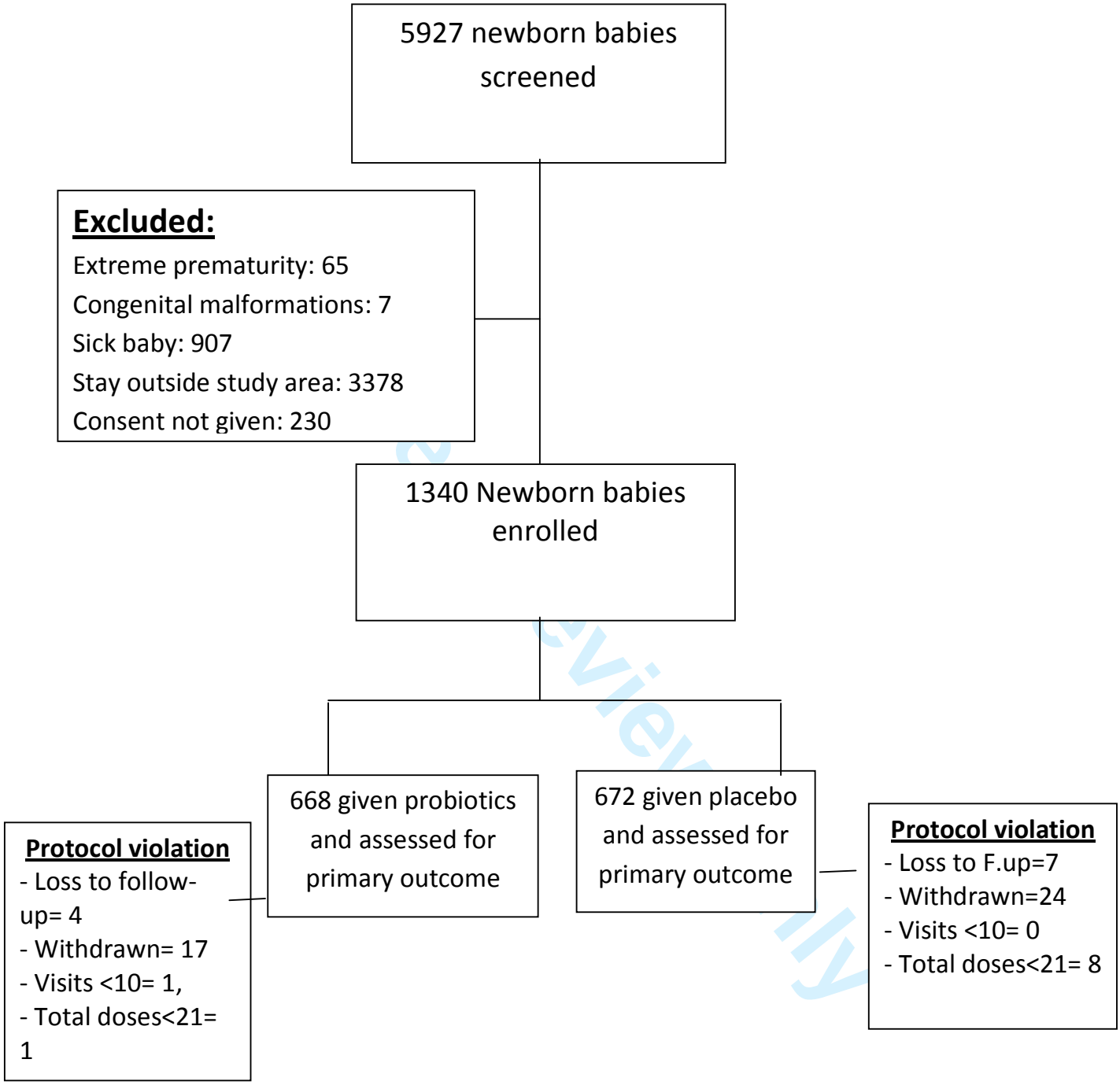
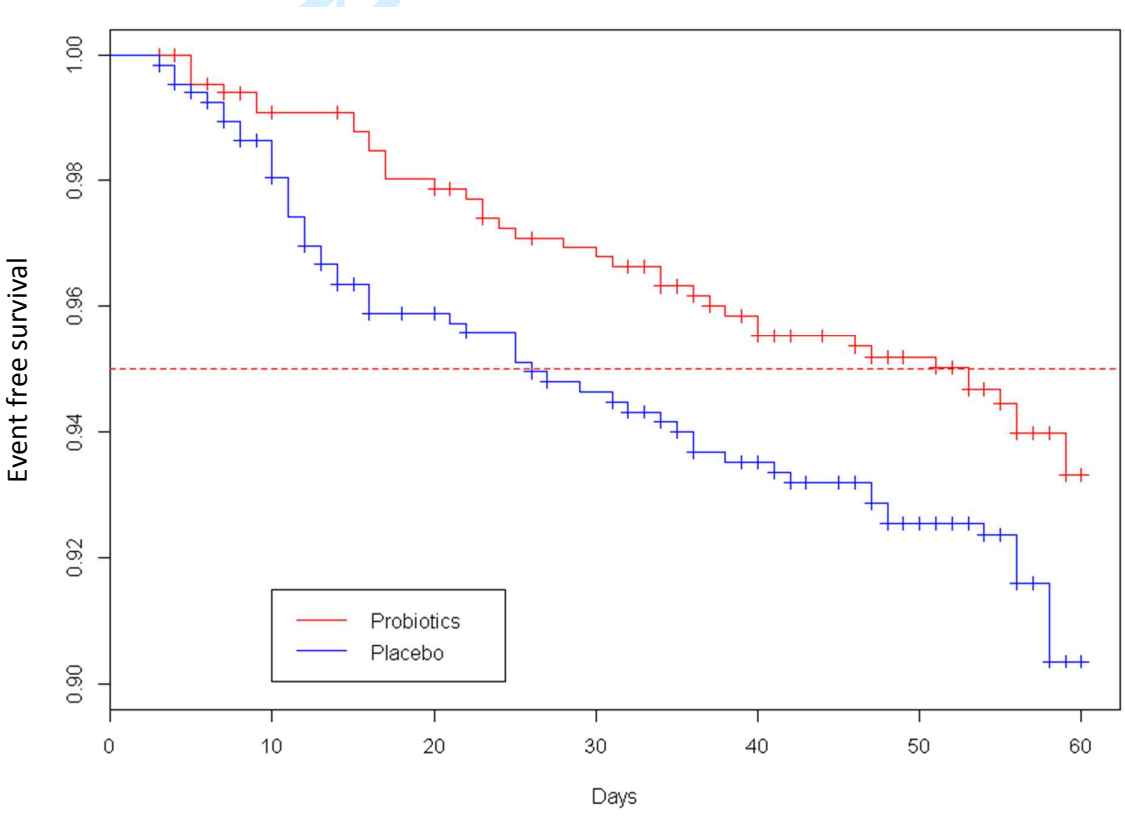
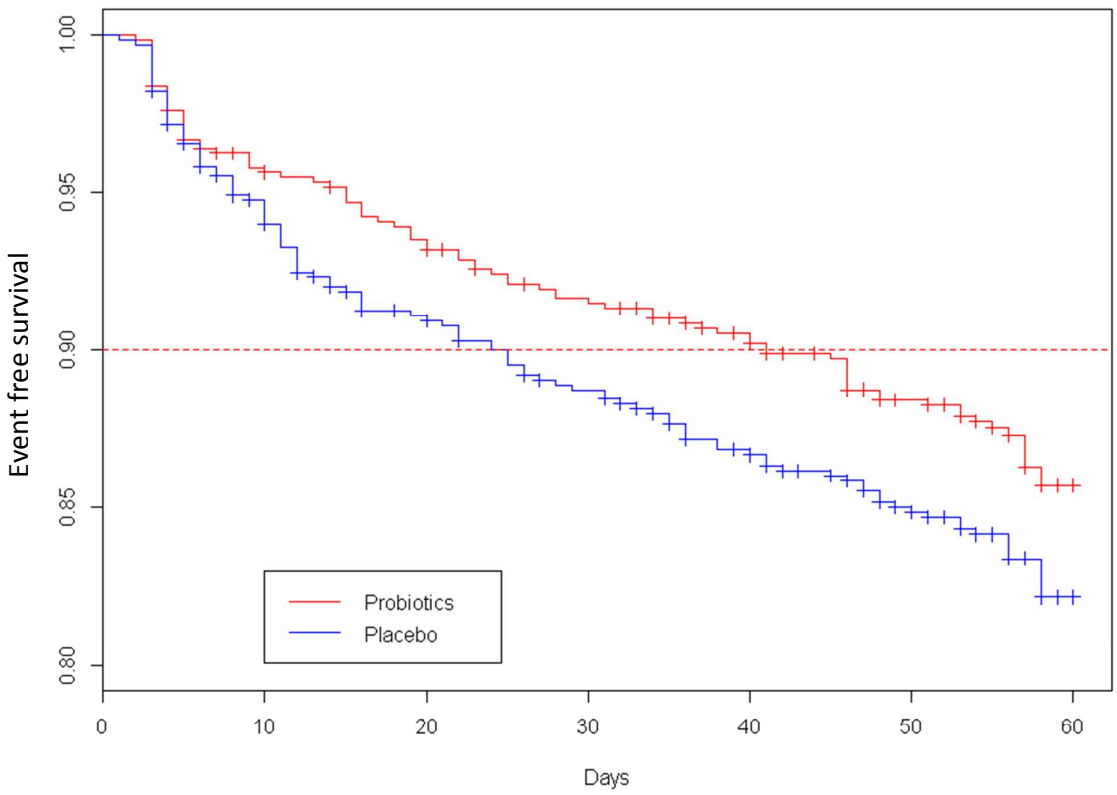


Figure 2 Kaplan-Meier curves for difference between event rates in probiotic and placebo groups. Clinically suspected sepsis by Physicians



B. Possible serious bacterial infection by field workers



For publication as web appendix only:

Methods: Duplicate visits were performed by a study supervisor in 10% of visits as a quality control measure. Field workers records were verified by study medical officer weekly before entering the data in the computer. Viability testing of the probiotics sachets collected from the field sites was done. A schedule for collection of these sachets was prepared such that every week 4 sachets were collected in reverse cold chain from infants who were in 1-4th week of follow up, over a six month period. These sachets were transported to an external lab (Micro s.r.l., Italy, accredited by SINAL [National System for accreditation of laboratories, Italy] and the International Laboratory accreditations cooperation ILAL-MRA]. The certificate of analysis received from the lab certified the adequacy of cell counts in the sachets, and based on the results extended the expiry of the batch by one year. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry in EPI Info version 6.0) with built in range and consistency checks. Hand checking on random samples was done and frequency distribution of important variables examined periodically to identify aberrant values. A program file developed in EPI 6 platform was run, the list of errors was sent to the sites for corrections.

IMNCI Algorithm: Presence of any of the following signs suggested possible serious bacterial infection: convulsions or fast breathing (60 breaths per minute or more); severe chest in-drawing or nasal flaring or grunting; 10 or more skin pustules or a large boil; axillary temperature 37.5 Celsius or above (or feels hot to touch); temperature less than 35.4 Celcius (or feels cold to touch); lethargic or unconscious or less than normal movements. (Ref: Training Modules 1 to 9 - Unicef. www.unicef.org/india/Training_Module_1-9.)

Title of the Project

"Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2 month period: A Randomized Controlled trial"

Indian Council of Medical research

Ansari Nagar, New Delhi 25.4.08

2. *Objectives:*

Primary

- To estimate reduction in the incidence of suspected sepsis in 0-2 month old LBW infants in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants**.**

Secondary

- To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.
- To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (to be done at one center)
- To monitor the side effects due to the probiotics VSL#3

3. Summary of the proposed research

Rationale:

Neonatal infections (pneumonia, septicemia, meningitis and diarrhea) are the commonest causes of mortality in neonates, accounting for almost half of deaths. It has also been identified as national priority area of research to achieve the Millennium Development Goals & National population policy goals.

The world Health Organization estimates that, globally, 32% of the estimated four million neonatal deaths each year are caused by infections, including sepsis, pneumonia, diarrhea and tetanus.[1] Another global review of neonatal infections estimated that annually there are approximately 29 million neonatal infections (including 800,000 cases of sepsis and 130,000 cases of meningitis) and as many as 1.5 million neonatal deaths due to infections[2].

Low Birth Weight is a very important indirect cause of death in neonates the world over. Globally, between 40 and 80% of neonatal deaths occur among LBW[3] (Bang et al). These neonates have poor cognitive function and compromised immune function[4]. In LBW infants infections are known to spread rapidly leading to severe disease and death. Prevention of infection in low birth weight babies would directly decrease the neonatal morbidity and mortality.

The increasing antibiotic resistance in community due to availability over the counter, indiscriminate and incomplete courses used by quacks aggravates the difficulty in management of Sepsis in the community. Problem of drug resistance outweighs the fast pace of newer generation antibiotic production. It is recommended not to use antibiotics relentlessly as antibiotics are not the final answer for infection. WHO recommends global programmes to reduce the use of antibiotics in animals, plants, fishes and in human medicine[5]

Use of better measures to prevent infection using immunomodulation/immunopotentiation with the use of probiotics may prove to be an alternative for prevention of (sepsis).

Aim:

To examine whether it is possible to prevent the morbidity due to neonatal sepsis (septicemia, pneumonia, meningitis) by supplementing the neonates with probiotics.

Objective:

To estimate reduction in the incidence of suspected sepsis in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants.

Hypothesis:

Use of probiotics VSL#3 during neonatal period may reduce morbidity due to clinically suspected sepsis in 0-2 month old LBW infants.

A randomized control trial would be undertaken to prove the hypothesis.

Sample Size:

Assumptions:

Hoyos AB showed a 60% reduction in necrotizing enterocolitis and overall mortality by treatment with *Bifidobacterium infantis* and *Lactobacillus acidophilus*.

Incidence of neonatal sepsis in the community as reported by Bang et al is 17%.

For 30% reduction at 5% significance and 80% power a sample of 670 per group is required including 10% attrition.

A total of 1340 newborns would be enrolled within a period of one year by two study sites.

Methodology:

A double blind randomized controlled trial would be conducted in a facility linked community setting.

Newborns in the Intervention arm would receive VSL#3 10 billion for thirty days. A physically similar preparation of placebo (containing Maltodextrin) would be given to the newborns in control arm. The research team and the PI would remain unaware of the group allocation of neonates.

Enrollment of subjects would be done at a Hospital. Trained field workers would visit the homes of these newborns (within the prescribed study area of 15-20 Kms) for supplementation and morbidity detection as per schedule of visitation.

A detailed manual of operations and data collection tools will be developed and provided to the site investigator.

Incidence rates of clinically suspected sepsis would be compared within the groups using the Chi2 test.

4. Present knowledge and relevant bibliography

Background & Rationale:

Neonatal infections are a major cause of morbidity and mortality worldwide. The world Health Organization estimates that, globally, 32% of the estimated four million neonatal deaths each year are caused by infections, including sepsis, pneumonia, diarrhea and tetanus.[1] Another global review of neonatal infections estimated that annually there are approximately 29 million neonatal infections (including 800,000 cases of sepsis and 130,000 cases of meningitis) and as many as 1.5 million neonatal deaths due to infections[2].

National Neonatal – Perinatal database has reported systemic sepsis as the predominant morbidity (39.7%) in extramural admissions. Septicemia (88.1%) was the most common clinical category of systemic infection, while pneumonia was diagnosed in 32.8% of infants with systemic sepsis. In the 645 culture positive infants, Klebsiella pneumonia was the commonest (30.1%), followed by Staphylococcus aureas (16.2%), E.coli (13%) and Pseudomonas species (9.3%). Sepsis was the commonest primary cause of death (37.6%)

Neonatal sepsis in the community:

It is a major cause of neonatal morbidity and mortality in the neonatal and young infant period. Bang et al[3] have estimated the incidence of clinically suspected sepsis to be 17% and case fatality without intervention to be 18.5%. The definition of clinically suspected sepsis in their study included Invasive bacterial infection of neonates includes septicemia, pneumonia and meningitis. It was responsible for more than 50% of the newborn deaths in the community.

Low Birth Weight defined as a birth weight <2500 g, is a very important indirect cause of death in neonates the world over. Globally, between 40 and 80% of neonatal deaths occur among LBW[3] (Bang et al). These neonates have poor cognitive function and compromised immune function[4]. In LBW infants infections are known to spread rapidly leading to severe disease and death. Prevention of infection in low birth weight babies would directly decrease the neonatal morbidity and mortality

The increasing antibiotic resistance in community due to availability over the counter, indiscriminate and incomplete courses used by quacks aggravates the difficulty in management of Sepsis in the community. Problem of drug resistance outweighs the fast pace of newer generation antibiotic production. It is recommended not to use antibiotics relentlessly as antibiotics are not the final answer for infection. WHO recommends global programmes to reduce the use of antibiotics in animals, plants, fishes and in human medicine[5]

Use of better infection control measures using immunomodulation/immunopotentiation with the use of probiotics may prove to be an alternative for prevention of (sepsis).

Infection control through Microbial interference treatment (MIT)

There are several reasons for renewed and more general interest in infection control through MIT. Antibiotic treatment deranges the protective flora and therby predisposes to later infections. Widespread overprescription and misuse of antibiotics gives rise to antibiotic resistant strains and industry is not able to develop effective antibiotics at a sufficient rate to compete with the development of microbial resistance to old antibiotics.

Probiotic bacteria are live microorganisms belonging to the natural flora with low or no pathogenicity, but with functions of importance to health and well being of the host. Maintenance of this ecological flora is important in preventing disease, especially infections[6].It is increasingly accepted that probiotic bacteria are effective tools for controlling overgrowth of PPMs of bacterial, viral and fungal origin.[7] Probiotic bacteria can control various enteric pathogens such as *Salmonella typhimurium*, *Shigella, Clostridium difficile, campylobacter jejuni* and *Eschirichia coli.* Much evidence thus supports the expectation that probiotic bacteria can be effective weapons for preventing and treating many microbial infections[6]

Probiotics:

The concept of probiotics was introduced by Metchnikoff (Russian Scientist). FAO/WHO defines probiotics as Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.

Benefits of Probiotics on Human Health

Probiotics are viable non-pathogenic microorganisms which, when ingested, exert a positive influence on host health or physiology[8]. The ingestion of probiotics is associated with various beneficial effects on human health and modification in physiological homeostasis of the intestinal flora[9]. The best evidence for efficacy of specific probiotics strains obtained with RCTs is there for prevention/treatment of antibiotic-associated disorders, gastroenteritis and acute diarrhea and in alleviation of lactose intolerance[8]. *L. casei* and *L. acidophilus* have been shown to be useful in management of persistent diarrhea[10]. LGG has been shown to promote clinical recovery from rotavirus gastroenteritis in children[11]. *L Plantarum* has been shown to be useful as a protective agent in the primary prevention of atherosclerosis in smokers[12]. Significant increase in weight and height in experimental group receiving fermented foods (*L. acidophilus*) to combat stunting and failure to thrive has also been reported[10]. Probiotics have also been found useful in prevention of atopic disease [14]

Mechanism of action of probiotics:

Probiotic microorganisms have particular characteristics such as human origin, safety in human use, bile acid resistance, survival in the intestine temporary colonization of human gut, adhesion to the mucosa and bacteriocine production. Thanks to these characteristics, probiotics block the invasion of human intestinal cells by the enteroinvasive bacteria[15].

Probiotic microbial agents and their components exert their protective activities through a variety of mechanisms[16]. Probiotic organisms suppress growth of conventional or potential pathogens as well as their epithelial attachment and /or invasion either directly by secreting antimicrobial substances or by stimulating host expression of protective molecules. Additionally, increased levels of probiotics may induce a “barrier” influence against common pathogens. They can stimulate host production of immunosuppressive molecules that down regulate inflammatory responses, or conversely stimulate host protective immunologic mechanisms that can prevent or accelerate clearance of pathogenic infections. Mechanisms of effect are excretion of acids (lactate, acetate), competition for nutrients and gut receptor sites, immune modulation and formation of specific antimicrobial agents. Mucosal immune stimulation induced by oral administration of LAB influences the balance Th1/Th2 (cellular or humoral response) due to different patterns of cytokine release[17]. LAB can interact with the immune cells of the gut and induce their activation signals. Cell wall structures of pathogenic Gram-positive bacteria act as excellent inducers of inflammatory cytokines TNF alpha, IFN gamma, IL-12. It has been shown that *L bulgaricus* and *L acidophilus* affect the systemic humoral immune response. Interference with pathogen adhesion and invasion. Probiotics likely also enhance the barrier function of naïve epithelial cells not exposed to any pathogen[18]. *L.B. Plantarum* reduces attachment of EPEC to CACO 2 cells. It reduces the in vitro secretary response of intestinal epithelial cells to enteropathogenic E.coli infection and can play an important role in reducing the secretary change in response to EPEC infection, possibly through inhibition of its binding[19]. However, the presence of the probiotic agent before the infection is required, as its role is more preventive rather than therapeutic. Up regulation of immune responses and increased mucosal barrier to translocation of bacteria and bacterial products have been cited as the mechanism for reduction of incidence of NEC in preterm infants[20].

Table: **Mechanism of action of probiotic agents**[16]

Inhibit growth of pathogenic enteric bacteria
Decrease luminal pH
Secrete bactericidal proteins

Stimulate defensin production by epithelial and Paneth cells
Resist colonization (occupy ecologic niche)

Block epithelial attachment or invasion by pathogens
Block epithelial binding by inducing of MUC 2
Stimulate mucus production to alter biofilm
Inhibit epithelial invasion, Rho dependent and independent pathways

Improve epithelial and mucosal barrier function
Produce short-chain fatty acids, including butyrate
Increase barrier integrity

Alter host immune response
Induce IL-10, TGF-b and Cox2 $(PGE)_2$ expression and secretion
Stimulate secretary IgA production
Decrease TN, IFN-y expression
Active regulatory T cells

Genetic engineering
Express and secrete IL-10 and trefoil factors

It has been suggested that some probiotics can help maintain remission in the inflammatory conditions, ulcerative colitis and pauchitis. They also repress enzymes responsible for genotoxin formation[21].. Lykoba[22] et al1 recorded a decrease in detection rate of endotoxinemia, which correlated with the tendency towards the normalization of defective intestinal microflora by inclusion of probiotics Bifidobacterium forte adsorbed on activated charcoal in therapy of digestive tract disease.

Effect of Lactobacillus on bacterial translocation in a neonatal animal model was demonstrated by Drongowski[23] et al. Neonatal rabbits receiving colonization by E.coli KIA,. Lactobacillus GG decreases the frequency of extra intestinal Bacterial translocation by 46% ($p<0.05$), 61%($p<0.05$) and 23% respectively in MNL, SPL & LIV. They showed that enterally-administered LactoGG decreases the frequency of E.coli KIA translocation.

Tsunoda et al[24] showed that pretreatment with heat killed *Lactobacillus Casei* (LC9018) developed a protective activity (peritoneal exudates cell accumulation observed 24 hrs after inj of LC9018) against fecal peritonitis induced after cecal ligation and tip resection surgery.

Sherman et al[25] showed that prophylactic therapy with recombinant human Lactoferrin and probiotics *Lactoacillus GG* act to enhance defenses against invasive *E.coli* in the nascent small intestine. They suggest that recombinant Lactoferrin (rhLF) & LGG are therapeutic agents that may reduce NEC and gut related sepsis in preterm human infants.

Other studies indicated that Bifidobacteria not only colonized the gut of animals, possibly helping to exclude pathogens: they also reduced endotoxemia and appeared to modulate the inflammatory cascade.

Perhaps the most impressive indication that probiotics could benefit newborns comes from a human trial with 2.5X 10^8 live *Lactobacillus acidophilus* and 2.5X10^8 live *Bifidobacterium infantis* in 1237 neonates in Colombia[26]. Compared with 1282 hospitalized patients seen during the previous year, treatment with these strains resulted in a 60% reduction in necrotizing enterocolitis and overall mortality. The positive results in this study support the need for further investigation of bacterial colonization and its role in neonates.

Intestinal translocation is considered important source of infection in adults (in event of stress, chemotherapy, reduced immunity when gut permeability increases[27,28] . In a prospective study it was shown by molecular techniques that the organism recovered from blood in preterm population was always identical to the one cultured from the stool[29] it is highly unlikely that the organism move from blood to the intestinal lumen, hence translocation from the gut to blood stream is a possibility. It was shown that organism present on the skin probably went through mouth and GI tract and eventually translocated from the intestine to the blood stream[30].

Safety of probiotics in neonatal period:

There are indications of prolonged use in infants upto 30 days of Bifidobacteria/Lactobacillus in Russia to create "benign" stool micro floral patterns to prevent/cure dysbacteriosis/sepsis. The entire neonatal population in Russia receives Bifidobacteria or Lactobacilli in an attempt to prevent/ cure dysbacteriosis/sepsis. No blinded controlled studies of this therapy have been performed but the evidence suggests that there is at least no risk involved in such treatment since thousands of infants have been so treated. Also the low incidence of sepsis in Russia argues in favors of its use in neonates (personal communication; A Kuznetsova, Kazan Institute for advanced Medical studies, Tatarstan, Russia)**.**

VSL#3: It is a patented combination of live lactic acid bacteria that have been cultivated, freeze-dried and mixed in high concentration (hundreds of billion per gram). It has been proven in clinical trials to be effective in serious gastrointestinal disorders, and in particular in the management and prevention of inflammation of the small bowel reservoir or pouch, is the most frequent long-term complication following colon removal and pouch creation surgery for ulcerative colitis.

Eight strains of bacteria have been selected cultivated and mixed proportionately to obtain the proven experimental and clinical efficacy[31-39] of VSL#3. All strains included in the product blend are known and accepted organisms in food

Bifidobaterium Breve
Bifidobacterium longum
Bifidobacterium infantis
Lactobacillus acidophilus
Lactobacillus plantarum
Lactobacillus casei
Lactobacillus bulgaricus
Streptococcus thermophilus

These eight beneficial strains act together like a **living shield.**

Lactobacillus Plantarum is expected to be the potential sepsis-preventive strain. It is highly resistant to acid and bile. It exhibits excellent adherence to Caco-2 cells and blocks *E. coli* adherence to Caco-2 cells. It reduces *E. coli* translocation in the transwell system and also in vivo into the blood of weaning rabbits. Being a plantarum strain it can grow in absence of iron. It appears to be completely safe in the closed ileal loop model, and is the predominant human gut flora.(Lab studies by Dr. Panigrahi)

It affects the gut immunity, expression of anti-inflammatory cytokines**.**

Safety ofVSL#3

The lactic acid bacteria in VSL#3 are Generally Recognized As safe (GRAS) Clinical studies have shown that VSL#3 can be taken safely for long periods of time without any problems[31-33]. There is no evidence that ingested probiotic lactic acid bacteria or Bifidobacteria pose any risk of infection greater than that associated with commensal strains. In quantitative terms, the existing data suggest that the risk of bacteremia, which is the most commonly reported of these infections, is <1 per million individuals, considered to be in the "negligible" range.

Adjustment of the intestinal flora after VSL#3 administration can take up to a month for the colonization of the gut to become optimally stable.

Recently Hung- Chin Lin et al have shown that *Lactobacillus acidophilus* and *Bifidobacterium infantis* (inforan) as probitics fed enterally with breast milk reduces the incidence and severity of NEC in VLBW infants[40].

Based on the above review of literature we hypothesize that use of probiotics preparation VSL#3 during the neonatal period may prevent occurrence of sepsis in low birth weight neonates and young infants.

We propose to conduct a Randomized control trial in a community setting.

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8. *Detailed research plan*

Aim:

To examine whether it is possible to prevent the morbidity due to neonatal sepsis (septicemia, pneumonia, meningitis) by supplementing the LBW neonates with probiotics

Objective:

To estimate reduction in the incidence of suspected sepsis in the intervention arm with a daily supplementation of VSL #3 over a period of 30 days in 0-2 month old LBW infants.

Hypothesis:

Daily supplementation of LBW neonates with VSL#3 will reduce the incidence of neonatal sepsis by 30%.

Assumptions:

Hoyos AB showed a 60% reduction in necrotizing enterocolitis and overall mortality by treatment with *Bifidobacterium infantis* and *Lactobacillus acidophilus*.

Incidence of neonatal sepsis in the community as reported by Bang et al is 17%.

For a 30% reduction in incidence of sepsis at a 5% level of significance, with 80% power a sample of 670 cases in each arm of intervention would be required (allowing for a 10% attrition rate). Incidence of LBW is 30%. In order to observe the required 1340 LBW newborns more than 4000 deliveries would be screened considering the fact that some may refuse to participate and some would belong to far off places that may not be possible to cover in the study).

The table below shows the required number of subjects with changing assumptions of power and effect size:

Reduction in incidence	Power	'n' Per group (incl. 10% attrition)	Total	No. to be screened for LBW
50%	80	265	530	1600
30%	**80**	**670**	**1340**	**4020**
50%	90	353	706	2118
30%	90	895	1790	5370

Study Methodology:

This would be a double blind randomized controlled trial. The research team as well as the PI would remain unaware of the group allocation of the neonates. The code would be kept at the INCLEN Trust office, New Delhi under lock and key.

Setting:

The study would be a facility-linked community study. It would be conducted at two sites; in the vicinity of a Delhi hospital, and at a district level hospital and adjoining community in Maharashtra. Screening and enrollment would be done in the hospital; follow-up visits would be carried out by the study staff in the community.

Intervention:

Oral administration of a probiotics preparation VSL#3 containing a dose of 10 billion live bacteria *per os* for 30 days during the neonatal period starting on third day of life.

Placebo:

A similar preparation in the same outer packing would be administered to the neonates in the control group. Content of the placebo has been decided in consultation with pharmaceutical company, keeping in mind the safety issue during neonatal period.

Research method:

A total of 1340 LBW neonates would be needed with 670 each in intervention and control arm. More than 4000 live births would be screened to enroll the required number of subjects, assuming a 30% incidence of LBW. Total number to be screened would be much more since some may refuse to participate and some may belong to far off areas that may be difficult to visit.

A detailed manual of operations and other research tools will be developed and provided to the site investigator.

Randomization

Randomization by permuted block with a block size of 4 would be used. It would ensure random allocation and high probability of balance between the groups at any point of subject recruitment. Computer generated table would be used; patient allocation would be indicated by a study number kept in a sealed-opaque envelope. In a double blind study neither the patient nor the investigator would be aware of the allocation. The code would remain with the INCLEN Trust, New Delhi..

Stratification

In order to achieve balance between the two study groups with regard to important characteristics such as sex and birth weight, randomization would be stratified by sex and birth weight. Two strata (1500 – 2000, and 2001-2500 gms) by sex males and females would be used. Thus there would be four strata as given below:

	Strata 1	**Strata 2**	**Strata 3**	**Strata 4**
Sex	**Male**	**Male**	**Female**	**Female**
B. Wt.	**1500-2000**	**2001-2500**	**1500-2000**	**2001-2500**
	B	**B**	**A**	**B**
	A	**B**	**A**	**A**
	B	**A**	**B**	**A**
	A	**A**	**B**	**B**
	A	**A**	**B**	**A**
	B	**A**	**A**	**B**
	A	**B**	**B**	**B**
	B	**B**	**A**	**A**
	A	**B**	**B**	**B**
	B	**A**	**A**	**B**
	B	**A**	**B**	**A**
	A	**B**	**A**	**A**
	B	**A**	**B**	**A**
	B	**B**	**A**	**B**
	A	**B**	**A**	**A**
	A	**A**	**B**	**B**
	.	.	.	.
	.	.	.	.

Four randomization lists would be prepared, one for each stratum using the proportionate allocation scheme

Selection of Subjects:

Information would be obtained regarding birth of LBW (<2500gms) babies in the hospital on a daily basis by the study staff. The case sheet would be examined to check the residential address of the delivered mother. For study purpose an area of about 15-20 Kms around the hospital would be

considered as study area. Mothers of all LBW neonates belonging to the study area would be approached by the study team (senior research fellow, field worker) for enrollment. The newborn would be assessed for eligibility criteria by using the study screening form. Enrolment would be done on third day of life in the presence of the study physician.

Eligibility: All live born LBW (≥1500 to ≤ 2500 gms) babies available in the hospital would be eligible for the study if they have the following inclusion criteria.

Inclusion criteria: 1. Birth weight ≥1500 gms to ≤ 2500 gms, 2. Residence within 15-20 Kms of the hospital, 3.The mother is planning to stay in Delhi/ study area for a period of at least two months and,

Exclusion criteria: 1. Extreme prematurity (<32 weeks) 2. Presence of a gross congenital malformation incompatible with life, 3. A mother who does not give consent, 4. mother going out of town with the baby,

Parents of babies fulfilling the inclusion criteria and not having any exclusion criteria would be explained about the study with the help of a patient information sheet and asked for consent.

Informed consent procedure:

A mother whose baby is eligible will be informed about the study by the study team (physician/field worker). She would be enquired whether she would allow her baby to be randomly allocated to one of the two groups of the study. If the patient agrees to participate, she will be asked to sign a consent form, which will be read aloud by the field worker for those who are illiterate.

Enrolment:

Enrolment will be done at hospital on 3rd day of life. In case of sick children it can be deferred upto 7th day but not later. Those who agree to give written informed consent would be enrolled and randomized to receive drug or placebo by opening the next in a consecutively numbered series of sealed opaque envelope. This envelope would contain the patient study number corresponding to the randomization list. The drug corresponding to the study number would be fed to the baby in presence of the physician. Administration of the daily dose would be subsequently done by the mother under supervision of the trained field worker. At the time of discharge from the hospital the designated field worker would escort the family to verify the address and note the exact location of residence. They will keep the study drug for the enrolled infant at home in a vaccine/day carrier. Follow up visits would be done by the field worker for supervising supplementation for a period of 30 days. Morbidities would be recorded on follow up forms during home visits as per a schedule.
Parents would be explained that participation in study is voluntary; withdrawal at any time during the course of the study is possible.

Supplement packaging:

The supplement would be prepared by CD Pharma India Pvt. Ltd. Identical packaging of the drug (containing 10 billion of the active ingredients of VSL#3) and placebo with similar consistency and color would be provided. The drugs can withstand a temperature upto 28 degrees Celsius. Therefore there is need for maintaining cold chain. Drug would be kept in suitable plastic packaging in a vaccine/day carrier at the residence of the baby. Mothers of enrolled infants would be instructed to open the lid of vaccine carrier just once daily to take out the required sachet and close the lid tightly thereafter.

Adverse Event Monitoring:
No major adverse events related to the intervention are expected, however continuous monitoring and reporting will be conducted by trained research staff. Any such events will be reported to the

local ethical committee/Data safety monitoring committee. This committee will be responsible for monitoring accrual, safety, outcome measurement and all aspects of the project and advocate continuation or termination of the study based on the results of the interim analyses.

Lab Investigations:

Gut Colonization study:

Gut colonization study of Probiotics VSL#3 is proposed to be conducted at the Department of Microbiology, Safdarjung Hospital in collaboration with The Institute of Pathology, ICMR. It is proposed to study the effect of VSL#3 supplementation on stool colonization patterns in the neonatal gut on a subset subjects. The stool samples for this purpose would be collected at the time of enrolment prior to feeding VSL#3, at the end of third week of supplementation, and at the end of follow-up (day 56-60). Method for this laboratory procedure will be detailed in the lab manual of operations.

Sample Size estimation –Gut Colonization study

Brigidi et al (International Journal of Food Microbiology 81 (2003) 203-209) in a study on patients with IBS have found VSL#3 strains *B. infantis* Y1 and *B. breve* Y8 to be present in 40% and 70% of patients at a concentration of $5x\ 10^5$ and 9×10^5 cells/g feces respectively. This colonization pattern was similar to that observed with the healthy subjects.

Assuming the anticipated proportion of infants likely to colonize (P) to be 40%, at 90% confidence level with a relative precision of 20% the required sample size to be studied is 101. Stool samples from 202 (101 each in intervention & placebo arm) enrolled infants would be collected on day '0', day '21' and at end study.

Additionally blood cultures would be performed on all suspected sepsis cases who give consent for it when they are referred to the facility by the field workers.

Overall morbidity pattern

During home visits other common morbidities of young infant period such as diarrhea, dehydration, dysentery, feeding problem, umbilical sepsis, skin pustules would be recorded and compared between the study groups.

Side effects

Although no major side effects are expected, however efforts would be made to record any side effects that the parents attribute to supplementation, and compared between study groups.

Phases of study implementation:

1. Preparatory phase – 3 months
2. Intervention phase – 15 months
3. Data analysis & reporting phase- 6 months

Activities of preparatory phase:

1. Orientation workshop at the hospital
2. Recruitment of Field workers (team of morbidity detectors)
3. Recruitment of senior research fellow and field attendant
4. Training of Staff (SRF, Field workers) In IMNCI algorithm of diagnosis for young infants
5. Preparation of randomization list by INCLEN Trust, New Delhi.
6. Procurement of drug & Placebo for the study site by CD Pharma

Intervention phase:

This would be the active phase of the study when the Randomized control trial would begin. During this phase enrolment of subjects after obtaining written or verbal informed consent would be done. Each enrolled newborn would be visited at home by a field worker who would supervise the daily supplementation of drug and placebo. The babies would be examined for morbidity detection during the two-month period as per the schedule.

Frequency of visitation:

All newborns would be visited for supplementation. For detection of morbidity the baby would be examined by the trained field worker daily during the first week of life and biweekly during 2-4th week of life. Thereafter during the second month of life weekly visits would be done. **Information would be recorded on a data recording form during all the visits.** Any sick infant would be advised referral to the hospital for treatment.

Staff requirement:

1. Field workers

To conduct a RCT in the community setting would pose many challenges. The entire area within 15-20 kilometers of the hospital would be under the study. The field workers would perform the functions of **intervention supplementation** and morbidity **detection.** The field worker would be required to visit the babies born in the current month as well as those born during the preceding month. On an average a worker would be able to cover 3 -5 babies in a day with some kind of transport support. Therefore a team of at least 6 field workers at each site would be required to conduct follow up visits in the study area. The localities within the study area would be listed and allocation of field workers for specific localities for visitation would be done. This would provide efficient functioning by saving time.

An enrollment card would be provided to each newborn enrolled in the study, mothers would be asked to carry this card whenever they seek treatment for the baby. Information about involvement of the baby in the study would be printed on the card.

Morbidity would be detected by active surveillance.

Active surveillance:

Field workers: Would perform the role of **Supplementors and Morbidity detectors**

There would be six field workers at each site recruited and suitably trained for recording morbidities during home visits. All newborns would be visited daily for supplementation. For detection of morbidity the baby would be examined by the trained field worker daily during the first week of life and biweekly during the first month of life. During the second month weekly visits would be done. Information would be recorded on a data recording forms during all the visits.

The Field workers would be trained in the IMNCI algorithm for detection of neonatal sepsis as described in Annexure1 during the preparatory phase. On detecting neonatal sepsis (possible serious bacterial infection) on the basis of the algorithm they would refer/ accompany the baby to the study clinic/hospital for treatment. At the facility blood culture would be requested and obtained after obtaining consent for the same.
Field workers would record information regarding morbidity conditions on the study forms and get it verified by the study medical officer on a weekly basis. During home visits field workers would replace the ice packs in the vaccine/day carrier containing study drug.

Study Clinic/Hospital:

There would be a clinic/dispensary/ hospital identified in the study area where the study physician would refer the patient for treatment. Proximity of the facility would ensure that there is no delay in treatment of a diagnosed patient. Blood cultures would be done preferably in all cases referred with suspected sepsis.

Quality assurance measures:

Measures to ensure correct administration of drug and placebo:

1. Daily visits to each baby enrolled in the study during the first seven days of life would be made. VSL#3 Probiotic would be administered to the baby in presence of the field worker.
2. During training of staff and initial interaction with the guardians of the subjects the importance of the study number and the corresponding drug packet would be explained. This would also be explained in the study information sheet.
3. Quality assurance of field implementation would be ensured, frequency checks (on 10% visits), surprise visits by study MO would be inbuilt in the procedures. These would be explained in detail in the Manual of operations.
4. Good clinical practice standards would be observed throughout the clinical and laboratory procedures.

Data Processing:

Data obtained by the field workers on the study forms would be checked by the project Medical officer on a weekly basis. The crosschecked forms would be entered in the computer at the study office. Range and frequency checks would be applied. Validated data would be transferred to CCU at ICMR electronically.

Data analysis:

Baseline variables such as mode of delivery, birth weight, gestation etc. will be compared to evaluate the comparability of the groups. The primary outcome measure in this study in a case of clinically suspected sepsis (possible serious bacterial infection), based on the algorithm for diagnosis (IMNCI). The two groups will be compared for the primary outcome.

Based on the study hypothesis a 30% reduction in the number of clinically suspected sepsis is expected in the intervention arm. Incidence rates of clinically suspected sepsis would be compared within the groups using the Chi2 test or Fisher's exact test as appropriate. Multivariate analysis will be used to adjust for potential confounding.

Incidence of other morbidities (diarrhoea, dysentery, feeding problem, skin infection and umbilical sepsis) would be compared in the intervention and control arms. Data on non compliers, protocol violators, study drop outs would be handled with an intention to treat analysis

Protection of Human subjects

The participating center will submit this protocol to its own Ethics Committee for local clearance and approval. The PI at the Coordinating center, ICMR has obtained certification of training in human subject protection. After collecting the information regarding birth of a low birth weight newborn (≤ 2500gms) the mother would be approached while she is still in the hospital. After screening and finding the baby eligible for inclusion in the trial, the parents would be requested for consent to participate. A written informed consent form would be read aloud in presence of a witness and signature/right thumb impression obtained on the form by the study team member. Each participant would be made aware that participation in the trial is voluntary and withdrawal at any point in time is possible without jeopardizing her access to care. Each participant will receive a copy of the consent form, which will contain the names and phone numbers of persons to contact in case of questions or concerns.

Risks to subjects participating in the trial are considered to be minimal. No studies have documented adverse effects related to the drug.

Benefits to the participants include the assurance that all subjects will receive close follow up visits to detect morbidity. Participation to the study may contribute important information, and add to scientific knowledge.

All participant level information would be entered in the computer using the enrollment number. Identity of the participants would not be revealed for any other purpose.

INDIAN COUNCIL OF MEDICAL RESEARCH

"Effect of Probiotics VSL#3 on prevention of sepsis during 0-2 month period in low birth weight infants: A Randomized Controlled trial"

Informed Consent Form

Neonatal Sepsis is a major cause of sickness and death during first two months of life in low birth weight infants. This is a research study conducted by ICMR, New Delhi to determine whether daily supplementation of probiotics VSL#3 to LBW newborns for a period of 30 days can reduce the occurrence of sepsis in 0-2 month period. In this study there are equal chances of your baby receiving either the probiotic or a similar looking substance without probiotics. A field worker would visit your house daily and supervise the administration of the drug. This would be done taking all hygienic precautions. The probiotics are considered beneficial for human health and there are no known risks involved. If this research study demonstrates that occurrence of sepsis in those receiving the probiotics is less than those receiving the placebo then the drug can be recommended for wider use in the community. Mothers milk is the best nutritious food for the baby during first six months of life. Give the baby only mothers milk and the drug during the study.

All the details provided by you would be kept confidential. For any queries during the study you can contact Dr. ------, at ------, Phone No.---. Your participation is completely voluntary and you can withdraw from the study at any time.

Signature of the guardian
Date:

Signature of witness
Date:

INDIAN COUNCIL OF MEDICAL RESEARCH

"Effect of Probiotics VSL#3 on prevention of sepsis during 0-2 month period in low birth weight infants: A Randomized Controlled trial"

Patient Information Sheet

Purpose:

This research study is being conducted by ICMR to understand whether giving VSL3# (a probiotic drug) to LBW newborn babies can be beneficial in reducing the occurrence of neonatal sepsis (meningitis, pneumonia, septicemia) during 0-2 month period. The study will be under the supervision of Dr. (name of concerned PI and institution).

Your participation in the study is completely voluntary.

Procedure:

Your newborn baby would qualify for the study if you are planning to stay at your residence for a period of at least two months, agree to provide the necessary medical information, if your baby is well and does not have any birth defects. If you agree to be a part of the study, then the baby would be given VSL#3 or a similar looking substance, once daily for 30 days. A field worker would visit your house daily during the first week of life and twice in a week during the first month of life and weekly subsequently till 60 days. During the visits He/She would enquire about the wellbeing of the baby since the last visit. The field worker would record information on a form. In case of any illness he would direct the baby to study physician for treatment. You would be instructed to look for danger signs indicating illness in babies 0-2 months given in the pamphlet and inform the field worker.

Risk/Discomforts:

Although exclusive breastfeeding is recommended upto 6 months of life we think that supplementing newborns with the drug VSL#3 would help minimize the risk of neonatal sepsis (meningitis, pneumonia, and septicemia). It is a safe product; no serious adverse events or side-effects have ever been observed. However, hygienic precautions should be taken during its administration to prevent any untoward effect.

Benefits:

The chances of your baby falling sick with sepsis may be reduced. If VSL#3 does reduce sickness during 0-2 month period, this knowledge may benefit both your and other babies throughout the world.

Alternative:

Even if you do not participate it will not lead to any loss in health care which is available to you under the programme of the Government of India

Voluntary participation:

Your participation in the study is completely voluntary. You have the right to withdraw your baby from the study at any point of time.

Privacy and confidentiality:

The information collected during home visits will be treated as confidential and your baby will not be identified as this information would be coded.

Authorization to publish results:

Results of this study may be published for scientific purposes and/or presented to scientific groups; however your identity will not be disclosed.

Person to contact:

In case of any difficulty experienced or in the event of any emergency, you can contact Dr. (PI/MO) at (address of PI and MO or by calling up at the following telephone No…….(Tel No on which the PI and MO can be contacted).

Dummy Tables:

Table1: Baseline characteristics comparison between probiotics and placebo group

Characteristics	Probiotics n %		Placebo n %	
Sex				
SLI				
Caste				
Education				
Religion				
gestation				

Table 2: Primary Outcome

Outcome	probiotic		placebo	
	n	%	n	%
Neonatal sepsis				
Possible serious bacterial infection				

Table 3: Secondary Outcomes

Outcome	probiotic		placebo	
	n	%	n	%
Diarrhea				
Oral thrush				
Cold Cough				
Number of Possible serious bacterial infection				
Number of Culture confirmed neonatal sepsis				
Number of Drop outs				
Non compliers				
Number alive at 60 days				
Number hospitalized				

Table 4: Side Effects:

	probiotic		placebo	
	n	%	n	%
vomiting				
?				
?				

Table 5: Rate of enrolment at interim analysis

	Safdarjung hospital	Wardha Hospital
Probiotics		
Placebo		
Total		

Table 6: Compliance & drop outs

	SJH				WH			
	n		%		n		%	
Non compliers								
Dropouts								

Amendment to the protocol

- Following the first meeting of the Data Safety Monitoring Committee meeting on 22 May 09 the following amendments to the protocol are made. It was stated that the two study sites should follow the **same method for screening the subjects.** All live births taking place in different labor rooms and O.Ts should be screened for detection of births eligible for the study ie; newborns with birth weight <= 2500 gms. All Screening forms should be preserved in a file. The Field Workers employed for the project should be trained and allowed to conduct screening.

- **A fixed protocol for investigations and treatment** of the referred infants should be prepared and followed by both centers. Even for cases of confirmed Sepsis defined doses for all common injections with number of days of prescribed treatments should be written down and adhered to. It was said that it was the Ethical responsibility of the study team to give best treatment at home to the enrolled infant at home if the mother refuses referral. The study physician should visit home of such sick infants to deliver injection treatment at home.

- Maintaining the study drug in **cold chain** was discussed. It was stated that the drug remains effective at 24 degrees temperature for about 18 months however in view of the high temperature during summer months there is a need to store the drug at 4-8 degrees Celsius. Additional budget has been provided to the centers for purchase of Vaccine carriers and deep freezers. It was suggested that a weekly dose of the drug should be provided to the mother instead of the monthly supply and proper SOP developed to monitor the cold chain including hours of electric supply available.

- It was recommended to undertake weekly viability tests on the samples and prepare graphs from the lab results. The CD Pharma company should be requested to help with this activity. It was also suggested to prepare thermostable labels that change color when exposed to heat and use it on the sachets. It was recommended to discuss this issue with the Company providing the Drug/Placebo.

- **Supervision for Quality Control**: It was recommended to hire a Research Assistant level person to work independent of the Field Worker team. These visits would be independent of the FW visits and at least 2 such visits should be conducted daily on a random basis. A list from the computer would be generated for this purpose.

- **Quality Assurance at CCU**:
  The CCU should monitor the study like a CRO organization with site visits every three months. Initially more number of visits are needed to check selection criteria, documentation and adherence to the protocol.

- It was stated that the IMNCI protocol is very sensitive and should be used as a screening test to detect cases of possible serious bacterial infection, however it can not remain the diagnostic criteria at the facility where the infant has been referred for care. In clinical trials specificity for the outcome is more important. At the facility the infant should be examined by the pediatrician and labeled as suspected sepsis if the pediatrician so decides. Blood culture should be performed on referred infants for final diagnosis by the gold standard method. Site PIs should be able to prepare the following table:
  - Suspected Sepsis Person making diagnosis IMNCI Definition Field Worker Clinical Screening positive Pediatrician Culture positive sepsis Laboratory
- A pediatrician should cross check all referred cases on the day of referral. If referral is refused SRF should visit and provide best possible treatment as given in the treatment protocol for the study.
- It was stated that validation of the IMNCI would be a by-product of the study.

Data Safety Monitoring Committee:

- Blood samples should be obtained from sick children who are referred to facility in two transport media, one for routine blood culture and other suitable for culture of probiotics bacteria. If in any sample same species of probiotics bacteria are cultured from the blood as contained in the VSL#3 the study will be stopped.
- All Serious Adverse Events should be immediately reported to Chairperson and members of the DSMC. The study forms of the case should be Xeroxed and sent to CCU by speed post where a summary of findings should be prepared and shared with the members of DSMC.
- An interim analysis would be indicated in the following situations:
    1. If one and a half time more number of deaths are reported from the study population as against the expected numbers.
    2. If confirm sepsis rates are increasing in the study population as against the expected rates.
    3. If loss to follow up exceeds the expected 10%. If 50% of babies completed 60 days of follow-up. (The CCU should look at literature to find out the expected death rates and rates of neonatal sepsis)

Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2monthperiod: A Randomized Controlled trial"

Justification of staff required at one site

One S.R.F- A senior Research office (Medical) is required for screening the all newborns delivered in the hospital on daily basis, to scrutinize their suitability for recruitment in the study. He will help in enrolment and supervise follow-up visits by the Field workers. He will ensure quality control by performing 5-10% visits in the field. He will be responsible for data collection, cleaning, entry and transfer.

Six Field workers- This is a facility-linked community study. The field workers are required for making home visits of enrolled babied at home for detection of morbidity, checking compliance of study drug/placebo. 670 babies x15 visits =10050 visits / 6FWx24daysx15 months =2160person days =4.65 visits per person per day.

One DEO is required for entering the data from the study forms to the computer software, do data cleaning as per corrections done by FW and SRF and send it to ICMR .

One Laboratory Technicians They are required for conducting the lab work for the study such as stool colonization, blood and stool culture etc.

One Statistician for ICMR HQ
A statistician is required at the head quarter to write the plan of analysis, conduct interim data analysis, monitor data and conduct the final data analysis for the study

Equipment: In the current budget there is no provision for providing any equipment to the centers. Although the lab work requires equipments, the centers are requested to utilize their in house facilities for lab work.

Justification of contingency required at one site
Contingency: Recurring contingency is required under the budget heads given in the budget to carry out the work related to the project. These amounts are minimum required without which the project can not be carried out.

Justification of T.A: The money under TA is required for field work. Visiting babies at home for detection of illness is an activity directly linked with the outcome of the study. Since there is no provision of vehicular support in the budget, this amount is the minimum required to carry out the field work.

Plan of Analyses document for

"Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2 month period: A Randomized Controlled trial"

Objectives:

Primary

- To estimate reduction in the incidence of suspected sepsis in 0-2 month old LBW infants in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants**.**

Secondary

- To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.
- To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (to be done at one center)
- To monitor the side effects if any, due to the probiotics VSL#3

Methodology:

A double blind randomized controlled trial is being conducted in a facility linked community setting.

Newborns in the Intervention arm receive VSL#3 10 billion for thirty days. A physically similar preparation of placebo (containing Maltodextrin) is given to the newborns in control arm. The research team and the PI are unaware of the group allocation of neonates.

Enrollment of subjects is done at a Hospital. Trained field workers visit the homes of these newborns (within the prescribed study area of 15-20 Kms) for supplementation and morbidity detection as per schedule of visitation. A detailed manual of operations and data collection tools will be developed and provided to the site investigator.

Data Collection Forms:

1. Baseline form: Collects Socio-demographic information from families of enrolled subjects.

2. Screening Form: Is used for initial screening of LBW live born babies for checking their eligibility.

3. Enrolment form: Is filled at the time of enrolment, for all eligible infants fulfilling inclusion criteria who enter the study.

4. Follow up Form: This is the main form filled during all home visits during the follow up period. It records information related to compliance, symptoms and signs of morbidity elicited by the Field worker. Temperature, Respiratory rate and Weekly weights of babies are measured and recorded.

5. Final outcome form: Filled for all enrolled subjects, it gives information about the status of the infant on day 60 whether alive or not, whether the infant was sick, hospitalized during the two month period and also records immunization status.

6. Referral & medicine form: Records all information regarding the treatment of referred infants and the drug doses received by them.

List of Definitions:

1. Suspected Sepsis: A case diagnosed by the field worker as per IMNCI criteria for severe possible bacterial infection.

2. Low Birth Weight: An infant weighing less than or equal to 2500 gms.

3. **Loss to follow up: Withdrew from study: drop out**: All subjects on whom less that 50% of expected visits could be completed (less than 10 visits)

4. Protocol violation study drug discontinuation will be treated as violation of study protocol.

5. **Adverse events**: All cases of hospitalizations and deaths among enrolled infants will be treated as adverse events.

6**. Morbidities**: As defined under IMNCI.

Plan of Analysis:

Data obtained by the field workers on the study forms would be checked by the project Medical officer on a weekly basis. The crosschecked forms would be entered in computer using Epi info software with built in range and frequency checks. Validated data would be transferred to the central coordinating unit at ICMR electronically. Analysis would be performed using SPSS version 17. Analysis would be done on pooled data from the two study sites.

Baseline variables such as mode of delivery, birth weight, gestation etc. will be compared to evaluate the comparability of the groups. Continuous variables would be compared using t-test, categorical variables would be compared using Chi square or Fisher's exact tests as appropriate.
Primary Analysis: The primary outcome measure in this study in a case of clinically suspected sepsis (possible serious bacterial infection), based on the algorithm for diagnosis (IMNCI). The two groups will be compared for the primary outcome.

Based on the study hypothesis a 30% reduction in the number of clinically suspected sepsis is expected in the intervention arm. Both absolute and relative measures of association would be computed. We would compute the following: risk reduction (effect size), number needed to treat, relative risk, and 95% CI for each of the outcome measures. In case of imbalance in two groups with respect to the baseline characterics, multivariate analysis method would be used to compute adjusted outcome measures.

Analysis would be by *intention to treat*. Data on non compliers, protocol violators, study drop outs would be handled in a way such that subject assigned to intervention arm will be considered as belonging to that arm even if he/she has not complied with the study protocol.

A separate *Per-Protocol* analysis will also be done including only the cases who have complied with the intervention. Compliance will be defined as those enrolled infants who ingested the study drug (for 25 days) and were followed up for more than 50% of scheduled visits.

Analysis 1: Proportion of suspected sepsis cases diagnosed by the IMNCI algorithm would be calculated for each intervention arm.

Decision rule for Analysis 1:

Numerator = No. of cases of suspected sepsis observed by IMNCI algorithm in one arm
Denominator = Number of subjects enrolled in the study arm
Primary outcome of suspected sepsis by IMNCI would be the answers marked as code 1 (possible serious bacterial infection) in Q. No. 24 in the Probiotics follow up form. Number of such cases in each arm would also be cross checked/verified from the final outcome form as well as monthly statistics prepared by the centers.

Since IMNCI is expected to over diagnose suspected sepsis cases, in a separate analysis we would compare the arms using more stringent definition of suspected sepsis such as 'when two or more signs are present'.

Test of proportions (Chi2 test or Fisher's exact test as appropriate) would be used to compare the two arms, Effect estimates and 95 percent confidence limits will be calculated by conventional method.

Although sample size was calculated for the primary objective as diagnosed by FW, however diagnoses by Pediatrician/physician are also being collected and blood cultures are also being done. A comparison between arms would also be done on sepsis as defined by these parameters. Data reported by the centers on the number of suspected sepsis cases as diagnosed by Physician/Pediatrician, and confirmed blood cultures would be obtained for this analysis and reported for hypothesis generation purposes only.

Analysis 2: We would also compare the Incidence rate ratios between the two arms since we would have the person time data collected during home visits. The following statistics would be computed:

		Disease		
		D+	D-	Person Time
	E+	a	b	N1
Exposure				
	E-	c	d	N2

Incidence rate among exposed = a/N1 = IR1

Incidence rate among unexposed = c/N2 = IR2

Incidence Rate Ratio = IR1/IR2

Incidence Rate Difference = IR1-IR2

Efficacy of probiotics = 1-RR x 100 (Incidence rate in control minus Incidence rate in intervention divided by Incidence rate in control multiplied by 100%)
Number Needed to Treat. = 1/Incidence Rate difference

Calculation of person time: From the probiotics follow up forms number of days contributed by each infant would be computed arm wise. Total number of person-months or years can be derived in each arm. Person-time will be expressed as 60 days; exposure for each infant would be calculated as the time from enrolment/ or first visit to time of detection of morbidity, death or completion of study. Incidence –density of suspected sepsis will be estimated by dividing the total incident cases by overall person-time, and expressed as incident cases per 100 young infant periods.

Numerator: Number of incident cases of suspected sepsis observed in each arm.

Denominator: Person-time.

An episode of sepsis would be defined as: A period of illness when the infant has one or more sign/symptom of illness continuously. Two episodes should be separated by at least 3-5 days Multivariate analysis: Would be done to look at the effect of probiotics after adjusting for confounding by sex, birth weight, Mother/fathers Education status, religion, SLI Score, mode of delivery, breastfeeding status, and premature rupture of membrane in mother. The dependent variable would be suspected sepsis.

Secondary Objectives:

1. **To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.**

Analysis: Incidence of other morbidities (diarrhea, dysentery, feeding problem, skin infection and umbilical sepsis) would be compared in the intervention and control arms using the Chi square or the Fisher's exact test as appropriate.

Morbidity	Q No. & Form	Numerator	Denominator	Statistic	test
Diarrhea	Q 17 Probiotics F.up form	All infants with Code 1 (yes) for Q 17	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Dysentery	Q 18 probiotics F.up form	All infants with Code 1 (yes) for Q 18	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Feeding problem	Q 23 & Q 24	All infants with Q23 code 'a', and Code 1 for Q 24	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Skin infection	Q 14	All infants with Code 1 for Q 14	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Umbilical sepsis	Q 13	All infants with Code 1 for Q 13	No. of infants enrolled in the arm%	% %	Chi square/Fisher's exact test
Local Bacterial infection	Q 24	All infants with Code 2 for Q 24	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Hospitalization	Q27	All infants with Code 1 for Q 27	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test

2. To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (done at Safdarjung hospital center only).

Laboratory study: Gut colonization rates of probiotics at three time points (day '0', day '21' and day '60' as described in the lab results would be compared using repeated measures ANOVA between the intervention and control arms.

To monitor the side effects if any, due to the probiotics VSL#3

Comparison of Adverse events and Loss to follow ups would also be done between the two study arms.

Dummy Tables:

1. **Flow Diagram of Trial Participants:**

   Numbers screened

   Number enrolled/randomized

   Drop outs, loss to follow up

   Non compliers

   Numbers included in intention to treat analysis= No. enrolled

Numbers included in per protocol analysis = No. receiving the intervention for 23? Days and available for 10 or more visits.

2. Table showing baseline characteristics in Probiotics and Control arms

Characteristic	**Probiotics**	**Control**	**'p' Value**
No. of Babies			
Sex			
B.Wt. 1500-2000			
2001-2500			
Mode of delivery			
Normal			
LSCS			
Forceps			
Mother' education			
1. Illiterate,			
2. I-VIIIth,			
3. X-12th,			
4. Graduation			
Religion Hindu			
Muslim			
Christian			
Other			
Standard of Living Index			
Low 0-14			
Medium 15-24			
High 25-67			

3. Intervention Coverage by treatment group

Household Visits	Probiotics	Placebo	'p' value
Mean			
Median			
Effective Coverage			

4. Suspected Sepsis by Treatment Group

Algorithm Suspected Sepsis diagnosed by F.W.(IMNCI)	**Infants**	**Cases**	**Person-time**	**Rate**	**IRR (95% CI)**
Probiotics					
Placebo					
Suspected Sepsis diagnosed by pediatrician					
Probiotics					
Placebo					
Blood Confirmed Sepsis					
Probiotics					
Placebo					

5. Other morbidities by treatment group

Morbidities	**Probiotics**	**Placebo**	**'p' value**
Diarrhea			
Dysentery			
Feeding problem			
Skin Infection			
Umbilical sepsis			
Local Bacterial Infection			
Hospitalization			

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Role of Probiotics VSL#3 in prevention of suspected sepsis in low birth weight infants in India: a randomized controlled trial

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Role of Probiotics VSL#3 in prevention of suspected sepsis in low birth weight infants in India: a randomized controlled trial

Brief Title: Probiotics in prevention of suspected sepsis in LBW infants

Anju Sinha[1](PhD), Subodh S. Gupta[2](MD), Harish Chellani[3](MD), Chetna Maliye[2](MD), Vidya Kumari[3](PGDMCH), Sugandha Arya[3](MD), B.S Garg[2](PhD), SunitaDixit[4](DNB), M.S. Dixit, Sunita (MD), Prasad[3](MD), Vijayshri Deotale[2](MD), Manish Taywade[2](MD),Vasantha Thavraj[1] (MD)Ajit Mukherjee[1](PhD), and Malabika Roy[1](MD)

1. Indian Council of Medical Research, 2. Mahatma Gandhi Institute of Medical Sciences, Sewagram, 3. Safdarjung Hospital, New Delhi. 4. District Hospital Wardha.

Corresponding author:

Anju Sinha, Scientist 'D', Indian Council of Medical Research, New Delhi 110029.Email: apradhandr@gmail.com

Telephone: +91 26589493

Mobile: +91 9811422241

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This trial is registered with Clinical Trial Registry of India (CTRI), number CTRI/2008/091/000049

Abstract

Objectives: To assess the effect of the probiotic VSL#3 in prevention of neonatal sepsis in low birth weight (LBW) infants. **Design**: Randomized, double-blind, placebo-controlled trial. **Setting**: Community setting in rural India. **Participants**: LBW infants aged 3-7 days. **Interventions**: Infants were randomized to receive probiotic (VSL#3, 10 billion cfu) or placebo for 30 days, and were followed up for two months. **Main outcome measure**: possible serious bacterial infection (PSBI) as per Integrated Management of Neonatal Childhood Illnesses algorithm, diagnosed by field workers/physicians. **Results**: 668 infants were randomized to probioticVSL#3 and 672 to placebo. By intention-to-treat analysis, the risk of PSBI among infants in the overall population of LBW infants was not statistically significant (RR 0.79 [95% CI 0.56 to 1.03]). Probiotics reduced median days of hospitalization (6 days vs. 3 days in probiotics, p=0.018) but not the risk of hospitalization (RR 0.66 [95% CI 0.42 to 1.04]. The onset of PSBI in 10% of infants occurred on the 40th day in the probiotics arm versus 25th day in control arm (p=0.063). **Conclusions**: Daily supplementation of LBW infants with probiotics VSL#3 (10 billion cfu) for 30 days led to a non-significant 21% reduction in risk of neonatal sepsis. A larger study with sufficient power and a more specific primary end point is warranted to confirm the preventive effect of VSL#3 on neonatal sepsis in LBW infants. **Trial registration:** The study is registered at the Clinical Trial Registry of India (Trial is registered at www.ctri.nic.in. Registration No. CTRI/2008/091/000049).

Funding: Indian Council of Medical Research.

Article summary: Strengths and limitations of this study

- Low birth weight (LBW) neonates are at high risk for infections, including neonatal sepsis.
- Probiotics are effective in preventing neonatal necrotising entero-colitis and nosocomial infections in preterm LBW babies
- In our study, daily supplementation of LBW infants with probiotics VSL#3 (10 billion cfu) for 30 days led to a non-significant 21% reduction in risk of neonatal sepsis. A significant

effect was observed among infants weighing 1.5-1.9 kg. Survival analysis showed 15 day delay in the onset of sepsis in the intervention arm.

- Our study used IMNCI algorithm for diagnosis of possible serious bacterial infection (PSBI-suspected sepsis) by field workers. A larger study with sufficient power and a more specific primary end point (such as physician's diagnosis of neonatal sepsis) is warranted to confirm the preventive effect of VSL#3 on neonatal sepsis in LBW infants.
- Our study was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 days of life, therefore the role of probiotics on early onset sepsis could not be evaluated.

Introduction

Neonatal infections are responsible for more than a quarter of the 1 million neonatal deaths every year in India.[1]Low Birth Weight (LBW) is a very important indirect cause of death in neonates, accounting for40%to 80% of neonatal deaths.[2]Infections (sepsis, pneumonia and meningitis) are known to evolve more rapidly in LBW infants, leading to severely increased disease and higher rate of death. Prevention of infection in LBW babies would directly decrease neonatal morbidity and mortality. Management of neonatal sepsis with antibiotics faces the problem of drug resistance, attributed to availability over the counter, indiscriminate use and incomplete courses in India. Researchers are evaluating immunotherapy (with immune globulin, myeloid colony stimulating factors, probiotics, glutamine supplementation, recombinant human protein C and lactoferrin) as adjuvants for the prevention of neonatal sepsis.[3]

attracted much interest and debate in the neonatal literature during the last decade.[4]FAO/WHO defines probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[5]Probiotic microorganisms have particular characteristics: human origin, safety in human use, bile acid resistance, survival in the intestine, temporary colonization of the gut, adhesion to the mucosa, and bacteriocine production. The ingestion of probiotics is associated with modification in physiological homeostasis of the intestinal flora, which is important in preventing disease, especially infections.[6]The best evidence for efficacy of specific probiotics strains has been obtained with randomized controlled trials and meta-analysisisin the prevention and treatment of antibiotic-associated diarrhoea,[7]gastroenteritis and acute diarrhoea,[8]and in alleviation of lactose intolerance.[9]

Clinical trials evaluating the role of probiotics (Infloran) in preterm very low birth weight infants[10-12] reported a reduction in incidence of necrotizing entero-colitis (NEC), overall mortality[10] and severity of NEC.[11]A meta-analysis[13]and systematic reviews[14,15]of randomized trialsuggested a beneficial effect of probiotic treatment on reducing the incidence and all-cause mortalitydue to NEC. Following on fromthe evidence on VLBW and premature infants, we hypothesized that the probiotic

preparationVSL#3 mightreduce morbidity due to sepsis in LBW infants.We aimed to estimate reduction in the incidence of suspected sepsis in 0-2 month old low birth weight infants in the intervention arm with a daily supplementation of probiotic VSL#3, 10 billion colony-formingunits (cfu) over a period of 30 days. Ifprovento be efficacious,itcould be an important public health intervention for prevention of neonatal infections.

Methods

Study design and participants

We undertook a randomized, double-blind, placebo-controlled (1:1) trial from January 2009 to November2011 at two tertiary care hospitals and the adjoining community areas (Safdarjung hospital in New Delhi and Mahatma Gandhi Institute of Medical Sciences Wardha, India). We screened newborn infants aged 3 days, born in the hospitals weighing 1500-2500 g, residing within 20-25 km of the hospital, and not planning to shift residence for at least the next two months. We excluded extremely premature infants (< 34 weeks), sick infants, those with congenital malformations incompatible with life, thosewith guardiansnot giving consentand belonging to out of study area. Eligible babies, for whom parents/guardians gave informed consent, were enrolled on days 3-7 of life. Participants were enrolled by a physician in the hospital and followed up in the community for two months for occurrence of neonatal sepsis and other morbidities.Baseline information on demographic characteristics was obtained for assessment of Standard of Living Index.[16]Ethical clearance was obtained from the two participating institutes. A Data Safety and Monitoring Committee (DSMC) met every six months and reviewed severe adverse events.

Study medication

Infants were randomly assigned to receive probiotic or placebo by the study physician. The intervention consisted of administration of the probiotic preparation VSL#3(a mix of 8 strains: *Streptococcus thermophilus, Bifidobacteriumbreve, B. Longum, B. Infantis, Lactobacillus acidophilus, L. Plantarum, L. Paracasei,* and *L. Delbrueckiisppbulgaricus,*at a dose of 10 billion colony-forming units (cfu) for 30 days, starting on third day of life. The content of the probiotic sachet was mixed in

expressed breast milk in a plastic cup and fed to the infant. Sterilized plastic cup and stirrer were provided along with the sachets. A similar-looking maltodextrin preparation in the same outer packing was administered to the control group. The supplement was prepared by CD Pharma India Pvt. Ltd. The preparations withstood a temperature up to 28 degrees Celsius and were therefore kept in a cold chain (refrigerators/vaccine carriers) at the homes of enrolled infants.

Randomization and masking

A computer generated stratified block randomization with permuted block size of 4 was used. We stratified infants by birth weight (1500-2000g, 2001-2500g) and sex. A team of scientists at INCLEN Trust, New Delhi, used a computer-generated table for subjectallocation. Allocation concealment was ensured by sequentially numbering the sachet packets containing VSL#3 or placebo after block randomization. Identical packaging of VSL#3 and a placebo with similar consistency and colour was provided. Parents of enrolled infants, investigators and field workers were masked to treatment allocation. Data analysis was performed in a blinded manner. The codes remained with the INCLEN Trust, and were disclosed to the DSMB and ICMR on completion of data analysis.

Follow-up and assessment

Follow-up visits were done by the field worker, for supervising supplementation over 30 days, and detection of morbidities over two months. Visitation was daily during the first week, biweekly in weeks 2-4 of life, and weekly in the second month. Detection of neonatal sepsis was performedduring visits,using the Integrated Management ofNeonatal and Childhood Illnesses (IMNCI) algorithm (www.unicef.org/india/Training_Module_1-9.) for detection of possible serious bacterial infection suggested by presence of any of the following signs of infection: convulsions or fast breathing (60 breaths per minute or more); severe chest in-drawingor nasal flaring or grunting; 10 or more skin pustules or a large boil; axillarytemperature 37.5 Celsius or above (or feels hot to touch); temperature less than 35.4 Celcius (or feels cold to touch); lethargic or unconscious or less than normal movements.Field workers referred and accompanied sick infants to the study hospital for treatment. At the hospital, the infants were examined by a physician, blood cultures were obtained, and treatment was carried out as per the protocol of the hospital.

Information on compliance and morbidities was recorded. An enrollment card was provided whichparents were asked to carry whenever they sought treatment for the infant in between study visits. Effort was made to contact local practitioners visited independently by parents of infants and collect the details of treatments prescribed. Study staffwere trained in the IMNCI algorithm and givenpractice on eliciting signs of neonatal sepsis. Study procedures were standardized and regular exercises were conducted so as to reduce inter- and intra-observer variability. Quality assurance measures included supervisory checks in the field work, data collection and data cleaning. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry (in EPI Info version 6.0) with built-in range and consistency checks.

The primaryoutcome was risk of possible serious bacterial infection (PSBI) as per the IMNCI algorithm, diagnosed by the field workers or physicians.Secondary outcomes were estimation of the effect of VSL#3 on overall morbidity pattern in 0-2 month-old LBW infants; stool colonization patterns in 10% of subjects (to be reported separately); and assessment of side effects due to the probiotic VSL#3, if any.On the recommendation ofthe DSMC, data on diagnosis of sepsis by a physician was also recorded as an amendment to the protocol.

Statistical analysis

Bang et al[2] reported a 17% incidence of neonatal sepsis in the community. Assuming a 10% loss to follow-up, 1340 infants were needed (670 in each group), toobserve a 30% reduction in incidence of sepsis at 5% significance with 80% power.Analyses were done by intention to treat. Software 'R' [17] (version 3.0.0) was used for calculation of PSBI risk, incidence rates, confidence intervals and incidence rate ratios. We used Kaplan-Meier survival analysis curves with Herrington Flemming variation[18] of the log rank test to compare the survival curves in the probiotic and placebo arms.

Role of the funding source

Funding source played no role in the study design, data collection, analysis and interpretation, writing of the report or decision to submit it for publication.

Results

Between January 2009 and November 2011, 5927 LBW newborn infants were screened and 1340 eligible LBW infants were enrolled (Figure1)**.**Of the 5927 screened, 4587 were excluded (reasons given in Fig.1). The probiotic and placebo groups were comparable with regard to baseline characteristics such as mode of delivery, mean birth weight, mother's schooling, religion of the family, standard of living index (SLI), andmaternal morbiditiesduring current pregnancy (Table 1).

The intervention and control groups were similar in mean number of fieldworkervisits performed (20.8 ± 3.7 in probiotic versus 20.5 ± 4.0 in placebo groups; p= 0.154), mean number of doses of interventional product consumed (29.1 ± 4.4 in probiotics versus 28.7 ± 5.2 in placebo; p= 0.129), and mean number of days of follow-up visits(56.3± 2.2 in probiotics versus 56.1±3.8 in placebo; p=0.239).

Primary outcome: Possible serious bacterial infection (PSBI)

Based on the intention-to-treat (ITT) analysis there was a non-significant 21% reduction in the overall risk of PSBI in the probiotic group (84 cases in 688 infants in the probiotic arm versus.107 cases in 672 in the placebo arm; RR 0.79 [95% CI 0.56 to 1.03]; p = 0.080) (Table 2**).** In the probiotic group there was a significant 71% reduction in the risk of unpre-specified sub-group of infants with birth weights 1.5-1.99 kg (4 cases in 74 infants in probiotics vs. 14 cases in 75 in the placebo arm; RR 0.29 [95% CI 0.10 to 0.84]; p = 0.014). A 32% reduction in the risk of PSBI among unpre-specified sub-group of female infants was observed (36 cases in 348 infants in probiotic vs. 53 cases in 349 in placebo group; RR 0.68 [95% CI 0.46 to 0.99]; p= 0.056).There was no evidence of an interaction effect in the un pre-specified sub-group analysis(p-value = 0.128 for the interaction term between treatment and birth weight group).

We also calculated the incidence rates of PSBI computed with the person-time data collected during home visits (**Table 3)**. The PSBI incidence rate in the probiotics arm was 2.61per 1000 days follow-up, versus 3.40 per 1000 days in the placebo arm (RR 0.77 [95% CI 0.59 to 0.99], p=0.0493). Among unpre-specified sub-group of babies weighing 1.50-1.99kg, the incidence rate of PSBI per 1000 days was 1.67 and 4.57 in the probiotic and placebo groups, respectively (RR 0.36 [95% CI 0.15, 0.87; p=0.008).

Secondary outcomes:

Other morbidities

There was no significant difference between the groups for proportion of babies who had local infection (3.0%; [95% CI 2.0% - 4.7%]in probiotic vs. 3.4%; [95% CI 2.2% - 5.0%] in placebo group, p=0.69), feeding problems (18.9%; [95% CI 16.0% - 22.0%] in probiotic vs. 16.4%; [95% CI 13.7% - 19.3%] in placebo group, p=0.21), or other morbidities (35.9%; [95% CI 32.4% - 39.6%] in probiotic vs. 34.2%; [95% CI 30.7% - 37.9%] in placebo group, p= 0.52).

Gut Colonization study

Results of this part of the study are being communicated elsewhere.

Post - Hoc Analyses:

A post-hoc analysis based on the ITTshowed a non-significant 29% reduction in the overall risk of physician-diagnosed sepsis in the probiotic group (38 cases in 688 infants in the probiotic vs.54 cases of 672 in the placebo group; RR 0.71 [95% CI 0.47 to 1.06], p = 0.091). There was no case of suspected sepsis diagnosed by physician in the group of 74 infants taking probiotics and weighing 1.50-1.99kg, as compared to 8 cases in 75 infants of this weight in the placebo group (RR & 95% CI not calculated due to no sepsis case in probiotics group, Fisher's Exact test p-value = 0.007).There was no evidence of an interaction effect in the un pre-specified sub-group analysis (p-value = 0. 974 for the interaction term between treatment and birth weight group).

In the post-hoc analysis of physician-diagnosed sepsis, the incidence rate in the probiotic arm was 1.07per 1000 days, versus 1.59 per 1000 days with placebo(RR 0.67; [95% CI 0.45 to 0.99], p

=0.048). In the 1.5-1.99 kg weight stratum, there was no case of sepsis diagnosed by the physician, versus an incidence rate of 2.40 per 1000 follow-up days in the placebo arm (RR 0.00 [95% CI 0.0, 0.35]; p = 0.002).

Comparison of event rates

Kaplan Meier survival analysis curves were plotted to compare the event rates in the probiotic and placebo arms (Figure 2).This shows a divergence between the curves for probiotic and placebo, starting after a week of supplementation and remaining throughout the follow-up period. The onset of first episode of PSBI in 10% of infants occurred on the 41st day in the probioticarm versus 24thday in control arm (p=0.063), and onset of first episode of suspected sepsis diagnosed by physician in 5% of infants occurred on 53rd day in probiotic arm versus 26th day in control arm (p=0.071).

Adverse outcomes:hospitalizations and deaths

Hospitalization and death in enrolled infants were considered as moderate and severe adverse outcomes respectively (Table 4). During the study 29 infants in the probiotic and 44 in the placebo arm needed to be hospitalized (p=0.075). Median number of hospitalizationwas of 3 days in the probiotic versus 6 days in the placebo arm(p <0.018). There were three deaths, one in the probiotic and two in the placebo arm. Verbal autopsy reports of deaths reviewed by the DSMB did not attributethem to the intervention. No side-effects of VSL#3 were reported.

Discussion

Overall, supplementation with the probiotic VSL#3 in LBW infants was associated with a 21% (non-significant) reduction in the riskof suspected sepsis (PSBI) diagnosed by the field worker. However, in the unpre-specified sub-group of infants weighing 1.5-1.99 kg, the reduction in risk of PSBI was statistically significant (reduction of 71%; p=0.014).The primary analysis in this study was based on PSBI classification by field worker as per the IMNCI algorithm as an indicator of neonatal sepsis. [19] The classification PSBI under IMNCI is described as sensitive but not specific for detection of

neonatal sepsis.[20]Prior to closure of the study, the DSMC recommended conducting post-hoc analyses using physician's diagnosis of sepsis as the outcome measure. In this analysis there is a 29% overall reduction in risk of sepsis. However, in the unpre-specified sub-group of infants weighing 1.5-1.99 kg, there is a 100% reduction, with no cases observed in the group receiving probiotic supplementation. Our findings of probiotics efficacy among infants 1.5-1.99 kg may be a chance finding, generating a hypothesis that this intervention may be useful for the most vulnerable of the LBW babies. As our power calculations did not consider this apriori, hence these findings need to be confirmed in future studies. Probiotic intervention significantly reduced the mean number of hospitalization days.The Kaplan Meier survival analysis shows a 15-day delay in the onset of sepsis in the intervention arm; this translates to a disease-free window during the 28-day period, crucial for neonatal survival.Moreover, considering a higher case fatality in sepsis at early ages, this becomes even more important. Our results may not be definitive or robust enough; however, there is a consistency in them, and we do not consider this as a "negative trial". Although our study is not large enough, it may be misleading to interpret it as proving that there is no effect of the probiotic intervention or no difference between the study groups. More evidence needs to be generated, since interpretation of no effect might discourage further studies.[21]

Physician's diagnosis of sepsis is more meaningful than PSBI, owing to its specificity. The reported post-hoc analyses increase our confidence in the results. However, physicians used their clinical judgement for diagnosing sepsis; there was no standardized definition used, and this is a limitation of the study. Future trials should evaluate the role of VSL#3 on incidence of sepsis with a precise definition of the outcome measure. The incidence of sepsis observed in the study was lower than the expected effect size used in determining the sample size of the study.Home visits,[22,23]health education messages about exclusive breastfeeding and hygiene, andreferralby field workers could improve care and care-seeking,resulting inlower morbidity and mortality and a type II error for the overall result of our study.Our study has several other limitations. It was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 day of life, so we cannot comment on the

role of probiotics on early onset sepsis. We followed infants for a period of two months, and cannot comment on the long term effects of probiotic supplementation.There are concerns regarding heterogeneity in probiotic products.The literature suggests greater protection with double or triple probiotic strains.[13]Probiotic VSL#3 is a mix of 8 strains namely-*Streptococcus thermophilus, Bifidobacteriumbreve, B. Longum, B. Infantis, Lactobacillus acidophilus, L. Plantarum, L. Paracasei,* and *L. Delbrueckiisppbulgaricus.*In a randomized placebo-controlled clinical trial in India, VSL#3 resulted in early recovery and reduced need for oral rehydration saltsin rotavirus-affected children aged 6 months to 2 years.[24]

In previous studies, probiotics have been found to prevent necrotizing entero-colitis (NEC) by preventing colonization of the gut by pathogens, promoting colonization with beneficial organisms, improving maturity and function of gut mucosal barrier and modulating the immune system to the advantage of the host.[11,12]A Cochrane review showed moderate to low quality evidence that oral lactoferrin with or without probiotics decreases sepsis and NEC in preterm infants[25]. The mechanism for efficacy of probiotics in reducing the incidence of sepsis in VLBW infants is probably similar to that for NEC.[26,11]However,in a further study by Lin et al[12] theeffect of reduction in incidence of sepsis was not confirmed. Thisstudy was conducted among severely ill hospitalized VLBW infants with central line, total parenteral nutrition and prolonged use of mechanical ventilation. Probiotics exert their effects by positively influencing normal microbe–microbe and host–microbe interactions and may augment the protection afforded by commensal flora through competitive interactions, direct antagonism of pathogens, and/or production of antimicrobial factors.The preventive mechanisms could fail in the face of severeconditions as in case of the study by Lin.[12]Probiotics alone would not overcome the infection induced by invasive procedures. However, in the community setting such as in our study, among LBW predominantly breastfed infants, probiotics could be effective in preventing sepsis, since the primary effect of orally administered probiotics is in the gastrointestinal tract with prevention of bacterial translocation.

Neonatal infection is a high priority area of research. Research on immunotherapy[3]has provided very few leads. To our knowledge, at present there are no proven interventions beneficial in preventing sepsis in LBW infants[27,] apart from exclusive breastfeeding and practice of hygiene. This study provides an indication that microbial interference by beneficial bacteria is helpful in decreasing neonatal morbidity. Considering a 30% prevalence of LBW in India[28] and 30% mortality due to sepsis in newborns,[29]even a modest decline in the incidence of sepsis due to preventive interventionwith probiotics could avert thousands of neonatal deaths.When produced at large scale it would be a cost-effective intervention for a major public health problem.

We observed a significant positive treatment effect in the subgroup of infants weighing 1.5-2.0 kg. This mandates conduct of a larger study with sufficient power to conclusively evaluate the role of probiotics among LBW infants in a population at high risk of mortality from sepsis.There is also a need to conduct this kind of study for all neonates to assess if probiotics could be beneficial even for children who are not LBW.

Contributions

AS conceptualized the study, prepared the protocol and drafted the report. All the authors reviewed and approved it. AS, SSG, HC, BSG were responsible for the design of the trial; AS SSG MSP were responsible for preparing the standard operating procedures and data collection instruments; SSG, HC,CM, VK, SA, SD, VD and MT were responsible for implementation of the trial and clinical management of subjects; SSG designed the database and managed the data; SSG and AS were responsible for the analyses and interpretation. AS, SSG, HC and SA edited the draft manuscript. VT closely monitored implementation, AM, MR and contributed at different stages of study implementation. All authorshad full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis, they approved the final version to be published, and agreed to be accountable for the accuracy and integrity of the manuscript.

Transparency statement

I, Dr. Anju Sinha, the lead author of this manuscript affirm that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Competing Interest Declaration

"All authors have completed the Unified Competing Interest form available atwww.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that (4) [AS, SSG, HC, CM, VK, SA, BSG, SD, MSP, VD, MT, VT, AM and MR] have no non-financial interests that may be relevant to the submitted work."

We declare that we do not have any conflict of interest.

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Table 1. Comparison of baseline characteristics,Intention-to-treat population

	Probiotics (N=668)		Placebo (N=672)	
Sex				
Male	319	47.8%	320	47.6%
Female	349	52.2%	352	52.4%
Birth weight groups				
1500 – 1999 gm	74	11.1%	75	11.2%
2000 – 2499 gm	594	88.9%	597	88.8%
Mean (SD)birth weight	2261 ± 179		2263 ± 179	
Mother's schooling				
≤ 8 years	292	43.7%	285	42.4%
> 8 years	376	56.3%	387	57.6%
Religion				
Hindu	489	73.2%	501	74.6%
Muslim	46	6.9%	41	6.1%
Others	133	19.9%	130	19.3%
Standard of Living Index				
Low	98	14.7%	85	12.6%
Medium	348	52.1%	382	56.8%
High	222	33.2%	205	30.5%
Mode of delivery				
Vaginal	633	94.8%	629	93.6%
LSCS + Others	35	5.2%	43	6.4%
Morbidities during pregnancy				
Hypertension	23	3.4%	18	2.7%
Anaemia	55	8.2%	63	9.4%
PROM	22	3.3%	30	4.5%
None	568	85.0%	561	83.5%
Mean SLI score	22.2 ± 7.9		22.3 ± 7.7	

Table 2. Cumulative riskof possible serious bacterial infection/clinically suspected sepsis

	Probiotics				Placebo				Cumulative risk ratio		p-value **
	N	N	Cumulative risk		N	N	Cumulative risk				
			(%)	95% CI			(%)	95% CI	RR	95% CI	
Possible serious bacterial infection (PSBI, by field investigator)											
All strata	84	668	12.6%	10.3,15.3	107	672	15.9%	13.3,18.9	0.79	0.56,1.03	0.080
1.5-1.99 Kg	4	74	5.4%	1.7,13.49	14	75	18.7%	11.3,29.1	0.29	0.10,0.84	**0.014**
2.0 – 2.49 Kg	80	594	13.5%	11.0,16.5	93	597	15.6%	12.9,18.7	0.86	0.66,1.14	0.303
Male	48	320	15.0%	11.5,19.4	54	323	16.7%	13.0,21.2	0.90	0.63,1.28	0.553
Female	36	348	10.3%	7.5,14.0	53	349	15.2%	11.8,19.4	0.68	0.46,0.99	0.056
Suspected sepsis (by physician)											
All strata	38	668	5.7%	4.2,7.7	54	672	8.0%	6.2,7.7	0.71	0.47,1.06	0.091
1.5-1.99 kg*	0	74	0.0%	0,5.9	8	75	10.7%	5.2,19.9	___	_____	**0.007**
2.0 – 2.49 kg	38	594	6.4%	4.7,8.7	46	597	7.7%	5.8,10.1	0.83	0.55,1.26	0.381
Male	21	320	6.6%	4.3,9.9	30	323	9.3%	6.6,13.0	0.71	0.41,1.21	0.205
Female	17	348	4.9%	3.0,7.7	24	349	6.9%	4.6,10.1	0.71	0.39,1.30	0.270

*RR and 95% confidence intervals could not be calculated due to no case of sepsis in probiotics group. Fisher exact test p-value.

** p-values less than 0.05 have been shown in bold.

Table 3. Incidence rate for PSBI clinically suspected sepsis per 1000 days of follow-up

	Probiotics				**Placebo**				Incidence rate ratio		p-value **
	n	Person-days	Incidence rate/ 1000 days		N	Person-days	Incidence rate/ 1000 days				
			Rate	95% CI			Rate	95% CI	RR	95% CI	
Possible serious bacterial infections (by field investigator) and sepsis by physician											
All strata	98	37532	2.61	2.12,3.18	128	37681	3.40	2.83,4.04	0.77	0.59, 0.99	**0.049**
1.5-1.99 Kg	6	4204	1.67	0.52,3.11	19	4159	4.57	2.75,7.13	0.36	0.15, 0.87	**0.008**
2.0 – 2.49 Kg	92	33328	2.19	2.23,3.39	109	33522	3.25	2.67,3.92	0.67	0.64, 1.12	0.248
Male	58	17946	3.23	2.45,4.18	69	18107	3.81	2.97,4.8	0.85	0.60, 1.20	0.357
Female	40	19586	2.04	1.46,2.78	59	19574	3.01	2.29,3.89	0.68	0.45, 1.01	0.056
Suspected sepsis by physician											
All strata	40	37532	1.07	0.76,1.45	60	37681	1.59	1.21,2.05	0.67	0.45, 0.99	**0.048**
1.5-1.99 Kg*	0	4204	0.00	0.00,1.11	10	4159	2.40	1.15,4.42	0.00	0.0, 0.35	**0.002**
2.0 – 2.49 Kg	40	33328	1.20	0.86,1.63	50	33522	1.49	1.11,1.97	0.80	0.53, 1.22	0.307
Male	23	17946	1.28	0.81,1.92	35	18107	1.93	1.35,2.69	0.66	0.39, 1.12	0.126
Female	17	19586	0.87	0.51, 1.39	25	19574	1.28	0.83,1.89	0.68	0.37, 1.26	0.221

* As there was no case among the exposed, the risk ratio and its confidence interval were calculated by adding 0.5 to each cell. Fisher's exact p-value was calculated instead of chi-squared test.

** p-values less than 0.05 have been shown in bold.

(Table 4) Comparison of Adverse outcomes between probiotics and placebo arms

	Probiotics	Placebo	Total	p-value*
Hospitalization required	29	44	73	**0.075**
Duration of hospitalization 25th centile Median 75th centile	 2 days 3 days 5 days	 3 days 6 days 8.75 days	73	**<0.018****
Deaths	1	2	3	NS

* p-values less than 0.05 have been shown in bold.

** p-values calculated using Mann-Whitney Wilcoxon test

Figure1 Participant flow through the trial

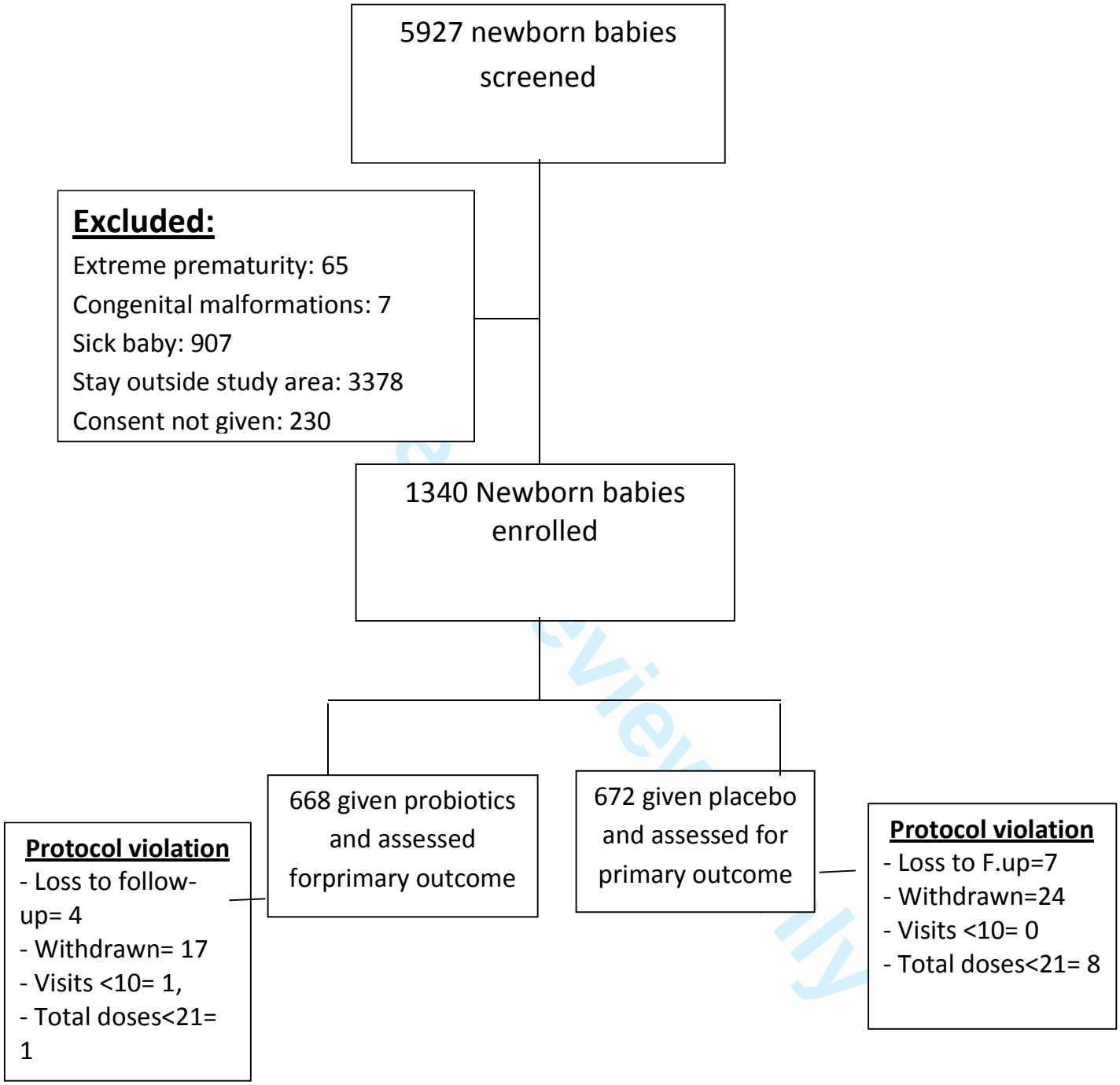
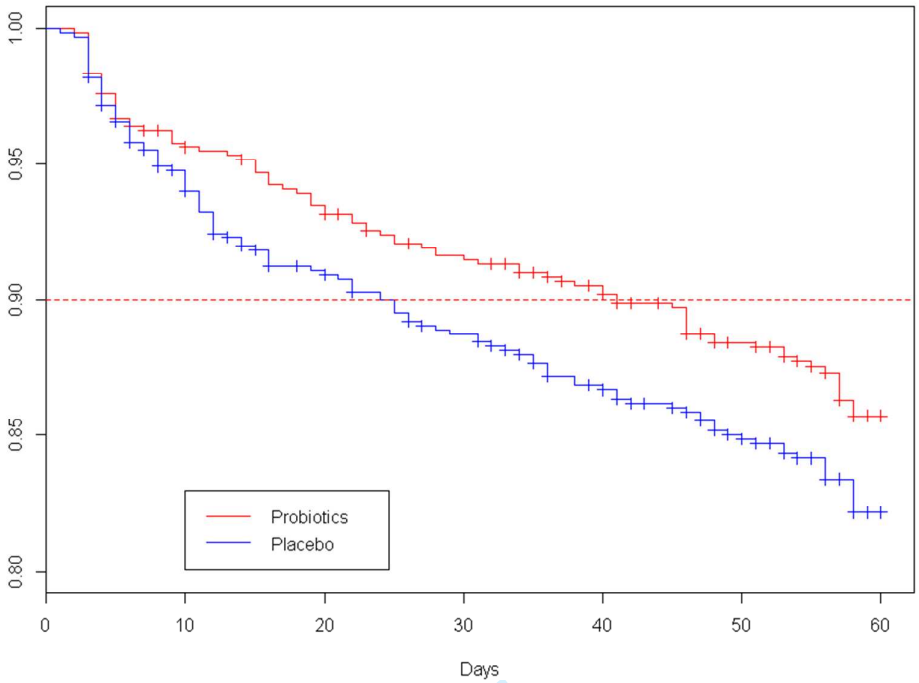


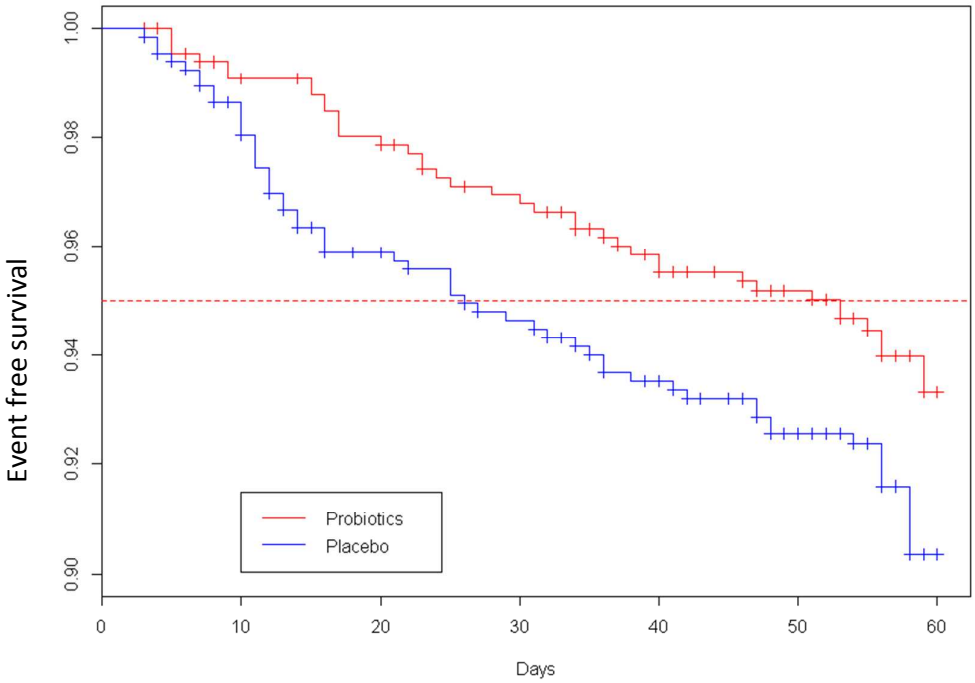
Figure 2: Kaplan-Meier curves for difference between event rates in probiotic and placebo groups.

A. Possible serious bacterial infection by field workers



Logrank test (Harrington Flemming Variation) for PSBI by Field workers. 'p' value 0.063

B. Clinically suspected sepsis by Physicians



Logrank test (Harrington Flemming Variation) for PSBI by Field workers for clinically suspected sepsis diagnosed by the physicians 'p' value 0.071

Title of the Project

"Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2 month period: A Randomized Controlled trial"

Indian Council of Medical research

Ansari Nagar, New Delhi 25.4.08

2. *Objectives:*

Primary

- To estimate reduction in the incidence of suspected sepsis in 0-2 month old LBW infants in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants**.**

Secondary

- To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.
- To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (to be done at one center)
- To monitor the side effects due to the probiotics VSL#3

3. Summary of the proposed research

Rationale:

Neonatal infections (pneumonia, septicemia, meningitis and diarrhea) are the commonest causes of mortality in neonates, accounting for almost half of deaths. It has also been identified as national priority area of research to achieve the Millennium Development Goals & National population policy goals.

The world Health Organization estimates that, globally, 32% of the estimated four million neonatal deaths each year are caused by infections, including sepsis, pneumonia, diarrhea and tetanus.[1] Another global review of neonatal infections estimated that annually there are approximately 29 million neonatal infections (including 800,000 cases of sepsis and 130,000 cases of meningitis) and as many as 1.5 million neonatal deaths due to infections[2].

Low Birth Weight is a very important indirect cause of death in neonates the world over. Globally, between 40 and 80% of neonatal deaths occur among LBW[3] (Bang et al). These neonates have poor cognitive function and compromised immune function[4]. In LBW infants infections are known to spread rapidly leading to severe disease and death. Prevention of infection in low birth weight babies would directly decrease the neonatal morbidity and mortality.

The increasing antibiotic resistance in community due to availability over the counter, indiscriminate and incomplete courses used by quacks aggravates the difficulty in management of Sepsis in the community. Problem of drug resistance outweighs the fast pace of newer generation antibiotic production. It is recommended not to use antibiotics relentlessly as antibiotics are not the final answer for infection. WHO recommends global programmes to reduce the use of antibiotics in animals, plants, fishes and in human medicine[5]

Use of better measures to prevent infection using immunomodulation/immunopotentiation with the use of probiotics may prove to be an alternative for prevention of (sepsis).

Aim:

To examine whether it is possible to prevent the morbidity due to neonatal sepsis (septicemia, pneumonia, meningitis) by supplementing the neonates with probiotics.

Objective:

To estimate reduction in the incidence of suspected sepsis in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants.

Hypothesis:

Use of probiotics VSL#3 during neonatal period may reduce morbidity due to clinically suspected sepsis in 0-2 month old LBW infants.

A randomized control trial would be undertaken to prove the hypothesis.

Sample Size:

Assumptions:

Hoyos AB showed a 60% reduction in necrotizing enterocolitis and overall mortality by treatment with *Bifidobacterium infantis* and *Lactobacillus acidophilus*.

Incidence of neonatal sepsis in the community as reported by Bang et al is 17%.

For 30% reduction at 5% significance and 80% power a sample of 670 per group is required including 10% attrition.

A total of 1340 newborns would be enrolled within a period of one year by two study sites.

Methodology:

A double blind randomized controlled trial would be conducted in a facility linked community setting.

Newborns in the Intervention arm would receive VSL#3 10 billion for thirty days. A physically similar preparation of placebo (containing Maltodextrin) would be given to the newborns in control arm. The research team and the PI would remain unaware of the group allocation of neonates.

Enrollment of subjects would be done at a Hospital. Trained field workers would visit the homes of these newborns (within the prescribed study area of 15-20 Kms) for supplementation and morbidity detection as per schedule of visitation.

A detailed manual of operations and data collection tools will be developed and provided to the site investigator.

Incidence rates of clinically suspected sepsis would be compared within the groups using the Chi2 test.

4. Present knowledge and relevant bibliography

Background & Rationale:

Neonatal infections are a major cause of morbidity and mortality worldwide. The world Health Organization estimates that, globally, 32% of the estimated four million neonatal deaths each year are caused by infections, including sepsis, pneumonia, diarrhea and tetanus.[1] Another global review of neonatal infections estimated that annually there are approximately 29 million neonatal infections (including 800,000 cases of sepsis and 130,000 cases of meningitis) and as many as 1.5 million neonatal deaths due to infections[2].

National Neonatal – Perinatal database has reported systemic sepsis as the predominant morbidity (39.7%) in extramural admissions. Septicemia (88.1%) was the most common clinical category of systemic infection, while pneumonia was diagnosed in 32.8% of infants with systemic sepsis. In the 645 culture positive infants, Klebsiella pneumonia was the commonest (30.1%), followed by Staphylococcus aureas (16.2%), E.coli (13%) and Pseudomonas species (9.3%). Sepsis was the commonest primary cause of death (37.6%)

Neonatal sepsis in the community:

It is a major cause of neonatal morbidity and mortality in the neonatal and young infant period. Bang et al[3] have estimated the incidence of clinically suspected sepsis to be 17% and case fatality without intervention to be 18.5%. The definition of clinically suspected sepsis in their study included Invasive bacterial infection of neonates includes septicemia, pneumonia and meningitis. It was responsible for more than 50% of the newborn deaths in the community.

Low Birth Weight defined as a birth weight <2500 g, is a very important indirect cause of death in neonates the world over. Globally, between 40 and 80% of neonatal deaths occur among LBW[3] (Bang et al). These neonates have poor cognitive function and compromised immune function[4]. In LBW infants infections are known to spread rapidly leading to severe disease and death. Prevention of infection in low birth weight babies would directly decrease the neonatal morbidity and mortality

The increasing antibiotic resistance in community due to availability over the counter, indiscriminate and incomplete courses used by quacks aggravates the difficulty in management of Sepsis in the community. Problem of drug resistance outweighs the fast pace of newer generation antibiotic production. It is recommended not to use antibiotics relentlessly as antibiotics are not the final answer for infection. WHO recommends global programmes to reduce the use of antibiotics in animals, plants, fishes and in human medicine[5]

Use of better infection control measures using immunomodulation/immunopotentiation with the use of probiotics may prove to be an alternative for prevention of (sepsis).

Infection control through Microbial interference treatment (MIT)

There are several reasons for renewed and more general interest in infection control through MIT. Antibiotic treatment deranges the protective flora and therby predisposes to later infections. Widespread overprescription and misuse of antibiotics gives rise to antibiotic resistant strains and industry is not able to develop effective antibiotics at a sufficient rate to compete with the development of microbial resistance to old antibiotics.

Probiotic bacteria are live microorganisms belonging to the natural flora with low or no pathogenicity, but with functions of importance to health and well being of the host. Maintenance of this ecological flora is important in preventing disease, especially infections[6].It is increasingly accepted that probiotic bacteria are effective tools for controlling overgrowth of PPMs of bacterial, viral and fungal origin.[7] Probiotic bacteria can control various enteric pathogens such as *Salmonella typhimurium*, *Shigella, Clostridium difficile, campylobacter jejuni* and *Eschirichia coli.* Much evidence thus supports the expectation that probiotic bacteria can be effective weapons for preventing and treating many microbial infections[6]

Probiotics:

The concept of probiotics was introduced by Metchnikoff (Russian Scientist). FAO/WHO defines probiotics as Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.

Benefits of Probiotics on Human Health

Probiotics are viable non-pathogenic microorganisms which, when ingested, exert a positive influence on host health or physiology[8]. The ingestion of probiotics is associated with various beneficial effects on human health and modification in physiological homeostasis of the intestinal flora[9]. The best evidence for efficacy of specific probiotics strains obtained with RCTs is there for prevention/treatment of antibiotic-associated disorders, gastroenteritis and acute diarrhea and in alleviation of lactose intolerance[8]. *L. casei* and *L. acidophilus* have been shown to be useful in management of persistent diarrhea[10]. LGG has been shown to promote clinical recovery from rotavirus gastroenteritis in children[11]. *L Plantarum* has been shown to be useful as a protective agent in the primary prevention of atherosclerosis in smokers[12]. Significant increase in weight and height in experimental group receiving fermented foods (*L. acidophilus*) to combat stunting and failure to thrive has also been reported[10]. Probiotics have also been found useful in prevention of atopic disease [14]

Mechanism of action of probiotics:

Probiotic microorganisms have particular characteristics such as human origin, safety in human use, bile acid resistance, survival in the intestine temporary colonization of human gut, adhesion to the mucosa and bacteriocine production. Thanks to these characteristics, probiotics block the invasion of human intestinal cells by the enteroinvasive bacteria[15].

Probiotic microbial agents and their components exert their protective activities through a variety of mechanisms[16]. Probiotic organisms suppress growth of conventional or potential pathogens as well as their epithelial attachment and /or invasion either directly by secreting antimicrobial substances or by stimulating host expression of protective molecules. Additionally, increased levels of probiotics may induce a “barrier” influence against common pathogens. They can stimulate host production of immunosuppressive molecules that down regulate inflammatory responses, or conversely stimulate host protective immunologic mechanisms that can prevent or accelerate clearance of pathogenic infections. Mechanisms of effect are excretion of acids (lactate, acetate), competition for nutrients and gut receptor sites, immune modulation and formation of specific antimicrobial agents. Mucosal immune stimulation induced by oral administration of LAB influences the balance Th1/Th2 (cellular or humoral response) due to different patterns of cytokine release[17]. LAB can interact with the immune cells of the gut and induce their activation signals. Cell wall structures of pathogenic Gram-positive bacteria act as excellent inducers of inflammatory cytokines TNF alpha, IFN gamma, IL-12. It has been shown that *L bulgaricus* and *L acidophilus* affect the systemic humoral immune response. Interference with pathogen adhesion and invasion. Probiotics likely also enhance the barrier function of naïve epithelial cells not exposed to any pathogen[18]. *L.B. Plantarum* reduces attachment of EPEC to CACO 2 cells. It reduces the in vitro secretary response of intestinal epithelial cells to enteropathogenic E.coli infection and can play an important role in reducing the secretary change in response to EPEC infection, possibly through inhibition of its binding[19]. However, the presence of the probiotic agent before the infection is required, as its role is more preventive rather than therapeutic. Up regulation of immune responses and increased mucosal barrier to translocation of bacteria and bacterial products have been cited as the mechanism for reduction of incidence of NEC in preterm infants[20]

Table: **Mechanism of action of probiotic agents**[16]

Inhibit growth of pathogenic enteric bacteria
Decrease luminal pH
Secrete bactericidal proteins

Stimulate defensin production by epithelial and Paneth cells
Resist colonization (occupy ecologic niche)

Block epithelial attachment or invasion by pathogens
Block epithelial binding by inducing of MUC 2
Stimulate mucus production to alter biofilm
Inhibit epithelial invasion, Rho dependent and independent pathways

Improve epithelial and mucosal barrier function
Produce short-chain fatty acids, including butyrate
Increase barrier integrity

Alter host immune response
Induce IL-10, TGF-b and Cox2 $(PGE)_2$ expression and secretion
Stimulate secretary IgA production
Decrease TN, IFN-y expression
Active regulatory T cells

Genetic engineering
Express and secrete IL-10 and trefoil factors

It has been suggested that some probiotics can help maintain remission in the inflammatory conditions, ulcerative colitis and pauchitis. They also repress enzymes responsible for genotoxin formation[21].. Lykoba[22] et al1 recorded a decrease in detection rate of endotoxinemia, which correlated with the tendency towards the normalization of defective intestinal microflora by inclusion of probiotics Bifidobacterium forte adsorbed on activated charcoal in therapy of digestive tract disease.

Effect of Lactobacillus on bacterial translocation in a neonatal animal model was demonstrated by Drongowski[23] et al. Neonatal rabbits receiving colonization by E.coli KIA,. Lactobacillus GG decreases the frequency of extra intestinal Bacterial translocation by 46% ($p<0.05$), 61%($p<0.05$) and 23% respectively in MNL, SPL & LIV. They showed that enterally-administered LactoGG decreases the frequency of E.coli KIA translocation.

Tsunoda et al[24] showed that pretreatment with heat killed *Lactobacillus Casei* (LC9018) developed a protective activity (peritoneal exudates cell accumulation observed 24 hrs after inj of LC9018) against fecal peritonitis induced after cecal ligation and tip resection surgery.

Sherman et al[25] showed that prophylactic therapy with recombinant human Lactoferrin and probiotics *Lactoacillus GG* act to enhance defenses against invasive *E.coli* in the nascent small intestine. They suggest that recombinant Lactoferrin (rhLF) & LGG are therapeutic agents that may reduce NEC and gut related sepsis in preterm human infants.

Other studies indicated that Bifidobacteria not only colonized the gut of animals, possibly helping to exclude pathogens: they also reduced endotoxemia and appeared to modulate the inflammatory cascade.

Perhaps the most impressive indication that probiotics could benefit newborns comes from a human trial with 2.5X 10^8 live *Lactobacillus acidophilus* and 2.5X10^8 live *Bifidobacterium infantis* in 1237 neonates in Colombia[26]. Compared with 1282 hospitalized patients seen during the previous year, treatment with these strains resulted in a 60% reduction in necrotizing enterocolitis and overall mortality. The positive results in this study support the need for further investigation of bacterial colonization and its role in neonates.

Intestinal translocation is considered important source of infection in adults (in event of stress, chemotherapy, reduced immunity when gut permeability increases[27,28] . In a prospective study it was shown by molecular techniques that the organism recovered from blood in preterm population was always identical to the one cultured from the stool[29] it is highly unlikely that the organism move from blood to the intestinal lumen, hence translocation from the gut to blood stream is a possibility. It was shown that organism present on the skin probably went through mouth and GI tract and eventually translocated from the intestine to the blood stream[30].

Safety of probiotics in neonatal period:

There are indications of prolonged use in infants upto 30 days of Bifidobacteria/Lactobacillus in Russia to create "benign" stool micro floral patterns to prevent/cure dysbacteriosis/sepsis. The entire neonatal population in Russia receives Bifidobacteria or Lactobacilli in an attempt to prevent/ cure dysbacteriosis/sepsis. No blinded controlled studies of this therapy have been performed but the evidence suggests that there is at least no risk involved in such treatment since thousands of infants have been so treated. Also the low incidence of sepsis in Russia argues in favors of its use in neonates (personal communication; A Kuznetsova, Kazan Institute for advanced Medical studies, Tatarstan, Russia)**.**

VSL#3: It is a patented combination of live lactic acid bacteria that have been cultivated, freeze-dried and mixed in high concentration (hundreds of billion per gram). It has been proven in clinical trials to be effective in serious gastrointestinal disorders, and in particular in the management and prevention of inflammation of the small bowel reservoir or pouch, is the most frequent long-term complication following colon removal and pouch creation surgery for ulcerative colitis.

Eight strains of bacteria have been selected cultivated and mixed proportionately to obtain the proven experimental and clinical efficacy[31-39] of VSL#3. All strains included in the product blend are known and accepted organisms in food

Bifidobaterium Breve
Bifidobacterium longum
Bifidobacterium infantis
Lactobacillus acidophilus
Lactobacillus plantarum
Lactobacillus casei
Lactobacillus bulgaricus
Streptococcus thermophilus

These eight beneficial strains act together like a **living shield.**

Lactobacillus Plantarum is expected to be the potential sepsis-preventive strain. It is highly resistant to acid and bile. It exhibits excellent adherence to Caco-2 cells and blocks *E. coli* adherence to Caco-2 cells. It reduces *E. coli* translocation in the transwell system and also in vivo into the blood of weaning rabbits. Being a plantarum strain it can grow in absence of iron. It appears to be completely safe in the closed ileal loop model, and is the predominant human gut flora.(Lab studies by Dr. Panigrahi)

It affects the gut immunity, expression of anti-inflammatory cytokines**.**

Safety ofVSL#3

The lactic acid bacteria in VSL#3 are Generally Recognized As safe (GRAS) Clinical studies have shown that VSL#3 can be taken safely for long periods of time without any problems[31-33]. There is no evidence that ingested probiotic lactic acid bacteria or Bifidobacteria pose any risk of infection greater than that associated with commensal strains. In quantitative terms, the existing data suggest that the risk of bacteremia, which is the most commonly reported of these infections, is <1 per million individuals, considered to be in the "negligible" range.

Adjustment of the intestinal flora after VSL#3 administration can take up to a month for the colonization of the gut to become optimally stable.

Recently Hung- Chin Lin et al have shown that *Lactobacillus acidophilus* and *Bifidobacterium infantis* (inforan) as probitics fed enterally with breast milk reduces the incidence and severity of NEC in VLBW infants[40].

Based on the above review of literature we hypothesize that use of probiotics preparation VSL#3 during the neonatal period may prevent occurrence of sepsis in low birth weight neonates and young infants.

We propose to conduct a Randomized control trial in a community setting.

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8. *Detailed research plan*

Aim:

To examine whether it is possible to prevent the morbidity due to neonatal sepsis (septicemia, pneumonia, meningitis) by supplementing the LBW neonates with probiotics

Objective:

To estimate reduction in the incidence of suspected sepsis in the intervention arm with a daily supplementation of VSL #3 over a period of 30 days in 0-2 month old LBW infants.

Hypothesis:

Daily supplementation of LBW neonates with VSL#3 will reduce the incidence of neonatal sepsis by 30%.

Assumptions:

Hoyos AB showed a 60% reduction in necrotizing enterocolitis and overall mortality by treatment with *Bifidobacterium infantis* and *Lactobacillus acidophilus*.

Incidence of neonatal sepsis in the community as reported by Bang et al is 17%.

For a 30% reduction in incidence of sepsis at a 5% level of significance, with 80% power a sample of 670 cases in each arm of intervention would be required (allowing for a 10% attrition rate). Incidence of LBW is 30%. In order to observe the required 1340 LBW newborns more than 4000 deliveries would be screened considering the fact that some may refuse to participate and some would belong to far off places that may not be possible to cover in the study).

The table below shows the required number of subjects with changing assumptions of power and effect size:

Reduction in incidence	Power	'n' Per group (incl. 10% attrition)	Total	No. to be screened for LBW
50%	80	265	530	1600
30%	**80**	**670**	**1340**	**4020**
50%	90	353	706	2118
30%	90	895	1790	5370

Study Methodology:

This would be a double blind randomized controlled trial. The research team as well as the PI would remain unaware of the group allocation of the neonates. The code would be kept at the INCLEN Trust office, New Delhi under lock and key.

Setting:

The study would be a facility-linked community study. It would be conducted at two sites; in the vicinity of a Delhi hospital, and at a district level hospital and adjoining community in Maharashtra. Screening and enrollment would be done in the hospital; follow-up visits would be carried out by the study staff in the community.

Intervention:

Oral administration of a probiotics preparation VSL#3 containing a dose of 10 billion live bacteria *per os* for 30 days during the neonatal period starting on third day of life.

Placebo:

A similar preparation in the same outer packing would be administered to the neonates in the control group. Content of the placebo has been decided in consultation with pharmaceutical company, keeping in mind the safety issue during neonatal period.

Research method:

A total of 1340 LBW neonates would be needed with 670 each in intervention and control arm. More than 4000 live births would be screened to enroll the required number of subjects, assuming a 30% incidence of LBW. Total number to be screened would be much more since some may refuse to participate and some may belong to far off areas that may be difficult to visit.

A detailed manual of operations and other research tools will be developed and provided to the site investigator.

Randomization

Randomization by permuted block with a block size of 4 would be used. It would ensure random allocation and high probability of balance between the groups at any point of subject recruitment. Computer generated table would be used; patient allocation would be indicated by a study number kept in a sealed-opaque envelope. In a double blind study neither the patient nor the investigator would be aware of the allocation. The code would remain with the INCLEN Trust, New Delhi..

Stratification

In order to achieve balance between the two study groups with regard to important characteristics such as sex and birth weight, randomization would be stratified by sex and birth weight. Two strata (1500 – 2000, and 2001-2500 gms) by sex males and females would be used. Thus there would be four strata as given below:

	Strata 1	**Strata 2**	**Strata 3**	**Strata 4**
Sex	**Male**	**Male**	**Female**	**Female**
B. Wt.	**1500-2000**	**2001-2500**	**1500-2000**	**2001-2500**
	B	**B**	**A**	**B**
	A	**B**	**A**	**A**
	B	**A**	**B**	**A**
	A	**A**	**B**	**B**
	A	**A**	**B**	**A**
	B	**A**	**A**	**B**
	A	**B**	**B**	**B**
	B	**B**	**A**	**A**
	A	**B**	**B**	**B**
	B	**A**	**A**	**B**
	B	**A**	**B**	**A**
	A	**B**	**A**	**A**
	B	**A**	**B**	**A**
	B	**B**	**A**	**B**
	A	**B**	**A**	**A**
	A	**A**	**B**	**B**
	.	.	.	.
	.	.	.	.

Four randomization lists would be prepared, one for each stratum using the proportionate allocation scheme

Selection of Subjects:

Information would be obtained regarding birth of LBW (<2500gms) babies in the hospital on a daily basis by the study staff. The case sheet would be examined to check the residential address of the delivered mother. For study purpose an area of about 15-20 Kms around the hospital would be

considered as study area. Mothers of all LBW neonates belonging to the study area would be approached by the study team (senior research fellow, field worker) for enrollment. The newborn would be assessed for eligibility criteria by using the study screening form. Enrolment would be done on third day of life in the presence of the study physician.

Eligibility: All live born LBW (≥1500 to ≤ 2500 gms) babies available in the hospital would be eligible for the study if they have the following inclusion criteria.

Inclusion criteria: 1. Birth weight ≥1500 gms to ≤ 2500 gms, 2. Residence within 15-20 Kms of the hospital, 3.The mother is planning to stay in Delhi/ study area for a period of at least two months and,

Exclusion criteria: 1. Extreme prematurity (<32 weeks) 2. Presence of a gross congenital malformation incompatible with life, 3. A mother who does not give consent, 4. mother going out of town with the baby,

Parents of babies fulfilling the inclusion criteria and not having any exclusion criteria would be explained about the study with the help of a patient information sheet and asked for consent.

Informed consent procedure:

A mother whose baby is eligible will be informed about the study by the study team (physician/field worker). She would be enquired whether she would allow her baby to be randomly allocated to one of the two groups of the study. If the patient agrees to participate, she will be asked to sign a consent form, which will be read aloud by the field worker for those who are illiterate.

Enrolment:

Enrolment will be done at hospital on 3rd day of life. In case of sick children it can be deferred upto 7th day but not later. Those who agree to give written informed consent would be enrolled and randomized to receive drug or placebo by opening the next in a consecutively numbered series of sealed opaque envelope. This envelope would contain the patient study number corresponding to the randomization list. The drug corresponding to the study number would be fed to the baby in presence of the physician. Administration of the daily dose would be subsequently done by the mother under supervision of the trained field worker. At the time of discharge from the hospital the designated field worker would escort the family to verify the address and note the exact location of residence. They will keep the study drug for the enrolled infant at home in a vaccine/day carrier. Follow up visits would be done by the field worker for supervising supplementation for a period of 30 days. Morbidities would be recorded on follow up forms during home visits as per a schedule.
Parents would be explained that participation in study is voluntary; withdrawal at any time during the course of the study is possible.

Supplement packaging:

The supplement would be prepared by CD Pharma India Pvt. Ltd. Identical packaging of the drug (containing 10 billion of the active ingredients of VSL#3) and placebo with similar consistency and color would be provided. The drugs can withstand a temperature upto 28 degrees Celsius. Therefore there is need for maintaining cold chain. Drug would be kept in suitable plastic packaging in a vaccine/day carrier at the residence of the baby. Mothers of enrolled infants would be instructed to open the lid of vaccine carrier just once daily to take out the required sachet and close the lid tightly thereafter.

Adverse Event Monitoring:
No major adverse events related to the intervention are expected, however continuous monitoring and reporting will be conducted by trained research staff. Any such events will be reported to the

local ethical committee/Data safety monitoring committee. This committee will be responsible for monitoring accrual, safety, outcome measurement and all aspects of the project and advocate continuation or termination of the study based on the results of the interim analyses.

Lab Investigations:

Gut Colonization study:

Gut colonization study of Probiotics VSL#3 is proposed to be conducted at the Department of Microbiology, Safdarjung Hospital in collaboration with The Institute of Pathology, ICMR. It is proposed to study the effect of VSL#3 supplementation on stool colonization patterns in the neonatal gut on a subset subjects. The stool samples for this purpose would be collected at the time of enrolment prior to feeding VSL#3, at the end of third week of supplementation, and at the end of follow-up (day 56-60). Method for this laboratory procedure will be detailed in the lab manual of operations.

Sample Size estimation –Gut Colonization study

Brigidi et al (International Journal of Food Microbiology 81 (2003) 203-209) in a study on patients with IBS have found VSL#3 strains *B. infantis* Y1 and *B. breve* Y8 to be present in 40% and 70% of patients at a concentration of $5x\ 10^5$ and 9×10^5 cells/g feces respectively. This colonization pattern was similar to that observed with the healthy subjects.

Assuming the anticipated proportion of infants likely to colonize (P) to be 40%, at 90% confidence level with a relative precision of 20% the required sample size to be studied is 101. Stool samples from 202 (101 each in intervention & placebo arm) enrolled infants would be collected on day '0', day '21' and at end study.

Additionally blood cultures would be performed on all suspected sepsis cases who give consent for it when they are referred to the facility by the field workers.

Overall morbidity pattern

During home visits other common morbidities of young infant period such as diarrhea, dehydration, dysentery, feeding problem, umbilical sepsis, skin pustules would be recorded and compared between the study groups.

Side effects

Although no major side effects are expected, however efforts would be made to record any side effects that the parents attribute to supplementation, and compared between study groups.

Phases of study implementation:

1. Preparatory phase – 3 months
2. Intervention phase – 15 months
3. Data analysis & reporting phase- 6 months

Activities of preparatory phase:

1. Orientation workshop at the hospital
2. Recruitment of Field workers (team of morbidity detectors)
3. Recruitment of senior research fellow and field attendant
4. Training of Staff (SRF, Field workers) In IMNCI algorithm of diagnosis for young infants
5. Preparation of randomization list by INCLEN Trust, New Delhi.
6. Procurement of drug & Placebo for the study site by CD Pharma

Intervention phase:

This would be the active phase of the study when the Randomized control trial would begin. During this phase enrolment of subjects after obtaining written or verbal informed consent would be done. Each enrolled newborn would be visited at home by a field worker who would supervise the daily supplementation of drug and placebo. The babies would be examined for morbidity detection during the two-month period as per the schedule.

Frequency of visitation:

All newborns would be visited for supplementation. For detection of morbidity the baby would be examined by the trained field worker daily during the first week of life and biweekly during 2-4th week of life. Thereafter during the second month of life weekly visits would be done. **Information would be recorded on a data recording form during all the visits.** Any sick infant would be advised referral to the hospital for treatment.

Staff requirement:

1. Field workers

To conduct a RCT in the community setting would pose many challenges. The entire area within 15-20 kilometers of the hospital would be under the study. The field workers would perform the functions of **intervention supplementation** and morbidity **detection.** The field worker would be required to visit the babies born in the current month as well as those born during the preceding month. On an average a worker would be able to cover 3 -5 babies in a day with some kind of transport support. Therefore a team of at least 6 field workers at each site would be required to conduct follow up visits in the study area. The localities within the study area would be listed and allocation of field workers for specific localities for visitation would be done. This would provide efficient functioning by saving time.

An enrollment card would be provided to each newborn enrolled in the study, mothers would be asked to carry this card whenever they seek treatment for the baby. Information about involvement of the baby in the study would be printed on the card.

Morbidity would be detected by active surveillance.

Active surveillance:

Field workers: Would perform the role of **Supplementors and Morbidity detectors**

There would be six field workers at each site recruited and suitably trained for recording morbidities during home visits. All newborns would be visited daily for supplementation. For detection of morbidity the baby would be examined by the trained field worker daily during the first week of life and biweekly during the first month of life. During the second month weekly visits would be done. Information would be recorded on a data recording forms during all the visits.

The Field workers would be trained in the IMNCI algorithm for detection of neonatal sepsis as described in Annexure1 during the preparatory phase. On detecting neonatal sepsis (possible serious bacterial infection) on the basis of the algorithm they would refer/ accompany the baby to the study clinic/hospital for treatment. At the facility blood culture would be requested and obtained after obtaining consent for the same.
Field workers would record information regarding morbidity conditions on the study forms and get it verified by the study medical officer on a weekly basis. During home visits field workers would replace the ice packs in the vaccine/day carrier containing study drug.

Study Clinic/Hospital:

There would be a clinic/dispensary/ hospital identified in the study area where the study physician would refer the patient for treatment. Proximity of the facility would ensure that there is no delay in treatment of a diagnosed patient. Blood cultures would be done preferably in all cases referred with suspected sepsis.

Quality assurance measures:

Measures to ensure correct administration of drug and placebo:

1. Daily visits to each baby enrolled in the study during the first seven days of life would be made. VSL#3 Probiotic would be administered to the baby in presence of the field worker.
2. During training of staff and initial interaction with the guardians of the subjects the importance of the study number and the corresponding drug packet would be explained. This would also be explained in the study information sheet.
3. Quality assurance of field implementation would be ensured, frequency checks (on 10% visits), surprise visits by study MO would be inbuilt in the procedures. These would be explained in detail in the Manual of operations.
4. Good clinical practice standards would be observed throughout the clinical and laboratory procedures.

Data Processing:

Data obtained by the field workers on the study forms would be checked by the project Medical officer on a weekly basis. The crosschecked forms would be entered in the computer at the study office. Range and frequency checks would be applied. Validated data would be transferred to CCU at ICMR electronically.

Data analysis:

Baseline variables such as mode of delivery, birth weight, gestation etc. will be compared to evaluate the comparability of the groups. The primary outcome measure in this study in a case of clinically suspected sepsis (possible serious bacterial infection), based on the algorithm for diagnosis (IMNCI). The two groups will be compared for the primary outcome.

Based on the study hypothesis a 30% reduction in the number of clinically suspected sepsis is expected in the intervention arm. Incidence rates of clinically suspected sepsis would be compared within the groups using the Chi2 test or Fisher's exact test as appropriate. Multivariate analysis will be used to adjust for potential confounding.

Incidence of other morbidities (diarrhoea, dysentery, feeding problem, skin infection and umbilical sepsis) would be compared in the intervention and control arms. Data on non compliers, protocol violators, study drop outs would be handled with an intention to treat analysis

Protection of Human subjects

The participating center will submit this protocol to its own Ethics Committee for local clearance and approval. The PI at the Coordinating center, ICMR has obtained certification of training in human subject protection. After collecting the information regarding birth of a low birth weight newborn (≤ 2500gms) the mother would be approached while she is still in the hospital. After screening and finding the baby eligible for inclusion in the trial, the parents would be requested for consent to participate. A written informed consent form would be read aloud in presence of a witness and signature/right thumb impression obtained on the form by the study team member. Each participant would be made aware that participation in the trial is voluntary and withdrawal at any point in time is possible without jeopardizing her access to care. Each participant will receive a copy of the consent form, which will contain the names and phone numbers of persons to contact in case of questions or concerns.

Risks to subjects participating in the trial are considered to be minimal. No studies have documented adverse effects related to the drug.

Benefits to the participants include the assurance that all subjects will receive close follow up visits to detect morbidity. Participation to the study may contribute important information, and add to scientific knowledge.

All participant level information would be entered in the computer using the enrollment number. Identity of the participants would not be revealed for any other purpose.

INDIAN COUNCIL OF MEDICAL RESEARCH

"Effect of Probiotics VSL#3 on prevention of sepsis during 0-2 month period in low birth weight infants: A Randomized Controlled trial"

Informed Consent Form

Neonatal Sepsis is a major cause of sickness and death during first two months of life in low birth weight infants. This is a research study conducted by ICMR, New Delhi to determine whether daily supplementation of probiotics VSL#3 to LBW newborns for a period of 30 days can reduce the occurrence of sepsis in 0-2 month period. In this study there are equal chances of your baby receiving either the probiotic or a similar looking substance without probiotics. A field worker would visit your house daily and supervise the administration of the drug. This would be done taking all hygienic precautions. The probiotics are considered beneficial for human health and there are no known risks involved. If this research study demonstrates that occurrence of sepsis in those receiving the probiotics is less than those receiving the placebo then the drug can be recommended for wider use in the community. Mothers milk is the best nutritious food for the baby during first six months of life. Give the baby only mothers milk and the drug during the study.

All the details provided by you would be kept confidential. For any queries during the study you can contact Dr. ------, at ------, Phone No.---. Your participation is completely voluntary and you can withdraw from the study at any time.

Signature of the guardian
Date:

Signature of witness
Date:

INDIAN COUNCIL OF MEDICAL RESEARCH

"Effect of Probiotics VSL#3 on prevention of sepsis during 0-2 month period in low birth weight infants: A Randomized Controlled trial"

Patient Information Sheet

Purpose:

This research study is being conducted by ICMR to understand whether giving VSL3# (a probiotic drug) to LBW newborn babies can be beneficial in reducing the occurrence of neonatal sepsis (meningitis, pneumonia, septicemia) during 0-2 month period. The study will be under the supervision of Dr. (name of concerned PI and institution).

Your participation in the study is completely voluntary.

Procedure:

Your newborn baby would qualify for the study if you are planning to stay at your residence for a period of at least two months, agree to provide the necessary medical information, if your baby is well and does not have any birth defects. If you agree to be a part of the study, then the baby would be given VSL#3 or a similar looking substance, once daily for 30 days. A field worker would visit your house daily during the first week of life and twice in a week during the first month of life and weekly subsequently till 60 days. During the visits He/She would enquire about the wellbeing of the baby since the last visit. The field worker would record information on a form. In case of any illness he would direct the baby to study physician for treatment. You would be instructed to look for danger signs indicating illness in babies 0-2 months given in the pamphlet and inform the field worker.

Risk/Discomforts:

Although exclusive breastfeeding is recommended upto 6 months of life we think that supplementing newborns with the drug VSL#3 would help minimize the risk of neonatal sepsis (meningitis, pneumonia, and septicemia). It is a safe product; no serious adverse events or side-effects have ever been observed. However, hygienic precautions should be taken during its administration to prevent any untoward effect.

Benefits:

The chances of your baby falling sick with sepsis may be reduced. If VSL#3 does reduce sickness during 0-2 month period, this knowledge may benefit both your and other babies throughout the world.

Alternative:

Even if you do not participate it will not lead to any loss in health care which is available to you under the programme of the Government of India

Voluntary participation:

Your participation in the study is completely voluntary. You have the right to withdraw your baby from the study at any point of time.

Privacy and confidentiality:

The information collected during home visits will be treated as confidential and your baby will not be identified as this information would be coded.

Authorization to publish results:

Results of this study may be published for scientific purposes and/or presented to scientific groups; however your identity will not be disclosed.

Person to contact:

In case of any difficulty experienced or in the event of any emergency, you can contact Dr. (PI/MO) at (address of PI and MO or by calling up at the following telephone No…….(Tel No on which the PI and MO can be contacted).

Dummy Tables:

Table1: Baseline characteristics comparison between probiotics and placebo group

Characteristics	Probiotics n %		Placebo n %	
Sex				
SLI				
Caste				
Education				
Religion				
gestation				

Table 2: Primary Outcome

Outcome	probiotic		placebo	
	n	%	n	%
Neonatal sepsis				
Possible serious bacterial infection				

Table 3: Secondary Outcomes

Outcome	probiotic		placebo	
	n	%	n	%
Diarrhea				
Oral thrush				
Cold Cough				
Number of Possible serious bacterial infection				
Number of Culture confirmed neonatal sepsis				
Number of Drop outs				
Non compliers				
Number alive at 60 days				
Number hospitalized				

Table 4: Side Effects:

	probiotic		placebo	
	n	%	n	%
vomiting				
?				
?				

Table 5: Rate of enrolment at interim analysis

	Safdarjung hospital	Wardha Hospital
Probiotics		
Placebo		
Total		

Table 6: Compliance & drop outs

	SJH				WH			
	n		%		n		%	
Non compliers								
Dropouts								

Amendment to the protocol

- Following the first meeting of the Data Safety Monitoring Committee meeting on 22 May 09 the following amendments to the protocol are made. It was stated that the two study sites should follow the **same method for screening the subjects.** All live births taking place in different labor rooms and O.Ts should be screened for detection of births eligible for the study ie; newborns with birth weight <= 2500 gms. All Screening forms should be preserved in a file. The Field Workers employed for the project should be trained and allowed to conduct screening.

- **A fixed protocol for investigations and treatment** of the referred infants should be prepared and followed by both centers. Even for cases of confirmed Sepsis defined doses for all common injections with number of days of prescribed treatments should be written down and adhered to. It was said that it was the Ethical responsibility of the study team to give best treatment at home to the enrolled infant at home if the mother refuses referral. The study physician should visit home of such sick infants to deliver injection treatment at home.

- Maintaining the study drug in **cold chain** was discussed. It was stated that the drug remains effective at 24 degrees temperature for about 18 months however in view of the high temperature during summer months there is a need to store the drug at 4-8 degrees Celsius. Additional budget has been provided to the centers for purchase of Vaccine carriers and deep freezers. It was suggested that a weekly dose of the drug should be provided to the mother instead of the monthly supply and proper SOP developed to monitor the cold chain including hours of electric supply available.

- It was recommended to undertake weekly viability tests on the samples and prepare graphs from the lab results. The CD Pharma company should be requested to help with this activity. It was also suggested to prepare thermostable labels that change color when exposed to heat and use it on the sachets. It was recommended to discuss this issue with the Company providing the Drug/Placebo.

- **Supervision for Quality Control**: It was recommended to hire a Research Assistant level person to work independent of the Field Worker team. These visits would be independent of the FW visits and at least 2 such visits should be conducted daily on a random basis. A list from the computer would be generated for this purpose.

- **Quality Assurance at CCU**:
  The CCU should monitor the study like a CRO organization with site visits every three months. Initially more number of visits are needed to check selection criteria, documentation and adherence to the protocol.

- It was stated that the IMNCI protocol is very sensitive and should be used as a screening test to detect cases of possible serious bacterial infection, however it can not remain the diagnostic criteria at the facility where the infant has been referred for care. In clinical trials specificity for the outcome is more important. At the facility the infant should be examined by the pediatrician and labeled as suspected sepsis if the pediatrician so decides. Blood culture should be performed on referred infants for final diagnosis by the gold standard method. Site PIs should be able to prepare the following table:
  - Suspected Sepsis Person making diagnosis IMNCI Definition Field Worker Clinical Screening positive Pediatrician Culture positive sepsis Laboratory
- A pediatrician should cross check all referred cases on the day of referral. If referral is refused SRF should visit and provide best possible treatment as given in the treatment protocol for the study.
- It was stated that validation of the IMNCI would be a by-product of the study.

Data Safety Monitoring Committee:

- Blood samples should be obtained from sick children who are referred to facility in two transport media, one for routine blood culture and other suitable for culture of probiotics bacteria. If in any sample same species of probiotics bacteria are cultured from the blood as contained in the VSL#3 the study will be stopped.
- All Serious Adverse Events should be immediately reported to Chairperson and members of the DSMC. The study forms of the case should be Xeroxed and sent to CCU by speed post where a summary of findings should be prepared and shared with the members of DSMC.
- An interim analysis would be indicated in the following situations:
    1. If one and a half time more number of deaths are reported from the study population as against the expected numbers.
    2. If confirm sepsis rates are increasing in the study population as against the expected rates.
    3. If loss to follow up exceeds the expected 10%. If 50% of babies completed 60 days of follow-up. (The CCU should look at literature to find out the expected death rates and rates of neonatal sepsis)

Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2monthperiod: A Randomized Controlled trial"

Justification of staff required at one site

One S.R.F- A senior Research office (Medical) is required for screening the all newborns delivered in the hospital on daily basis, to scrutinize their suitability for recruitment in the study. He will help in enrolment and supervise follow-up visits by the Field workers. He will ensure quality control by performing 5-10% visits in the field. He will be responsible for data collection, cleaning, entry and transfer.

Six Field workers- This is a facility-linked community study. The field workers are required for making home visits of enrolled babied at home for detection of morbidity, checking compliance of study drug/placebo. 670 babies x15 visits =10050 visits / 6FWx24daysx15 months =2160person days =4.65 visits per person per day.

One DEO is required for entering the data from the study forms to the computer software, do data cleaning as per corrections done by FW and SRF and send it to ICMR .

One Laboratory Technicians They are required for conducting the lab work for the study such as stool colonization, blood and stool culture etc.

One Statistician for ICMR HQ
A statistician is required at the head quarter to write the plan of analysis, conduct interim data analysis, monitor data and conduct the final data analysis for the study

Equipment: In the current budget there is no provision for providing any equipment to the centers. Although the lab work requires equipments, the centers are requested to utilize their in house facilities for lab work.

Justification of contingency required at one site
Contingency: Recurring contingency is required under the budget heads given in the budget to carry out the work related to the project. These amounts are minimum required without which the project can not be carried out.

Justification of T.A: The money under TA is required for field work. Visiting babies at home for detection of illness is an activity directly linked with the outcome of the study. Since there is no provision of vehicular support in the budget, this amount is the minimum required to carry out the field work.

Plan of Analyses document for

"Effect of Probiotics VSL#3 on prevention of sepsis in LBW infants during 0-2 month period: A Randomized Controlled trial"

Objectives:

Primary

- To estimate reduction in the incidence of suspected sepsis in 0-2 month old LBW infants in the intervention arm with a daily supplementation of VSL#3 over a period of 30 days in 0-2 month old LBW infants**.**

Secondary

- To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.
- To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (to be done at one center)
- To monitor the side effects if any, due to the probiotics VSL#3

Methodology:

A double blind randomized controlled trial is being conducted in a facility linked community setting.

Newborns in the Intervention arm receive VSL#3 10 billion for thirty days. A physically similar preparation of placebo (containing Maltodextrin) is given to the newborns in control arm. The research team and the PI are unaware of the group allocation of neonates.

Enrollment of subjects is done at a Hospital. Trained field workers visit the homes of these newborns (within the prescribed study area of 15-20 Kms) for supplementation and morbidity detection as per schedule of visitation. A detailed manual of operations and data collection tools will be developed and provided to the site investigator.

Data Collection Forms:

1. Baseline form: Collects Socio-demographic information from families of enrolled subjects.

2. Screening Form: Is used for initial screening of LBW live born babies for checking their eligibility.

3. Enrolment form: Is filled at the time of enrolment, for all eligible infants fulfilling inclusion criteria who enter the study.

4. Follow up Form: This is the main form filled during all home visits during the follow up period. It records information related to compliance, symptoms and signs of morbidity elicited by the Field worker. Temperature, Respiratory rate and Weekly weights of babies are measured and recorded.

5. Final outcome form: Filled for all enrolled subjects, it gives information about the status of the infant on day 60 whether alive or not, whether the infant was sick, hospitalized during the two month period and also records immunization status.

6. Referral & medicine form: Records all information regarding the treatment of referred infants and the drug doses received by them.

List of Definitions:

1. Suspected Sepsis: A case diagnosed by the field worker as per IMNCI criteria for severe possible bacterial infection.

2. Low Birth Weight: An infant weighing less than or equal to 2500 gms.

3. **Loss to follow up: Withdrew from study: drop out**: All subjects on whom less that 50% of expected visits could be completed (less than 10 visits)

4. Protocol violation study drug discontinuation will be treated as violation of study protocol.

5. **Adverse events**: All cases of hospitalizations and deaths among enrolled infants will be treated as adverse events.

6**. Morbidities**: As defined under IMNCI.

Plan of Analysis:

Data obtained by the field workers on the study forms would be checked by the project Medical officer on a weekly basis. The crosschecked forms would be entered in computer using Epi info software with built in range and frequency checks. Validated data would be transferred to the central coordinating unit at ICMR electronically. Analysis would be performed using SPSS version 17. Analysis would be done on pooled data from the two study sites.

Baseline variables such as mode of delivery, birth weight, gestation etc. will be compared to evaluate the comparability of the groups. Continuous variables would be compared using t-test, categorical variables would be compared using Chi square or Fisher's exact tests as appropriate.
Primary Analysis: The primary outcome measure in this study in a case of clinically suspected sepsis (possible serious bacterial infection), based on the algorithm for diagnosis (IMNCI). The two groups will be compared for the primary outcome.

Based on the study hypothesis a 30% reduction in the number of clinically suspected sepsis is expected in the intervention arm. Both absolute and relative measures of association would be computed. We would compute the following: risk reduction (effect size), number needed to treat, relative risk, and 95% CI for each of the outcome measures. In case of imbalance in two groups with respect to the baseline characterics, multivariate analysis method would be used to compute adjusted outcome measures.

Analysis would be by *intention to treat*. Data on non compliers, protocol violators, study drop outs would be handled in a way such that subject assigned to intervention arm will be considered as belonging to that arm even if he/she has not complied with the study protocol.

A separate *Per-Protocol* analysis will also be done including only the cases who have complied with the intervention. Compliance will be defined as those enrolled infants who ingested the study drug (for 25 days) and were followed up for more than 50% of scheduled visits.

Analysis 1: Proportion of suspected sepsis cases diagnosed by the IMNCI algorithm would be calculated for each intervention arm.

Decision rule for Analysis 1:

Numerator = No. of cases of suspected sepsis observed by IMNCI algorithm in one arm
Denominator = Number of subjects enrolled in the study arm
Primary outcome of suspected sepsis by IMNCI would be the answers marked as code 1 (possible serious bacterial infection) in Q. No. 24 in the Probiotics follow up form. Number of such cases in each arm would also be cross checked/verified from the final outcome form as well as monthly statistics prepared by the centers.

Since IMNCI is expected to over diagnose suspected sepsis cases, in a separate analysis we would compare the arms using more stringent definition of suspected sepsis such as 'when two or more signs are present'.

Test of proportions (Chi2 test or Fisher's exact test as appropriate) would be used to compare the two arms, Effect estimates and 95 percent confidence limits will be calculated by conventional method.

Although sample size was calculated for the primary objective as diagnosed by FW, however diagnoses by Pediatrician/physician are also being collected and blood cultures are also being done. A comparison between arms would also be done on sepsis as defined by these parameters. Data reported by the centers on the number of suspected sepsis cases as diagnosed by Physician/Pediatrician, and confirmed blood cultures would be obtained for this analysis and reported for hypothesis generation purposes only.

Analysis 2: We would also compare the Incidence rate ratios between the two arms since we would have the person time data collected during home visits. The following statistics would be computed:

		Disease		
		D+	D-	Person Time
	E+	a	b	N1
Exposure				
	E-	c	d	N2

Incidence rate among exposed = a/N1 = IR1

Incidence rate among unexposed = c/N2 = IR2

Incidence Rate Ratio = IR1/IR2

Incidence Rate Difference = IR1-IR2

Efficacy of probiotics = 1-RR x 100 (Incidence rate in control minus Incidence rate in intervention divided by Incidence rate in control multiplied by 100%)
Number Needed to Treat. = 1/Incidence Rate difference

Calculation of person time: From the probiotics follow up forms number of days contributed by each infant would be computed arm wise. Total number of person-months or years can be derived in each arm. Person-time will be expressed as 60 days; exposure for each infant would be calculated as the time from enrolment/ or first visit to time of detection of morbidity, death or completion of study. Incidence –density of suspected sepsis will be estimated by dividing the total incident cases by overall person-time, and expressed as incident cases per 100 young infant periods.

Numerator: Number of incident cases of suspected sepsis observed in each arm.

Denominator: Person-time.

An episode of sepsis would be defined as: A period of illness when the infant has one or more sign/symptom of illness continuously. Two episodes should be separated by at least 3-5 days Multivariate analysis: Would be done to look at the effect of probiotics after adjusting for confounding by sex, birth weight, Mother/fathers Education status, religion, SLI Score, mode of delivery, breastfeeding status, and premature rupture of membrane in mother. The dependent variable would be suspected sepsis.

Secondary Objectives:

1. **To estimate the effect of VSL#3 administration on overall morbidity pattern in 0-2 month old LBW infants.**

Analysis: Incidence of other morbidities (diarrhea, dysentery, feeding problem, skin infection and umbilical sepsis) would be compared in the intervention and control arms using the Chi square or the Fisher's exact test as appropriate.

Morbidity	Q No. & Form	Numerator	Denominator	Statistic	test
Diarrhea	Q 17 Probiotics F.up form	All infants with Code 1 (yes) for Q 17	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Dysentery	Q 18 probiotics F.up form	All infants with Code 1 (yes) for Q 18	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Feeding problem	Q 23 & Q 24	All infants with Q23 code 'a', and Code 1 for Q 24	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Skin infection	Q 14	All infants with Code 1 for Q 14	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Umbilical sepsis	Q 13	All infants with Code 1 for Q 13	No. of infants enrolled in the arm%	% %	Chi square/Fisher's exact test
Local Bacterial infection	Q 24	All infants with Code 2 for Q 24	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test
Hospitalization	Q27	All infants with Code 1 for Q 27	No. of infants enrolled in the arm	%	Chi square/Fisher's exact test

2. To study stool colonization patterns at baseline, during third week of supplementation and at end study in 10% of subjects (done at Safdarjung hospital center only).

Laboratory study: Gut colonization rates of probiotics at three time points (day '0', day '21' and day '60' as described in the lab results would be compared using repeated measures ANOVA between the intervention and control arms.

To monitor the side effects if any, due to the probiotics VSL#3

Comparison of Adverse events and Loss to follow ups would also be done between the two study arms.

Dummy Tables:

1. **Flow Diagram of Trial Participants:**

   Numbers screened

   Number enrolled/randomized

   Drop outs, loss to follow up

   Non compliers

   Numbers included in intention to treat analysis= No. enrolled

Numbers included in per protocol analysis = No. receiving the intervention for 23? Days and available for 10 or more visits.

2. Table showing baseline characteristics in Probiotics and Control arms

Characteristic	**Probiotics**	**Control**	**'p' Value**
No. of Babies			
Sex			
B.Wt. 1500-2000			
2001-2500			
Mode of delivery			
Normal			
LSCS			
Forceps			
Mother' education			
1. Illiterate,			
2. I-VIIIth,			
3. X-12th,			
4. Graduation			
Religion Hindu			
Muslim			
Christian			
Other			
Standard of Living Index			
Low 0-14			
Medium 15-24			
High 25-67			

3. Intervention Coverage by treatment group

Household Visits	Probiotics	Placebo	'p' value
Mean			
Median			
Effective Coverage			

4. Suspected Sepsis by Treatment Group

Algorithm Suspected Sepsis diagnosed by F.W.(IMNCI)	**Infants**	**Cases**	**Person-time**	**Rate**	**IRR (95% CI)**
Probiotics					
Placebo					
Suspected Sepsis diagnosed by pediatrician					
Probiotics					
Placebo					
Blood Confirmed Sepsis					
Probiotics					
Placebo					

5. Other morbidities by treatment group

Morbidities	**Probiotics**	**Placebo**	**'p' value**
Diarrhea			
Dysentery			
Feeding problem			
Skin Infection			
Umbilical sepsis			
Local Bacterial Infection			
Hospitalization			

CONSORT 2010 checklist of information to include when reporting a randomized trial*

Section/Topic	Item No	Checklist item	Reported on page no
Title and abstract			
	1a	Identification as a randomised trial in the title	**Title page 1**
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	**page 2**
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	**page 4**
	2b	Specific objectives or hypotheses	**page 4-5**
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	**page 5**
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	**Not Applicable**
Participants	4a	Eligibility criteria for participants	**page 5 sentence 8**
	4b	Settings and locations where the data were collected	**Page 5 Sentences 6-7**
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	**Page 5-6 under Study Methodology**
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	**page 7**
	6b	Any changes to trial outcomes after the trial commenced, with reasons	**Not Applicable**
Sample size	7a	How sample size was determined	**Page 7 under Statistical Analyses**
	7b	When applicable, explanation of any interim analyses and stopping guidelines	**Not Applicable***
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	**Page 6 under Randomization & Masking**
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	**Page 6, under Randomization & Masking**
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	**Page 6, under Randomization & Masking**
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	**Page 6 INCLEN Trust**
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	**Page 6 under Randomization & Masking**

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	**Page 6 under Randomization & Masking**
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	**Page 7, Statistical analyses**
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	**Not Done**
Results Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	**Page 21 Flow Diagram**
	13b	For each group, losses and exclusions after randomisation, together with reasons	**Page 21 Flow Diagram**
Recruitment	14a	Dates defining the periods of recruitment and follow-up	**Page 5**
	14b	Why the trial ended or was stopped	**Completion of sample size**
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	**Page 18 Table1**
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	**Yes Pages 18-20,Tables 1,2,3,**
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	**Pages 19-20, Tables 2,3.**
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	**Page 19-29, Table 2,3**
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	**Kaplan Meir Survival analysis Pages 22-23**
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	**Not applicable**
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	**Pages 10-12**
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	**Pages 10-12**
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	**Pages 10-12**
Other information			
Registration	23	Registration number and name of trial registry	**CTRI/2008/091/000049**
Protocol	24	Where the full trial protocol can be accessed, if available	**Is attached, available from ICMR website**
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	**Page 7 & Page 14**

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

For publication as web appendix only:

Methods: Duplicate visits were performed by a study supervisor in 10% of visits as a quality control measure. Field workers records were verified by study medical officer weekly before entering the data in the computer. Viability testing of the probiotics sachets collected from the field sites was done. A schedule for collection of these sachets was prepared such that every week 4 sachets were collected in reverse cold chain from infants who were in 1-4th week of follow up, over a six month period. These sachets were transported to an external lab (Micro s.r.l., Italy, accredited by SINAL [National System for accreditation of laboratories, Italy] and the International Laboratory accreditations cooperation ILAL-MRA]. The certificate of analysis received from the lab certified the adequacy of cell counts in the sachets, and based on the results extended the expiry of the batch by one year. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry in EPI Info version 6.0) with built in range and consistency checks. Hand checking on random samples was done and frequency distribution of important variables examined periodically to identify aberrant values. A program file developed in EPI 6 platform was run, the list of errors was sent to the sites for corrections.

IMNCI Algorithm: Presence of any of the following signs suggested possible serious bacterial infection: convulsions or fast breathing (60 breaths per minute or more); severe chest in-drawing or nasal flaring or grunting; 10 or more skin pustules or a large boil; axillary temperature 37.5 Celsius or above (or feels hot to touch); temperature less than 35.4 Celcius (or feels cold to touch); lethargic or unconscious or less than normal movements. (Ref: Training Modules 1 to 9 - Unicef. www.unicef.org/india/Training_Module_1-9.)

BMJ Open

Role of Probiotics VSL#3 in prevention of suspected sepsis in low birth weight infants in India: a randomized controlled trial

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Complete List of Authors:	Sinha, Anju; Indian Council of Medical Research, Reproductive and Child Health Gupta, Subodh; Mahatma Gandhi Instituite of Medical Sciences, Chellani, Harish; Safdarjung Hospital, Maliye, Chetna; Mahatma Gandhi Institute of Medical Sciences, Kumari, Vidya; Safdarjung Hospital, Arya, Sugandha Garg, Bishan; Mahatma Gandhi Institute of Medical sciences, Dixit, sunita; District Hospital Wardha, Gaind, Rajni; Safdarjung hospital, Delhi, Microbiology DEOTALE, VIJAYASHRI; MGIMS,SEVAGRAM, MICROBIOLOGY; MGIMS,SEVAGRAM, MICROBIOLOGY TAYWADE, MANISH; MGIMS, SEWAGRAM, COMMUNITY MEDICINE Prasad, M.S; Safdarjung Hospital , Thavraj, Vasantha; Indian Council of Medical Research, Mukherjee, Ajit; Mukherjeeajit@hotmail.com, Roy, Malabika; Indian Council of Medical Research,
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Role of Probiotics VSL#3 in prevention of suspected sepsis in low birth weight infants in India: a randomized controlled trial

Brief Title: Probiotics in prevention of suspected sepsis in LBW infants

Anju Sinha[1](PhD), Subodh S. Gupta[2](MD), Harish Chellani[3](MD), Chetna Maliye[2](MD), Vidya Kumari[3](PGDMCH), Sugandha Arya[3](MD), B.S Garg[2](PhD), Sunita Dixit[4](DNB), Gaind Rajni[3](MD), Vijayshri Deotale[2](MD), Manish Taywade[2](MD), M.S. Prasad[3](MD), Vasantha Thavraj[1] (MD) Ajit Mukherjee[1](PhD), and Malabika Roy[1](MD)

1. Indian Council of Medical Research, 2. Mahatma Gandhi Institute of Medical Sciences,Sewagram, 3. Safdarjung Hospital, New Delhi. 4. District Hospital Wardha.

Corresponding author:

Anju Sinha, Scientist 'D', Indian Council of Medical Research, New Delhi 110029.Email: apradhandr@gmail.com

Telephone: +91 26589493

Mobile: +91 9811422241

Keywords: Neonatal sepsis, Low Birth weight, Prevention, Probiotics.

This trial is registered with Clinical Trial Registry of India (CTRI), number CTRI/2008/091/000049

Abstract

Objectives: To assess the effect of the probiotic VSL#3 in prevention of neonatal sepsis in low birth weight (LBW) infants. **Design**: Randomized, double-blind, placebo-controlled trial. **Setting**: Community setting in rural India. **Participants**: LBW infants aged 3-7 days. **Interventions**: Infants were randomized to receive probiotic (VSL#3, 10 billion cfu) or placebo for 30 days, and were followed up for two months. **Main outcome measure**: possible serious bacterial infection (PSBI) as per Integrated Management of Neonatal Childhood Illnesses algorithm, diagnosed by field workers/physicians. **Results**: 668 infants were randomized to VSL#3 and 672 to placebo. By intention-to-treat analysis, the risk of PSBI among infants in the overall population of LBW infants was not statistically significant (RR 0.79 [95% CI 0.56 to 1.03]). Probiotics reduced median days of hospitalization (6 days vs. 3 days in probiotics) [p= 0.018] but not the risk of hospitalization (RR 0.66 [95% CI 0.42 to 1.04]. The onset of PSBI in 10% of infants occurred on the 40th day in the probiotics arm versus 25th day in control arm (p=0.063). **Conclusions**: Daily supplementation of LBW infants with probiotics VSL#3 (10 billion cfu) for 30 days led to a non-significant 21% reduction in risk of neonatal sepsis. A larger study with sufficient power and a more specific primary end point is warranted to confirm the preventive effect of VSL#3 on neonatal sepsis in LBW infants. **Trial registration:** The study is registered at the Clinical Trial Registry of India (Trial is registered at www.ctri.nic.in. Registration No. CTRI/2008/091/000049).

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Article summary: Strengths and limitations of this study

- Low birth weight (LBW) neonates are at high risk for infections, including neonatal sepsis.
- Probiotics are effective in preventing neonatal necrotising entero-colitis and nosocomial infections in preterm LBW babies
- In our study, daily supplementation of LBW infants with probiotics VSL#3 (10 billion cfu) for 30 days led to a non-significant 21% reduction in risk of neonatal sepsis. A significant

effect was observed among infants weighing 1.5-1.9 kg. Survival analysis showed 15 day delay in the onset of sepsis in the intervention arm.

- Our study used IMNCI algorithm for diagnosis of possible serious bacterial infection (PSBI-suspected sepsis) by field workers. A larger study with sufficient power and a more specific primary end point (such as physician's diagnosis of neonatal sepsis) is warranted to confirm the preventive effect of VSL#3 on neonatal sepsis in LBW infants.
- Our study was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 days of life, therefore the role of probiotics on early onset sepsis could not be evaluated.

Introduction

Neonatal infections are responsible for more than a quarter of the 1 million neonatal deaths every year in India.[1]Low Birth Weight (LBW) is a very important indirect cause of death in neonates, accounting for40%to 80% of neonatal deaths.[2]Infections (sepsis, pneumonia and meningitis) are known to evolve more rapidly in LBW infants, leading to severely increased disease and higher rate of death. Prevention of infection in LBW babies would directly decrease neonatal morbidity and mortality. Management of neonatal sepsis with antibiotics faces the problem of drug resistance, attributed to availability over the counter, indiscriminate use and incomplete courses in India. Researchers are evaluating immunotherapy (with immune globulin, myeloid colony stimulating factors, probiotics, glutamine supplementation, recombinant human protein C and lactoferrin) as adjuvants for the prevention of neonatal sepsis.[3]

Probiotics have attracted much interest and debatein the neonatal literature during the last decade.[4]FAO/WHO defines probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[5]Probiotic microorganisms have particular characteristics: human origin, safety in human use, bile acid resistance, survival in the intestine, temporary colonization of the gut, adhesion to the mucosa, and bacteriocine production. The ingestion of probiotics is associated with modification in physiological homeostasis of the intestinal flora, which is important in preventing disease, especially infections.[6]The best evidence for efficacy of specific probiotics strains has been obtained with randomized controlled trials and meta-analysisis in the prevention and treatment of antibiotic-associated diarrhoea,[7] gastroenteritis and acute diarrhoea,[8]and in alleviation of lactose intolerance.[9]

Clinical trials evaluating the role of probiotics (Infloran) in preterm very low birth weight infants[10-12] reported a reduction in incidence of necrotizing entero-colitis (NEC), overall mortality[10] and severity of NEC.[11]A meta-analysis[13]and systematic reviews[14,15]of randomized trial suggested a beneficial effect of probiotic treatment on reducing the incidence and all-cause mortality due to NEC. Following on from the evidence on VLBW and premature infants, we hypothesized that the probiotic

preparationVSL#3 might reduce morbidity due to sepsis in LBW infants. We aimed to estimate reduction in the incidence of suspected sepsis in 0-2 month old low birth weight infants in the intervention arm with a daily supplementation of probiotic VSL#3, 10 billion colony-forming units (cfu) over a period of 30 days. If proven to be efficacious, it could be an important public health intervention for prevention of neonatal infections.

Methods

Study design and participants

We undertook a randomized, double-blind, placebo-controlled (1:1) trial from January 2009 to November 2011 at two tertiary care hospitals and the adjoining community areas (Safdarjung hospital in New Delhi and Mahatma Gandhi Institute of Medical Sciences Wardha, India). We screened newborn infants aged 3 days, born in the hospitals weighing 1500-2500 g, residing within 20-25 km of the hospital, and not planning to shift residence for at least the next two months. We excluded extremely premature infants (< 34 weeks), sick infants, those with congenital malformations incompatible with life, those with guardians not giving consent and belonging to out of study area. Eligible babies, for whom parents/guardians gave informed consent, were enrolled on days 3-7 of life. Participants were enrolled by a physician in the hospital and followed up in the community for two months for occurrence of neonatal sepsis and other morbidities. Baseline information on demographic characteristics was obtained for assessment of Standard of Living Index.[16]Ethical clearance was obtained from the two participating institutes. A Data Safety and Monitoring Committee (DSMC) met every six months and reviewed severe adverse events.

Study medication

Infants were randomly assigned to receive probiotic or placebo by the study physician. The intervention consisted of administration of the probiotic preparation VSL#3 (a mix of 8 strains: *Streptococcus thermophilus, Bifidobacterium breve, B. Longum, B. Infantis, Lactobacillus acidophilus, L. Plantarum, L. Paracasei,* and *L. Delbrueckii spp bulgaricus,* at a dose of 10 billion colony-forming units (cfu) for 30 days, starting on third day of life. The content of the probiotic

sachet was mixed in expressed breast milk in a plastic cup and fed to the infant. Sterilized plastic cup and stirrer were provided along with the sachets. A similar-looking maltodextrin preparation in the same outer packing was administered to the control group. The supplement was prepared by CD Pharma India Pvt. Ltd. The preparations withstood a temperature up to 28 degrees Celsius and were therefore kept in a cold chain (refrigerators/vaccine carriers) at the homes of enrolled infants.

Randomization and masking

A computer generated stratified block randomization with permuted block size of 4 was used. We stratified infants by birth weight (1500-2000g, 2001-2500g) and sex. A team of scientists at INCLEN Trust, New Delhi, used a computer-generated table for subject allocation. Allocation concealment was ensured by sequentially numbering the sachet packets containing VSL#3 or placebo after block randomization. Identical packaging of VSL#3 and a placebo with similar consistency and colour was provided. Parents of enrolled infants, investigators and field workers were masked to treatment allocation. Data analysis was performed in a blinded manner. The codes remained with the INCLEN Trust, and were disclosed to the DSMB and ICMR on completion of data analysis.

Follow-up and assessment

Follow-up visits were done by the field worker, for supervising supplementation over 30 days, and detection of morbidities over two months. Visitation was daily during the first week, biweekly in weeks 2-4 of life, and weekly in the second month. Detection of neonatal sepsis was performed during visits, using the Integrated Management of Neonatal and Childhood Illnesses (IMNCI) algorithm (www.unicef.org/india/Training_Module_1-9.) for detection of possible serious bacterial infection suggested by presence of any of the following signs of infection: convulsions or fast breathing (60 breaths per minute or more); severe chest in-drawing or nasal flaring or grunting; 10 or more skin pustules or a large boil; axillary temperature 37.5 Celsius or above (or feels hot to touch); temperature less than 35.4 Celcius (or feels cold to touch); lethargic or unconscious or less than normal movements. Field workers referred and accompanied sick infants to the study hospital for treatment. At the hospital, the infants were examined by a physician, blood cultures were obtained, and treatment was carried out as per the protocol of the hospital.

Information on compliance and morbidities was recorded. An enrollment card was provided which parents were asked to carry whenever they sought treatment for the infant in between study visits. Effort was made to contact local practitioners visited independently by parents of infants and collect the details of treatments prescribed. Study staff were trained in the IMNCI algorithm and given practice on eliciting signs of neonatal sepsis. Study procedures were standardized and regular exercises were conducted so as to reduce inter- and intra-observer variability. Quality assurance measures included supervisory checks in the field work, data collection and data cleaning. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry (in EPI Info version 6.0) with built-in range and consistency checks. (Details on quality assurance given as web-appendix).

The primary outcome was risk of possible serious bacterial infection (PSBI) as per the IMNCI algorithm, diagnosed by the field workers or physicians. Secondary outcomes were estimation of the effect of VSL#3 on overall morbidity pattern in 0-2 month-old LBW infants; stool colonization patterns in 10% of subjects; and assessment of side effects due to the probiotic VSL#3, if any. On the recommendation of the DSMC, data on diagnosis of sepsis by a physician was also recorded as an amendment to the protocol.

Gut Colonization sub-study

Data on gut colonization was important to substantiate the clinical findings. Stool samples from 202 (101 each in intervention & placebo arm) enrolled infants were collected on day '1', day '21' and day '60' to correspond with end of follow up

The samples were collected in sterile specimen jars (plastic containers) and transported to the lab at 4 ºC, and stored at -20ºC. Processing was done within 10 days to evaluate their bacterial micro flora composition and enzymatic activities. Sequencing and Real time PCR were conducted on DNA samples extracted from stool.

Statistical analysis

Bang et al[2] reported a 17% incidence of neonatal sepsis in the community. Assuming a 10% loss to follow-up, 1340 infants were needed (670 in each group), to observe a 30% reduction in incidence of sepsis at 5% significance with 80% power. Analyses were done by intention to treat. Software 'R' [17] (version 3.0.0) was used for calculation of PSBI risk, incidence rates, confidence intervals and incidence rate ratios. We used Kaplan-Meier survival analysis curves with Herrington Flemming variation[18] of the log rank test to compare the survival curves in the probiotic and placebo arms. We used 't' test for comparing the groups in the gut colonization sub-study.

Role of the funding source

Funding source played no role in the study design, data collection, analysis and interpretation, writing of the report or decision to submit it for publication.

Results

Between January 2009 and November 2011, 5927 LBW newborn infants were screened and 1340 eligible LBW infants were enrolled (Figure legends

Figure*1*)**.** Of the 5927 screened, 4587 were excluded (reasons given in Fig.1). The probiotic and placebo groups were comparable with regard to baseline characteristics such as mode of delivery, mean birth weight, mother's schooling, religion of the family, standard of living index (SLI), and maternal morbidities during current pregnancy (Table 1).

The intervention and control groups were similar in mean number of field worker visits performed (20.8 ± 3.7 in probiotic versus 20.5 ± 4.0 in placebo groups; p= 0.154), mean number of doses of interventional product consumed (29.1 ± 4.4 in probiotics versus 28.7 ± 5.2 in placebo; p= 0.129), and mean number of days of follow-up visits (56.3± 2.2 in probiotics versus 56.1±3.8 in placebo; p=0.239).

Primary outcome: Possible serious bacterial infection (PSBI)

Based on the intention-to-treat (ITT) analysis there was a non-significant 21% reduction in the overall risk of PSBI in the probiotic group (84 cases in 688 infants in the probiotic arm versus. 107 cases in 672 in the placebo arm; RR 0.79 [95% CI 0.56 to 1.03]; p = 0.080) (Table 2**).** In the probiotic group there was a significant 71% reduction in the risk of un pre-specified sub-group of infants with birth weights 1.5-1.99 kg (4 cases in 74 infants in probiotics vs. 14 cases in 75 in the placebo arm; RR 0.29 [95% CI 0.10 to 0.84]; p = 0.014). A 32% reduction in the risk of PSBI among un pre-specified sub-group of female infants was observed (36 cases in 348 infants in probiotic vs. 53 cases in 349 in placebo group; RR 0.68 [95% CI 0.46 to 0.99]; p= 0.056). There was no evidence of an interaction effect in the un pre-specified sub-group analysis (p-value = 0.128 for the interaction term between treatment and birth weight group).

We also calculated the incidence rates of PSBI computed with the person-time data collected during home visits (**Table 3)**. The PSBI incidence rate in the probiotics arm was 2.61per 1000 days follow-up, versus 3.40 per 1000 days in the placebo arm (RR 0.77 [95% CI 0.59 to 0.99], p=0.0493). Among un pre-specified sub-group of babies weighing 1.50-1.99kg, the incidence rate of PSBI per 1000 days was 1.67 and 4.57 in the probiotic and placebo groups, respectively (RR 0.36 [95% CI 0.15, 0.87; p=0.008).

Secondary outcomes:

Other morbidities

There was no significant difference between the groups for proportion of babies who had local infection (3.0%; [95% CI 2.0% - 4.7%] in probiotic vs. 3.4%; [95% CI 2.2% - 5.0%] in placebo group, p=0.69), feeding problems (18.9%; [95% CI 16.0% - 22.0%] in probiotic vs. 16.4%; [95% CI 13.7% - 19.3%] in placebo group, p=0.21), or other morbidities (35.9%; [95% CI 32.4% - 39.6%] in probiotic vs. 34.2%; [95% CI 30.7% - 37.9%] in placebo group, p= 0.52).

Gut colonization

There were differences in absolute colony counts in the two groups on day 1, 21 and 60, however these differences were not significant statistically.

The difference between colony counts in probiotic and placebo groups (day 21- day 1) was statistically significant (p = 0.0476) for L. acidophilus, however, it was not significant for S. Thermophilus (p= 0.9964) and B. longum (p= 0.3872). Colonization was also observed in the placebo arm, likely due to exclusive breast-feeding.

Post - Hoc Analyses:

A post-hoc analysis based on the ITT showed a non-significant 29% reduction in the overall risk of physician-diagnosed sepsis in the probiotic group (38 cases in 688 infants in the probiotic vs.54 cases of 672 in the placebo group; RR 0.71 [95% CI 0.47 to 1.06], p = 0.091). There was no case of suspected sepsis diagnosed by physician in the group of 74 infants taking probiotics and weighing 1.50-1.99kg, as compared to 8 cases in 75 infants of this weight in the placebo group (RR & 95% CI not calculated due to no sepsis case in probiotics group, Fisher's Exact test p-value = 0.007). There was no evidence of an interaction effect in the un pre-specified sub-group analysis (p-value = 0. 974 for the interaction term between treatment and birth weight group).

In the post-hoc analysis of physician-diagnosed sepsis, the incidence rate in the probiotic arm was 1.07 per 1000 days, versus 1.59 per 1000 days with placebo (RR 0.67; [95% CI 0.45 to 0.99], p =0.048). In the 1.5-1.99 kg weight stratum, there was no case of sepsis diagnosed by the physician, versus an incidence rate of 2.40 per 1000 follow-up days in the placebo arm (RR 0.00 [95% CI 0.0, 0.35]; p = 0.002).

Comparison of event rates

Kaplan Meier survival analysis curves were plotted to compare the event rates in the probiotic and placebo arms (Figure 2).This shows a divergence between the curves for probiotic and placebo, starting after a week of supplementation and remaining throughout the follow-up period. The onset of first episode of PSBI in 10% of infants occurred on the 41st day in the probiotic arm versus 24thday in control arm (p=0.063), and onset of first episode of suspected sepsis diagnosed by physician in 5% of infants occurred on 53rd day in probiotic arm versus 26th day in control arm (p=0.071).

Adverse outcomes: hospitalizations and deaths

Hospitalization and death in enrolled infants were considered as moderate and severe adverse outcomes respectively (Table 4). During the study 29 infants in the probiotic and 44 in the placebo arm needed to be hospitalized (p=0.075). Median number of hospitalization was of 3 days in the probiotic versus 6 days in the placebo arm (p <0.018). There were three deaths, one in the probiotic and two in the placebo arm. Verbal autopsy reports of deaths reviewed by the DSMB did not attribute them to the intervention. No side-effects of VSL#3 were reported.

Discussion

Overall, supplementation with the probiotic VSL#3 in LBW infants was associated with a 21% (non-significant) reduction in the risk of suspected sepsis (PSBI) diagnosed by the field worker. However, in the un-pre-specified sub-group of infants weighing 1.5-1.99 kg, the reduction in risk of PSBI was statistically significant (reduction of 71%; p=0.014). The primary analysis in this study was based on PSBI classification by field worker as per the IMNCI algorithm as an indicator of neonatal sepsis.[19] The classification PSBI under IMNCI is described as sensitive but not specific for detection of neonatal sepsis.[20] Prior to closure of the study, the DSMC recommended conducting post-hoc analyses using physician's diagnosis of sepsis as the outcome measure. In this analysis there is a 29% overall reduction in risk of sepsis. However, in the un pre-specified sub-group of infants weighing 1.5-1.99 kg, there is a 100% reduction, with no cases observed in the group receiving probiotic supplementation. Our findings of probiotics efficacy among infants 1.5-1.99 kg may be a chance finding, generating a hypothesis that this intervention may be useful for the most vulnerable of the LBW babies. As our power calculations did not consider this a priori, and hence these findings need to be confirmed in future studies. Probiotic intervention significantly reduced the mean number of hospitalization days. The Kaplan Meier survival analysis shows a 15-day delay in the onset of sepsis in the intervention arm; this translates to a disease-free window during the 28-day period, crucial for neonatal survival. Moreover, considering a higher case fatality in sepsis at early ages, this becomes

even more important. Our results may not be definitive or robust enough; however, there is a consistency in them, and we do not consider this as a "negative trial". Although our study is not large enough, it may be misleading to interpret it as proving that there is no effect of the probiotic intervention or no difference between the study groups. More evidence needs to be generated, since interpretation of no effect might discourage further studies.[21]

In the current study, a consistent difference between the intervention and control groups A and B in all the tables as well as the Kaplan Meier survival analysis curves was observed. The difference between the groups was marked in most of the tables when physician diagnosis of sepsis was considered. After evaluating the coded results, the DSMB even considered amendment of the original protocol to change the primary outcome variable to sepsis as diagnosed by the physician so that the study conclusively finds out the role of VSL#3 in preventing neonatal sepsis. It was said that physician's diagnosis of sepsis would be widely acceptable owing to its accuracy as compared to diagnosis by Field workers. The Committee advised to extend the study to enroll more infants for the revised primary outcome. However, a 'Technical Advisory Group' (including clinical trialists, biostatistician, public health experts), formed on recommendations of the DSMB, suggested that the present study should be closed and another study planned, in view that the trial was registered and the statistical analytical plan specified the primary outcome as PSBI, it was suggested to present the findings on Physician's diagnosis as post-hoc.

Physician's diagnosis of sepsis is more meaningful than PSBI, owing to its specificity. The reported post-hoc analyses increase our confidence in the results. However, physicians used their clinical judgement for diagnosing sepsis; there was no standardized definition used, and this is a limitation of the study. Future trials should evaluate the role of VSL#3 on incidence of sepsis with a precise definition of the outcome measure. The incidence of sepsis observed in the study was lower than the expected effect size used in determining the sample size of the study. Home visits,[22,23]health education messages about exclusive breastfeeding and hygiene, and referral by field workers could improve care

and care-seeking, resulting in lower morbidity and mortality and a type II error for the overall result of our study. Our study has several other limitations. It was not powered to assess the role of probiotics on neonatal mortality. The enrolments were done during 3-7 day of life, so we cannot comment on the role of probiotics on early onset sepsis. We followed infants for a period of two months, and cannot comment on the long term effects of probiotic supplementation. There are concerns regarding heterogeneity in probiotic products.The literature suggests greater protection with double or triple probiotic strains.[13]Probiotic VSL#3 is a mix of 8 strains namely-*Streptococcus thermophilus, Bifidobacterium breve, B. Longum, B. Infantis, Lactobacillus acidophilus, L. Plantarum, L. Paracasei,* and *L. Delbrueckii spp bulgaricus.* In a randomized placebo-controlled clinical trial in India, VSL#3 resulted in early recovery and reduced need for oral rehydration salts in rotavirus-affected children aged 6 months to 2 years.[24]

In previous studies, probiotics have been found to prevent necrotizing entero-colitis (NEC) by preventing colonization of the gut by pathogens, promoting colonization with beneficial organisms, improving maturity and function of gut mucosal barrier and modulating the immune system to the advantage of the host.[11,12]A Cochrane review showed moderate to low quality evidence that oral lactoferrin with or without probiotics decreases sepsis and NEC in preterm infants[25]. The mechanism for efficacy of probiotics in reducing the incidence of sepsis in VLBW infants is probably similar to that for NEC.[26,11]However, in a further study by Lin et al[12] the effect of reduction in incidence of sepsis was not confirmed. This study was conducted among severely ill hospitalized VLBW infants with central line, total parenteral nutrition and prolonged use of mechanical ventilation. Probiotics exert their effects by positively influencing normal microbe–microbe and host–microbe interactions and may augment the protection afforded by commensal flora through competitive interactions, direct antagonism of pathogens, and/or production of antimicrobial factors. The preventive mechanisms could fail in the face of severe conditions as in case of the study by Lin.[12]Probiotics alone would not overcome the infection induced by invasive procedures. However, in the community setting such as in our study, among LBW predominantly breastfed infants, probiotics could be effective in preventing

sepsis, since the primary effect of orally administered probiotics is in the gastrointestinal tract with prevention of bacterial translocation.

Neonatal infection is a high priority area of research. Research on immunotherapy[3]has provided very few leads. To our knowledge, at present there are no proven interventions beneficial in preventing sepsis in LBW infants[27,] apart from exclusive breastfeeding and practice of hygiene. This study provides an indication that microbial interference by beneficial bacteria is helpful in decreasing neonatal morbidity. Considering a 30% prevalence of LBW in India[28] and 30% mortality due to sepsis in newborns,[29]even a modest decline in the incidence of sepsis due to preventive intervention with probiotics could avert thousands of neonatal deaths. When produced at large scale it would be a cost-effective intervention for a major public health problem.

We observed a significant positive treatment effect in the subgroup of infants weighing 1.5-2.0 kg. This mandates conduct of a larger study with sufficient power to conclusively evaluate the role of probiotics among LBW infants in a population at high risk of mortality from sepsis. There is also a need to conduct this kind of study for all neonates to assess if probiotics could be beneficial even for children who are not LBW.

Contributions

AS conceptualized the study, prepared the protocol and drafted the report. All the authors reviewed and approved it. AS, SSG, HC, BSG were responsible for the design of the trial; AS SSG MSP were responsible for preparing the standard operating procedures and data collection instruments; SSG, HC,CM, VK, SA, SD, VD and MT were responsible for implementation of the trial and clinical management of subjects; SSG designed the database and managed the data; SSG and AS were responsible for the analyses and interpretation. AS, SSG, HC and SA edited the draft manuscript. VT closely monitored implementation, AM, MR and contributed at different stages of study implementation. All authorshad full access to all of the data (including statistical reports and tables) in

the study and can take responsibility for the integrity of the data and the accuracy of the data analysis, they approved the final version to be published, and agreed to be accountable for the accuracy and integrity of the manuscript.

Transparency statement

I, Dr. Anju Sinha, the lead author of this manuscript affirm that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Competing Interest Declaration

"All authors have completed the Unified Competing Interest form available atwww.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that (4) [AS, SSG, HC, CM, VK, SA, BSG, SD, MSP, VD, MT, VT, AM and MR] have no non-financial interests that may be relevant to the submitted work."

We declare that we do not have any conflict of interest.

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Table 1. Comparison of baseline characteristics, Intention-to-treat population

	Probiotics (N=668)		Placebo (N=672)	
Sex				
Male	319	47.8%	320	47.6%
Female	349	52.2%	352	52.4%
Birth weight groups				
1500 – 1999 gm	74	11.1%	75	11.2%
2000 – 2499 gm	594	88.9%	597	88.8%
Mean (SD)birth weight	2261 ± 179		2263 ± 179	
Mother's schooling				
≤ 8 years	292	43.7%	285	42.4%
> 8 years	376	56.3%	387	57.6%
Religion				
Hindu	489	73.2%	501	74.6%
Muslim	46	6.9%	41	6.1%
Others	133	19.9%	130	19.3%
Standard of Living Index				
Low	98	14.7%	85	12.6%
Medium	348	52.1%	382	56.8%
High	222	33.2%	205	30.5%
Mode of delivery				
Vaginal	633	94.8%	629	93.6%
LSCS + Others	35	5.2%	43	6.4%
Morbidities during pregnancy				
Hypertension	23	3.4%	18	2.7%
Anaemia	55	8.2%	63	9.4%
PROM	22	3.3%	30	4.5%
None	568	85.0%	561	83.5%
Mean SLI score	22.2 ± 7.9		22.3 ± 7.7	

Table 2. Cumulative riskof possible serious bacterial infection/clinically suspected sepsis

	Probiotics				Placebo				Cumulative risk ratio		p-value **
	N	N	Cumulative risk		N	N	Cumulative risk				
			(%)	95% CI			(%)	95% CI	RR	95% CI	
Possible serious bacterial infection (PSBI, by field investigator)											
All strata	84	668	12.6%	10.3,15.3	107	672	15.9%	13.3,18.9	0.79	0.56,1.03	0.080
1.5-1.99 Kg	4	74	5.4%	1.7,13.49	14	75	18.7%	11.3,29.1	0.29	0.10,0.84	**0.014**
2.0 – 2.49 Kg	80	594	13.5%	11.0,16.5	93	597	15.6%	12.9,18.7	0.86	0.66,1.14	0.303
Male	48	320	15.0%	11.5,19.4	54	323	16.7%	13.0,21.2	0.90	0.63,1.28	0.553
Female	36	348	10.3%	7.5,14.0	53	349	15.2%	11.8,19.4	0.68	0.46,0.99	0.056
Suspected sepsis (by physician)											
All strata	38	668	5.7%	4.2,7.7	54	672	8.0%	6.2,7.7	0.71	0.47,1.06	0.091
1.5-1.99 kg*	0	74	0.0%	0,5.9	8	75	10.7%	5.2,19.9	___	_____	**0.007**
2.0 – 2.49 kg	38	594	6.4%	4.7,8.7	46	597	7.7%	5.8,10.1	0.83	0.55,1.26	0.381
Male	21	320	6.6%	4.3,9.9	30	323	9.3%	6.6,13.0	0.71	0.41,1.21	0.205
Female	17	348	4.9%	3.0,7.7	24	349	6.9%	4.6,10.1	0.71	0.39,1.30	0.270

*RR and 95% confidence intervals could not be calculated due to no case of sepsis in probiotics group, Yate's corrected p-value.

** p-values less than 0.05 have been shown in bold.

Table 3. Incidence rate for PSBI clinically suspected sepsis per 1000 days of follow-up

	Probiotics				Placebo				Incidence rate ratio		p-value **
	n	Person-days	Incidence rate/ 1000 days		N	Person-days	Incidence rate/ 1000 days				
			Rate	95% CI			Rate	95% CI	RR	95% CI	
Possible serious bacterial infections (by field investigator) and sepsis by physician											
All strata	98	37532	2.61	2.12,3.18	128	37681	3.40	2.83,4.04	0.77	0.59, 0.99	**0.049**
1.5-1.99 Kg	6	4204	1.67	0.52,3.11	19	4159	4.57	2.75,7.13	0.36	0.15, 0.87	**0.008**
2.0 – 2.49 Kg	92	33328	2.19	2.23,3.39	109	33522	3.25	2.67,3.92	0.67	0.64, 1.12	0.248
Male	58	17946	3.23	2.45,4.18	69	18107	3.81	2.97,4.8	0.85	0.60, 1.20	0.357
Female	40	19586	2.04	1.46,2.78	59	19574	3.01	2.29,3.89	0.68	0.45, 1.01	0.056
Suspected sepsis by physician											
All strata	40	37532	1.07	0.76,1.45	60	37681	1.59	1.21,2.05	0.67	0.45, 0.99	**0.048**
1.5-1.99 Kg*	0	4204	0.00	0.00,1.11	10	4159	2.40	1.15,4.42	0.00	0.0, 0.35	**0.002**
2.0 – 2.49 Kg	40	33328	1.20	0.86,1.63	50	33522	1.49	1.11,1.97	0.80	0.53, 1.22	0.307
Male	23	17946	1.28	0.81,1.92	35	18107	1.93	1.35,2.69	0.66	0.39, 1.12	0.126
Female	17	19586	0.87	0.51, 1.39	25	19574	1.28	0.83,1.89	0.68	0.37, 1.26	0.221

* As there was no case among the exposed, the risk ratio and its confidence interval were calculated by adding 0.5 to each cell. Fisher's exact p-value was calculated instead of chi-squared test.

** p-values less than 0.05 have been shown in bold.

(Table 4) Comparison of Adverse outcomes between probiotics and placebo arms

	Probiotics	Placebo	Total	p-value*
Hospitalization required	29	44	73	**0.075**
Duration of hospitalization 25th centile Median 75th centile	 2 days 3 days 5 days	 3 days 6 days 8.75 days	73	**<0.018****
Deaths	1	2	3	NS

* p-values less than 0.05 have been shown in bold.

** p-values calculated using Mann-Whitney Wilcoxon test

Figure legends

Figure1 Participant flow through the trial

Figure 2: Kaplan-Meier curves for difference between event rates in probiotic and placebo groups.

CONSORT 2010 checklist of information to include when reporting a randomized trial*

Section/Topic	Item No	Checklist item	Reported on page no
Title and abstract			
	1a	Identification as a randomised trial in the title	**Title page 1**
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	**page 2**
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	**page 4**
	2b	Specific objectives or hypotheses	**page 4-5**
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	**page 5**
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	**Not Applicable**
Participants	4a	Eligibility criteria for participants	**page 5 sentence 8**
	4b	Settings and locations where the data were collected	**Page 5 Sentences 6-7**
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	**Page 5-6 under Study Methodology**
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	**page 7**
	6b	Any changes to trial outcomes after the trial commenced, with reasons	**Not Applicable**
Sample size	7a	How sample size was determined	**Page 7 under Statistical Analyses**
	7b	When applicable, explanation of any interim analyses and stopping guidelines	**Not Applicable***
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	**Page 6 under Randomization & Masking**
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	**Page 6, under Randomization & Masking**
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	**Page 6, under Randomization & Masking**
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	**Page 6 INCLEN Trust**
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	**Page 6 under Randomization & Masking**

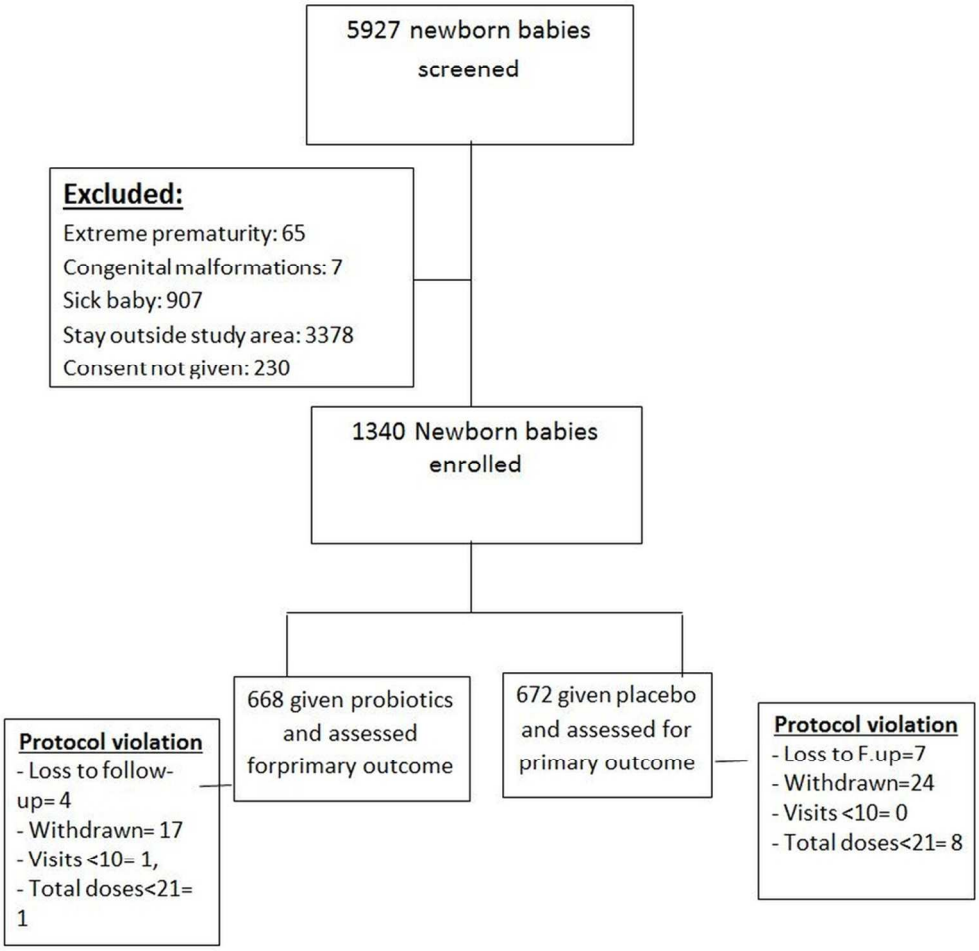
		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	**Page 6 under Randomization & Masking**
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	**Page 7, Statistical analyses**
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	**Not Done**
Results Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	**Page 21 Flow Diagram**
	13b	For each group, losses and exclusions after randomisation, together with reasons	**Page 21 Flow Diagram**
Recruitment	14a	Dates defining the periods of recruitment and follow-up	**Page 5**
	14b	Why the trial ended or was stopped	**Completion of sample size**
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	**Page 18 Table1**
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	**Yes Pages 18-20,Tables 1,2,3,**
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	**Pages 19-20, Tables 2,3.**
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	**Page 19-29, Table 2,3**
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	**Kaplan Meir Survival analysis Pages 22-23**
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	**Not applicable**
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	**Pages 10-12**
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	**Pages 10-12**
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	**Pages 10-12**
Other information			
Registration	23	Registration number and name of trial registry	**CTRI/2008/091/000049**
Protocol	24	Where the full trial protocol can be accessed, if available	**Is attached, available from ICMR website**
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	**Page 7 & Page 14**

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

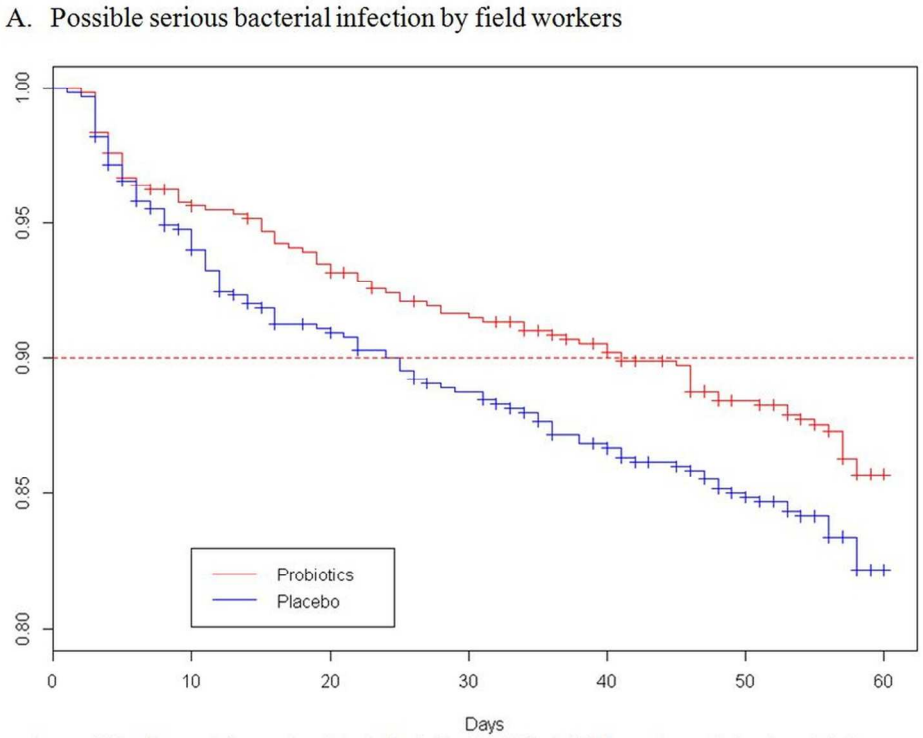
For publication as web appendix only:

Methods: Duplicate visits were performed by a study supervisor in 10% of visits as a quality control measure. Field workers records were verified by study medical officer weekly before entering the data in the computer. Viability testing of the probiotics sachets collected from the field sites was done. A schedule for collection of these sachets was prepared such that every week 4 sachets were collected in reverse cold chain from infants who were in 1-4th week of follow up, over a six month period. These sachets were transported to an external lab (Micro s.r.l., Italy, accredited by SINAL [National System for accreditation of laboratories, Italy] and the International Laboratory accreditations cooperation ILAL-MRA]. The certificate of analysis received from the lab certified the adequacy of cell counts in the sachets, and based on the results extended the expiry of the batch by one year. All case record forms were cross-checked by supervisors and medical officers before being sent for double data entry in EPI Info version 6.0) with built in range and consistency checks. Hand checking on random samples was done and frequency distribution of important variables examined periodically to identify aberrant values. A program file developed in EPI 6 platform was run, the list of errors was sent to the sites for corrections.

IMNCI Algorithm: Presence of any of the following signs suggested possible serious bacterial infection: convulsions or fast breathing (60 breaths per minute or more); severe chest in-drawing or nasal flaring or grunting; 10 or more skin pustules or a large boil; axillary temperature 37.5 Celsius or above (or feels hot to touch); temperature less than 35.4 Celcius (or feels cold to touch); lethargic or unconscious or less than normal movements. (Ref: Training Modules 1 to 9 - Unicef. www.unicef.org/india/Training_Module_1-9.)



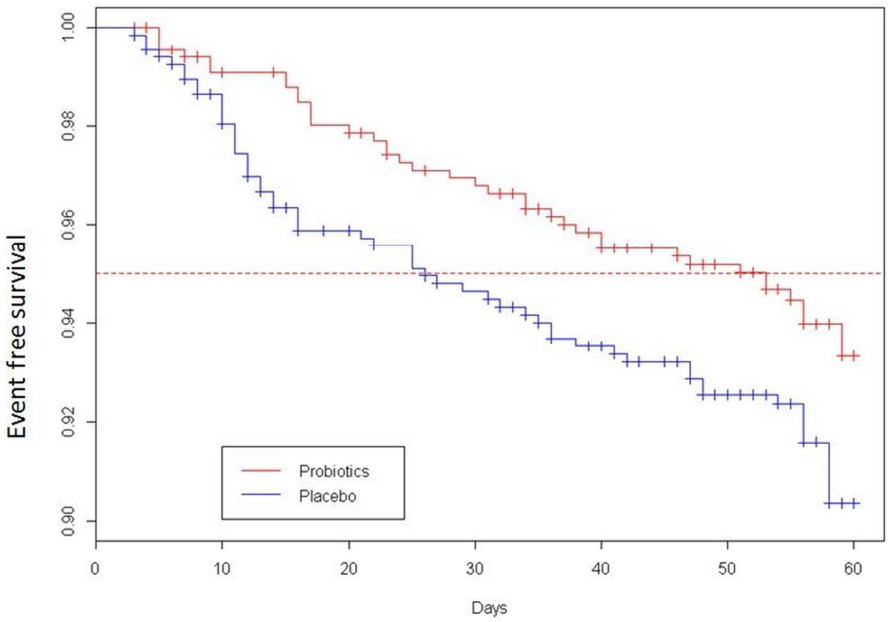
91x90mm (300 x 300 DPI)



Logrank test (Harrington Flemming Variation) for PSBI by Field workers. 'p' value 0.063

115x90mm (300 x 300 DPI)

B. Clinically suspected sepsis by Physicians



Logrank test (Harrington Flemming Variation) for PSBI by Field workers for clinically suspected sepsis diagnosed by the physicians 'p' value 0.071

113x90mm (300 x 300 DPI)