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A randomized synbiotic trial to prevent sepsis among infants in rural India

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Sepsis in early infancy results in one million annual deaths worldwide, most of them in developing countries. No efficient means of prevention is currently available. Here we report on a randomized, double-blind, placebo-controlled trial of an oral synbiotic preparation (*Lactobacillus plantarum* plus fructooligosaccharide) in rural Indian newborns. We enrolled 4,556 infants that were at least 2,000 g at birth, at least 35 weeks of gestation, and with no signs of sepsis or other morbidity, and monitored them for 60 days. We show a significant reduction in the primary outcome (combination of sepsis and death) in the treatment arm (risk ratio 0.60, 95% confidence interval 0.48–0.74), with few deaths (4 placebo, 6 synbiotic). Significant reductions were also observed for culture-positive and culture-negative sepsis and lower respiratory tract infections. These findings suggest that a large proportion of neonatal sepsis in developing countries could be effectively prevented using a synbiotic containing *L. plantarum* ATCC-202195.

Sepsis is a clinical syndrome characterized by systemic inflammation and circulatory compromise initiated by an infection. It is a major cause of neonatal morbidity and mortality, with case fatality rates ranging from 5 to 60%, even with antibiotic treatment[1]. Of the 6.3 million children worldwide who died before the age of five in 2013, 2.76 million were in the neonatal period, with the bulk of these in developing countries[2]. Over 0.6 million of these neonatal deaths were due to possible severe bacterial infection (pSBI) alone[3]. The disease burden of pSBI, at 6.9 million cases per year in moderate-to-late preterm neonates (>32 weeks of gestation) in sub-Saharan Africa, south Asia (3.5 million of these cases[4]), and Latin America, accounts for a loss of disability-adjusted life years similar to that of HIV and AIDS[5]. While the term 'sepsis' in the developed world refers to culture-confirmed bacterial or fungal infection, it is often used interchangeably with pSBI in the developing-world setting. Lacking culture and other diagnostic facilities, all infections (including viral) are grouped into one category, and modalities for the empirical management of neonatal sepsis decided.

Although the death rate of children worldwide (1–59 months) decreased by 3.4% annually from 1990 to 2012, neonatal mortality in that period only dropped by 2% per year[6]. In addition to prematurity, sepsis (37%) and pneumonia (5%) continue to be major contributors to deaths in the neonatal period[7].

Low birth weight and preterm birth predispose the infant to sepsis, and even in the United States, one-quarter of very low birth weight infants (less than 1,500 g) experience culture-positive sepsis, adding to the considerable total burden of morbidity, extended hospital stay, and mortality[8]. Given the scale of this problem, even modestly effective interventions would save several hundreds of thousands of lives, and many more millions of dollars, every year.

Trials targeting endogenous mediators of adult sepsis to ameliorate systemic inflammation have generally not shown effectiveness[9]. As well as the negative results of intravenous immunoglobulin treatment in neonatal sepsis in humans[10], lysozyme, lactoferrin, and vitamin A have all shown some promise as prophylactic agents in animal studies, but have not moved into the clinical arena. In developing-world settings, exclusive breastfeeding and applying antiseptics to the umbilical stump[11] are two interventions that have proven helpful.

The use of probiotic bacteria to prevent sepsis has been proposed, but only a few trials have focused on neonates, whose largely naive immune system and less complex intestinal microbiome should make colonization with probiotic strains more feasible[12]. Several small studies have examined the efficacy of probiotics in necrotizing enterocolitis in premature infants; some, but not all, of these have shown efficacy against neonatal sepsis[13].

Synbiotics are combinations of probiotics with a prebiotic, the latter added to promote growth and sustain colonization of the probiotic strain. Two prevention trials of synbiotics conducted in Turkey—in congenital heart disease[14] and in very low birth weight infants[15]—detected a reduction in sepsis. However, an Indian hospital-based trial in premature infants did not statistically significantly lower the risk of sepsis, although it reduced the incidence of necrotizing enterocolitis[16].

Strain selection and colonizing capacity have not been addressed in depth in the clinical trial literature, or, with some exceptions[17,18], most newborn trials did not perform blood cultures, use precise outcome measures, or use adequate sample sizes. The synbiotic trial described here was preceded by our experience in pilot hospital-based trials of different strains of probiotics in India[19], in which *Lactobacillus GG* (LGG) and *Lactobacillus sporogenes* (at different doses) showed minimal or no stool colonization. Conversely, colonization lasted for up to four months when *Lactobacillus plantarum* ATCC strain 202195 in combination with fructooligosaccharide was orally administered to neonates in the first week of life[20].

We conducted this community-based, double-blind, placebo-controlled randomized trial in 149 randomly chosen villages in Odisha state (Extended Data Fig. 1), where neonatal and infant mortality rates are among the highest in India[21], using a three-tier field management

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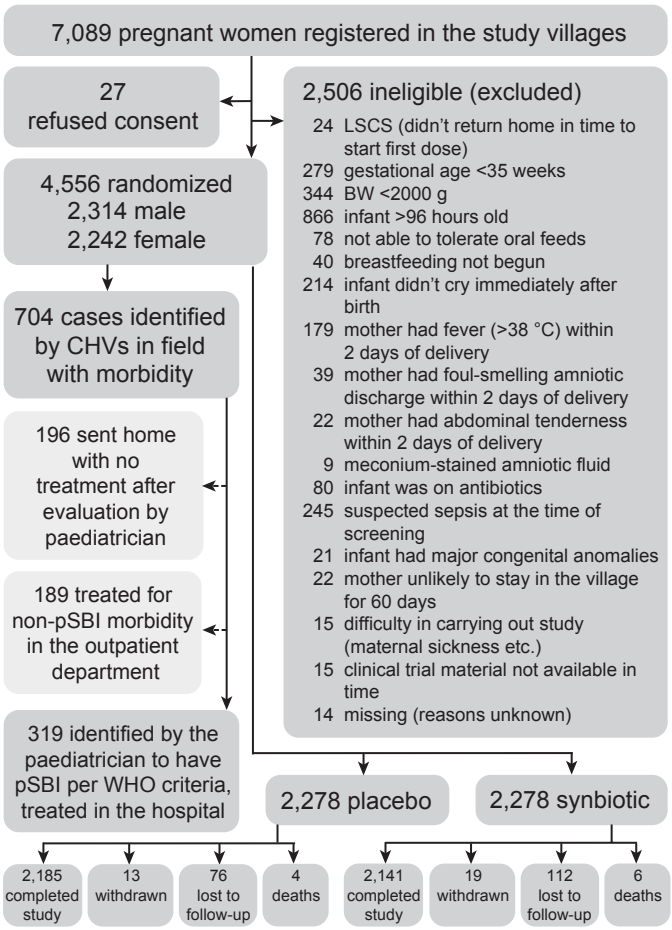

Figure 1 | Screening and enrolment of study infants in the sepsis trial. In total, 2,278 in each group were included in the current intent to treat analysis. pSBI, possible severe bacterial infection; CHVs, community health volunteers; WHO, World Health Organization; BW, birth weight.

Table 1 | Baseline and postnatal study variables ($n = 4,556$)

Variables	Placebo $n = 2,278$ *n* (%)*	Synbiotic $n = 2,278$ *n* (%)*
Baseline infant and mother characteristics		
Mother's age ((years; mean (s.d))	24.8 (3.8)	24.8 (3.9)
Mother's parity/gravida ((mean (s.d.))	2.0 (1.0)	2.0 (1.1)
Neonate place of birth		
Hospital	1,933 (84.9)	1,952 (85.7)
Nurse midwife centre	47 (2.1)	42 (1.8)
Home	298 (13.1)	284 (12.5)
Individual delivering neonate		
Physician	1,938 (85.1)	1,955 (85.8)
Traditional birth attendant	123 (5.4)	130 (5.7)
Family member	160 (7.0)	150 (6.6)
Paramedical worker	57 (2.5)	43 (1.9)
Type of delivery		
Unassisted vaginal delivery	2,273 (99.8)	2,272 (99.7)
C-section/assisted vaginal delivery	5 (0.2)	6 (0.3)
Infant birth weight (g; mean (s.d.))	2,799.9 (355.6)	2,803.7 (354.0)
Gestational age		
35–36 weeks	229 (10.0)	212 (9.3)
≥37 weeks	2,049 (90.0)	2,066 (90.7)
Infant feeding practices during first 60 days of life		
Initiation of breastfeeding since delivery†		
<1 h	1,433 (62.9)	1,451 (63.7)
1–12 h	813 (35.7)	796 (35.0)
13–24 h	20 (0.9)	17 (0.7)
>24 h	12 (0.5)	13 (0.6)
Received cow's milk	8 (0.4)	14 (0.6)
Received honey	16 (0.7)	20 (0.9)
Received formula	6 (0.3)	10 (0.4)
Received boiled water	51 (2.2)	43 (1.9)
Received vitamin drops	11 (0.5)	18 (0.8)
Treatment administration time since delivery‡		
Two days or less	1,078 (47.4)	1,110 (48.8)
Three days or more	1,196 (52.6)	1,166 (51.2)

*Results are expressed as *n* (%) unless otherwise stated. Percentages may not add up to 100% owing to rounding. There were no missing observations unless otherwise stated.
†One observation was missing.
‡Six observations were missing.
All chi-square and Satterthwaite two-sample *t*-tests comparing placebo and synbiotic groups had $P \geq 0.2$.

structure (Extended Data Fig. 2). The synbiotic was administered orally to the newborns for 7 days beginning on day 2–4 of life.

We screened 7,089 births in study villages, of which 4,556 (64%) were enrolled in the study (Fig. 1). All infant and maternal variables of interest (Table 1) and age at presentation of primary outcome (Supplementary Information 1A) were similarly distributed between placebo and treatment groups at the baseline. Of the 4,556 infants enrolled, 704 were identified by community health volunteers (CHVs) to have suspected sepsis/pSBI and referred to the study hospital for further evaluation by the paediatrician. Of these suspected cases of sepsis, 5% were in week 1, 13% in week 2, with the remainder (72%) during weeks 3 to 8 of life. Upon clinical examination by the paediatrician, 196 infants were sent home without any treatment, and an additional 189 were treated in an ambulatory clinic (hospital outpatients) or in the community during field visits by physicians for morbidities other than sepsis. Of all referred cases, 319 infants were recorded as cases of sepsis upon physician diagnosis using WHO (World Health Organization) criteria, admitted to the hospital, and treated with intravenous antibiotics. Again, only 3% and 14% of these infants were in their first and second weeks of life, respectively, showing that the bulk of the sepsis disease burden is after the first two weeks of life. CHVs or physicians did not identify any infant with pSBI in their first three days of life. Only two infants with pSBI were admitted on day four. The highest numbers of cases were identified in the field (at least 30%) and by the physician (23–24%) when infants were 5–6 weeks old. There were only four pairs of twins and three individual neonates from a twin birth. None of these infants had an adverse event.

In this trial, blood culture was performed for 182 out of 319 infants admitted to the hospital—in all cases of suspected sepsis and for those with overlapping or unclear signs or symptoms. Blood culture was not performed for infants with clear evidence of lower respiratory tract infection (LRTI) supported by chest X-ray or strong auscultatory findings (uneven or reduced breath sounds, crackles). There were 88 culture-positive and 94 culture-negative cases. All 319 infants were treated with intravenous antibiotics for at least 5 days. The 189 infants not admitted to the hospital received oral antibiotics and other symptomatic treatment prescribed by the paediatrician. Infection-related secondary outcomes (such as diarrhoea, omphalitis and skin infection) were recorded in 152 out of the 189 non-sepsis cases.

Treatment effects on primary and secondary outcomes

We observed a significant (40%) reduction in the primary combined outcome of death and neonatal sepsis, from 9% in the placebo arm to 5.4% in the treatment arm; 27 infants would need to be treated to prevent one primary outcome. This effect was entirely due to the impact on sepsis. Death during the study period was rare; ten infants died, six in the treatment arm and four in the placebo arm (Table 2). Causes of death and bacterial species identified are described in Table 3.

Each of the three components of the sepsis/pSBI syndrome—culture-positive sepsis, culture-negative sepsis and LRTIs—was significantly reduced in the treatment arm. Among infants documented to have positive microbial cultures, 27 were in the placebo arm, and just 6

Table 2 | Effect of synbiotic treatment on sepsis and other morbidities in the first 60 days of life

Outcome variables	Control $n=2,278$ (%)	Synbiotic $n=2,278$ (%)	RR (95% CI)	NNT (95% CI)	*P* value
Death and sepsis (primary outcome)	206 (9.0)	123 (5.4)	0.60 (0.48, 0.74)	27 (19, 47)	<0.001
Deaths	4 (0.2)	6 (0.3)	1.50 (0.42, 5.31)	NA*	0.526†
Sepsis (A + B + C)	202 (8.9)	117 (5.1)	0.58 (0.46, 0.72)	27 (19, 44)	<0.001
A. Sepsis/pSBI—culture-positive septicaemia	27 (1.2)	6 (0.3)	0.22 (0.09, 0.53)	108 (71, 232)	<0.001
Gram-negative sepsis	16 (0.7)	4 (0.2)	0.25 (0.08, 0.75)	190 (110, 699)	0.007
Gram-positive sepsis	11 (0.5)	2 (0.1)	0.18 (0.04, 0.82)	253 (142,1,169)	0.012
B. Sepsis/pSBI— culture-negative sepsis (Culture-negative clinical sepsis warranting hospitalization and IV antibiotics)	36 (1.6)	19 (0.8)	0.53 (0.30, 0.92)	134 (72, 890)	0.021
C. Sepsis/pSBI—LRTI (LRTIs requiring antibiotic therapy)	139 (6.1)	92 (4.0)	0.66 (0.51, 0.88)	48 (30, 126)	0.002
Diarrhoea	59 (2.6)	12 (0.5)	0.20 (0.11, 0.38)	48 (36, 74)	<0.001
Local infections (including >10 pustules, oral thrush, conjunctivitis)	33 (1.5)	16 (0.7)	0.48 (0.27, 0.88)	134 (74, 677)	0.015
Abscess/ otitis media	11 (0.5)	5 (0.2)	0.45 (0.16, 1.33)	NA*	0.133*
Omphalitis	13 (0.6)	3 (0.1)	0.23 (0.07, 0.81)	228 (128,1,045)	0.014

All comparisons showed a significant difference with $P < 0.05$, except death. A total of 182 blood cultures were performed for cases of suspect sepsis (culture-negative and culture-positive, $n=88$), and in an additional 94 cases with unclear presentations. Blood culture was not performed for infants that had clear evidence of LRTI.
pSBI, possible severe bacterial infection; LRTI, lower respiratory tract infection; IV, intravenous;
RR, risk ratio; NNT, number needed to treat; CI, confidence interval.
*No significant difference.
†Fisher's exact test. All other P values were obtained from the chi-square test.

in the treatment arm (risk ratio (RR) = 0.22, 95% confidence interval (CI) = 0.09–0.53). The risk reduction was 82% for Gram-positive infections and 75% for Gram-negative infections. One culture-positive case was diagnosed in the first week of life, two each in weeks 2 and 3, and the remaining 28 in weeks 4 or later. *Lactobacillus* was not grown from any blood culture. Culture-negative sepsis was reduced by 47% (RR = 0.53, 95% CI = 0.30–0.92). LRTI was identified in 231 study subjects (5%), and was significantly reduced, by 34%, in the synbiotic group (number needed to treat (NNT) = 48).

Among secondary outcomes, local infections were diagnosed in 1% of infants, and were reduced by 52% in the synbiotic arm. The incidence of other infections was low, but had statistically significant risk reductions, ranging from 55% to 80%, in the treatment arm. The weights of infants in the two study arms did not differ at birth, enrolment, or at 7 or 28 days, but at 60 days infants in the synbiotic group showed a greater increase in weight since birth compared to the placebo arm (mean difference between the two arms: 31.4 g; 95% CI = 3.5–59.2; $P = 0.03$).

Table 3 | Causes of death and organisms identified from sick infants

	Control	Synbiotic	Total
Causes of death			
Congenital varicella		1	1
Congenital heart disease		1	1
Necrotizing fasciitis		1	1
Pneumonia		1	1
Sepsis and pneumonia	1	1	2
Sepsis	1		1
Omphalitis	1		1
Hypothermia	1	1	2
Total	4	6	10
Bacterial species identified			
*Staphylococcus aureus**	7	1	8
Escherichia coli	12	4	16
Streptococcus viridans	3	1	4
Klebsiella pneumoniae	2		2
Citrobacter freundii	1		1
Enterococcus faecium	1		1
Acinetobacter sp.	1		1
Total	27	6	33

*Staphylococcus aureus was isolated from two death cases (one each in placebo and treatment arm) and was not included in the organism count.
P values were not significant for all.

Tolerance of the synbiotics and adverse events

This study was monitored tightly in the field and all adverse events were recorded and reported within the stipulated time frames, whether related to study intervention or not. All hospitalizations (including 319 cases of sepsis/pSBI) were considered serious adverse events. Several unrelated events were recorded as expected in the population, including one case each of hydrocephalus, biliary atresia and laryngomalacia, and two non-fatal cases of neonatal malaria. Gastrointestinal adverse events were surprisingly low, with only six cases of abdominal distention (five in the placebo group and one in the treatment group), and the preparation was well tolerated.

Protocol violations and sensitivity analysis

A protocol violation was defined as any notable deviation from the study protocol procedures, the details of which are described in the Methods. A total of 26 protocol violations were recorded. At the final status recording by the CHV scheduled on day 60, owing to unavailability of the infant, 188 (around 4.0%) of the study infants were recorded as lost to follow-up (LFU) per protocol. This was primarily due to maternal relocation outside of the study area (details in Supplementary Information). Although small, the percentage of these LFU infants differed by treatment arm (5.7% and 3.6%). To account for this, we conducted sensitivity analysis to examine the effect on the risk ratio for various hypothetical scenarios. Even when a majority (80%) of LFUs were considered to have had the primary outcome, our analysis demonstrated a continued lower risk of sepsis or death in the treatment arm (RR = 0.83, 95% CI = 0.70–0.98). We conducted additional sensitivity analysis to address any potential bias introduced owing to the fixed block design.

Although there was a shift of the risk ratio towards 1 when the last two treatments in each block (third and fourth) were removed, the RR remained reduced (RR = 0.65, 95% CI = 0.48–0.86) in the treatment arm, demonstrating that the direction of the overall trend did not change.

Results of sensitivity analyses for a range of LFUs and after removal of blocks are presented in Supplementary Information 1B and 1C.

The 42% reduction in sepsis observed in the current study was more than twice the 20% reduction anticipated, and permitted the data safety and monitoring board (DSMB) to terminate the trial early. LRTI constituted 72% of our study outcomes. The significant reduction in LRTI found in the trial is as important as the prevention of clinical or culture-positive sepsis. The substantial reduction in a variety of other infections further lends support to the value of this intervention. The week-long treatment costs about US$1, and given our finding of an NNT of 27, the investment required to prevent one case of sepsis is about $27, showing that this approach is cost-effective even in resource-constrained settings.

Neither one of two large ($n > 1{,}000$) probiotic trials in the literature that targeted sepsis in very preterm infants showed efficacy[17,18], although one found a benefit in a subset of infants born after 28 weeks' gestation[18]. In a trial conducted among low birth weight infants in India, Sinha *et al.* showed a non-significant reduction in sepsis/pSBI, but that trial did not use blood cultures[22]. The differences in findings may reflect the choice of probiotic. Although we used *L. plantarum*, the trial by Jacobs *et al.*[17] used a mixture of *Streptococcus thermophilus*, *Bifidobacterium infantis*, and *Bifidobacterium lactis*, and the trial by Costeloe *et al.*[18] used *Bifidobacterium breve* BBG001. Sinha *et al.* used a combination of eight probiotics (VSL-3)[22]. None of these three trials used any prebiotic supplement. Our trial was conducted in healthy newborns of approximately normal weight (thus likely near-term) in rural Indian communities, while the other three trials focused on premature or low birth weight infants. The differing findings among the trials suggest that probiotic interventions will need to be tailored (and tested) in the target population of interest. The choice of probiotic may be of utmost importance, and results may not be generalizable to all settings and populations.

We had launched this trial with the assumption that in India, our study locale, the predominant cause of neonatal sepsis would be Gram-negative organisms of intestinal origin. This was supported by data from our Indian laboratory, where a high rate of concordance by molecular typing was seen between Gram-negative isolates in blood and stools of the infant (unpublished observations), and by the literature[23]. Supported by *in vitro* and *in vivo* data from our laboratory[24,25], we had further hypothesized that Gram-positive organisms (such as *L. plantarum*) could adhere to gut mucosal cells and effectively block adherence and translocation of Gram-negative bacteria from the intestinal lumen into the bloodstream. While such mechanisms can explain a reduction in Gram-negative sepsis in our population, the mechanisms behind the reduction of Gram-positive sepsis in our treatment group have not yet been fully elucidated.

Our finding of a reduction in LRTIs was completely unexpected, and strongly suggests that host–probiotic interactions enhanced not just local gastrointestinal mucosal but also systemic host immunity. This has already been shown to occur in animal studies, in which enteral probiotic administration increased protection from inflammation in a model of *Pseudomonas aeruginosa* pneumonia, via the increased induction of regulatory T cells[26]. A similar observation was made with the probiotic *Bifidobacterium breve*, which produces polysaccharide A that induces Toll-like receptor 2 (TLR2)-mediated development of regulatory T cell responses[27], while the presence of gut-microbe-derived peptidoglycan increases the killing of both *Streptococcus pneumoniae* and *Staphylococcus aureus* by neutrophils[27]. Of particular interest to our study is the finding that the administration of beneficial microbes to neonatal mice promotes maintenance of neutrophils and other myeloid cells, enhancing both host resistance to sepsis in a TLR4- and myeloid differentiation factor 88 (MyD88)-dependent pathway via IL-17 production in group 3 innate lymphoid cells[28–30]. Lactobacilli in particular have also been shown to promote immune-mediated health. In mouse models, *Lactobacillus pentosus* provides protection against H1N1 influenza infection via the modulation of antiviral gene expression[31], and ameliorates *Streptococcus pneumoniae* LRTI in mice with a significant reduction in the number of pneumococci, increased expression of TLRs, and reduced secretion of inflammatory cytokines and chemokines[32]. These and other reports on the effect of *Lactobacillus* strains on LRTIs suggest a potent immunomodulatory role for probiotic bacteria.

Our study raises several interesting questions in newborn biology. Many years ago, we selected our strain (*L. plantarum*) purely on the basis of its superior ability to colonize the infant gut over the longer term, and in blocking adherence and translocation of Gram-negative bacteria. We are now intrigued to discover a recent report describing a higher prevalence of *L. plantarum* in meconium samples in the first days after birth than in those obtained after the first week of life in Spanish infants[33], suggesting that naturally occurring *L. plantarum* strains may protect the infant during the early weeks of life. This phenomenon may be analogous to the presence of a microbiome comprising nonpathogenic commensal bacteria in the sterile placenta[34]. If indeed these observations have physiological relevance, our study would suggest that such *L. plantarum* strains are not uniformly present in sufficient numbers in all Indian infants to provide protection.

These early life events lead us to believe that the timing of the intervention is probably crucial. There is likely to be a critical perinatal window during which the interaction of the host with the microbiome must occur for optimal immune development, and during which exposure to specific probiotics may enhance immune mediated homeostasis[35]. Colonization of germ-free or antibiotic-treated mice early in life with high doses of probiotics restores systemic immune function, while colonization of adult germ-free or antibiotic-treated mice fails to do so[27,36].

Another interesting observation in our community-based study is the presence of *Streptococcus viridans* sepsis. Although generally not considered to be common, infections due to *S. viridans* in the neonatal intensive care unit setting have been observed for quite some time[37–39]. More recently, after decades of group B *Streptococcus* prophylaxis, a US-based statewide epidemiological study has shown *S. viridans* to be a predominant cause (alongside group B streptococci and *E. coli*), of invasive newborn early-onset sepsis[40]. The presence and the role of *S. viridans* in invasive infant disease remains to be elucidated in India, where group B *Streptococcus* appears to be rare and antibiotics are available without a prescription, and used widely.

In the era of worldwide concern about antimicrobial resistance, probiotics may offer an attractive preventive or adjunct to therapy in treating infections. However, several reports have suggested that probiotics could be a vehicle for transmission of resistance genes. Studies have shown frequent transfer of a native plasmid from *Lactobacillus* to *Enterococcus in vitro*[41] and in the intestines of gnotobiotic rats[42] and streptomycin-treated mice[43]. *Lactobacillus* can also acquire vancomycin A resistance plasmids from *Enterococcus*. We do not have experimental evidence of resistance gene transfers in this study. Interestingly, most lactobacilli, including the current *L. plantarum*, are intrinsically resistant to vancomycin[44] and ciprofloxacin[45] and such intrinsic resistance traits are not transferable in mating experiments[44]. During preclinical investigations (included in the protocol), our chosen strain has shown sensitivity to many common antibiotics except for vancomycin and ciprofloxacin. Strains similar to the one used in this study with no plasmids and their intrinsic resistance may reduce the selective pressure to acquire transferable resistance plasmids from other organisms, thus offering some assurance on safety.

Limitations of our study include the exclusion of 2,506 infants (of 7,089 births in the study area) with major causes of morbidity and mortality in the early neonatal period; hence, we cannot comment on the effect of such synbiotic intervention on the entire population. We did not enrol premature (<35 weeks of gestation) or low birth weight (<2,000 g) infants, who are more prone to lethal sepsis. We also do not know the effect of our synbiotic on the developing gut microbiota. We did not record micronutrient status, vitamin A or D levels,

vaccination status, exposure to suboptimal water sanitation and hygiene, indoor smoke, or maternal body mass index, all of which may impact newborn infections in the region in which we conducted the study. We are unable to determine how the intestinal milieu (such as breast milk, other bacteria, luminal constituents) help or hinder continued colonization by probiotic strains in the newborn period. We have incomplete data on the aetiology of LRTIs in our study population. Blood culture was not performed in these infants because of very low yields of positive cultures (sometimes more contaminants than real isolates), lack of its effect on the adjustment of empirical antibiotic treatment, and concerns about increased length of hospital stay[46,47]. Also, the most recent studies attempting to ascertain the aetiology of infection have examined respiratory secretions (not blood) by culture and PCR in many settings, including developing countries[48]. It was outside the scope of this trial to collect data on possible atypical bacterial (for example, mycoplasmal) or viral origins of LRTI, but future studies on nasopharyngeal and oropharyngeal swabs in conjunction with such trials will help to elucidate the precise nature of probiotic effects on diverse infections of early infancy.

Conclusions

The current trial identifies a single synbiotic preparation as protective in a term or late-preterm population in the developing world, but other populations may require different formulations. Also, in our study cohort, only a small subset of cases identified by paediatricians as pSBI were of bacterial origin. If future studies suggest that they are largely viral, a major paradigm shift in our assumptions of probiotic-mediated protection will be required. This study was performed in a research setting under stringent daily follow-up at home. In the real-life situation, even if appropriate formulations are available, the maintenance of a cold chain and the final distribution to all newborns in a culturally acceptable manner in areas that are not easily accessible will need attention. Finally, although follow-up and Bactec culture data in this large population provides a level of reassurance, additional safety assessments in similar populations and in preterm and low birth weight infants will enable policymakers to launch successful scale-up programs.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions P.P. and I.G. conceived the study and P.P. wrote the first draft. P.P., S.N.P., I.G., N.P. and J.G.M. designed the study with help from the other authors. N.N., R.S., L.P. and A.M. were responsible for clinical operations. S.S.M. managed the field activities. J.J. and R.C. were in charge of developing microbiology protocols and standard operating procedures. R.C. was in charge of the reference laboratory and supervised the final strain designation of blood isolates. H.C. and L.B. conducted the data management and statistical analyses. D.C. managed the protocol from the principal investigator's laboratory and handled data acquisition, microbiology quality assurance, and institutional review board matters. All authors contributed in writing different sections of the manuscripts.

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METHODS

The study was launched in October 2008 and closed in May 2012. From four revenue blocks in two districts, 149 villages were randomly selected with a total population of 164,778 (Fig. 1). All pregnancies were registered and live-born infants in the study villages were eligible for randomization.

Primary outcome. A composite of sepsis or death, the former composed of septicaemia, meningitis, culture-negative sepsis, and LRTI.

Secondary outcomes. Other infections (including diarrhoea, omphalitis, local infections, abscess, and otitis media) and weight gain.

We followed the WHO/UNICEF guidelines[49] for neonatal sepsis, also referred to as pSBI. Categorization into culture-positive, culture-negative, and LRTI was done post-hoc, whereas all infants in these three groups were identified and enrolled as having 'clinical sepsis' on the basis of WHO criteria for pSBI. Apart from the signs in the WHO clinical algorithm, we collected additional clinical auscultatory signs recorded by the paediatrician at the hospital. WHO definitions were also used for omphalitis, abscess, acute otitis media, pustules, and diarrhoea. These infections were not included in the primary outcome, but recorded as secondary outcomes.

Septicaemia, meningitis or sepsis-pSBI. Positive for bacterial or fungal culture of blood or cerebrospinal fluid, or culture-positive sepsis.

Culture-negative sepsis or sepsis-pSBI. Presence of any of the following signs warranting hospitalization and use of antibiotics for five or more days (based on the decision of the paediatrician). Refusal to feed; lethargy (absent movement, or movement with stimulation only); respiratory rate greater than 60 breaths per minute, nasal flaring; axillary temperature $\geq$37.5 °C/99.5 °F; axillary temperature $\leq$35.5 °C/95.9 °F; convulsions; severe lower chest wall in-drawing (retractions).

LRTI (including pneumonia) or sepsis-pSBI. Presence of expiratory grunting, reduced breath sounds, uneven breath sounds, or crackles on auscultation by the paediatrician in conjunction with any of the above-mentioned signs categorized the infants under LRTI.

For analysis, infants were put into the above three categories post-hoc. There was no overlap between the three groups and they were mutually exclusive.

Infants were followed daily at home for signs or symptoms of pSBI. Although the study protocol called for a 60-day follow-up, in order to comply with new local institutional review board (IRB) stipulations, a laborious case-finding protocol was instituted to cover 18 months of life of the enrolled infants, using the help of local managers and supervisors.

Eligibility criteria. Inclusion criteria included: neonate >24 h and <96 h old, $\geq$2,000 g at birth, breastfeeding begun by 24 h of life, ability to tolerate oral feeds, informed consent by parent or guardian.

Exclusion criteria were: evidence or suspicion of clinical sepsis before the infant was randomized, gestational age reported voluntarily by the mother to be <35 weeks, infant >96 h old, infant did not cry immediately after birth, mother had fever (>38 °C) within 2 days of delivery, mother had foul-smelling amniotic discharge within 2 days of delivery, mother had abdominal tenderness within 2 days of delivery, amniotic fluid was meconium-stained, infant was on antibiotics, mother unlikely to stay in the village for 60 days, difficulty in carrying out study (maternal sickness etc.), or presence of major congenital anomalies (defined as any malformation that was felt to be life-threatening or that required surgical intervention).

Study design, randomization and masking. Newborns were individually randomized within each of the 149 villages. Since we anticipated enrolling over 8,400 subjects, we generated 152 consecutively numbered trial-arm assignments for each village to ensure sufficient assignments. Each assignment was the product of a random permutation scheme that assigned 2 intervention and 2 placebo slots to each of 38 consecutive blocks of 4 assignments for each village. This numbered list and corresponding bar codes were created by the GCRC (General Clinical Research Center) at the University of Maryland with assistance from the Department of Bioinformatics and given to the clinical trial supplier (Laxai USA, South Plainfield, New Jersey, USA) for labelling of the synbiotics and to prepare packages for each village to be assigned consecutively to enrolled subjects. Neonates from multiple births were randomized individually. Clinical trial material was stored at −20 °C at study hospitals, and distributed in a thermo-cool box with coolant packs every week to the field offices where they were stored in a fridge. Staff, investigators and parents of participants were blinded to treatment allocation. The clinical trial supplier's representatives distributed the labelled boxes (trial material) to the field where they were stored under refrigeration until randomization and initiation of oral administration to an average of 1–2 infants per month per village. The latter was done by field supervisors and CHVs. During the follow-up in the village, all infants with suspected cases of sepsis and other major morbidities were referred to the hospital. The paediatricians at the study hospitals received these patients from hundreds of villages and made the final pSBI criteria-based judgement on enrolment of infants into the study during their first 60 days of post-natal life. The physicians had no access to randomization, distribution, or administration of the intervention making them completely blinded to the intervention.

Clinical trial material. The synbiotic preparation consisted of a capsule containing $\sim 10^9$ *Lactobacillus plantarum* ATCC strain 202195 and 150 mg of fructooligosaccharide with 100 mg maltodextrin as excipient. Placebo capsules contained only 250 mg of maltodextrin. In the powder form or when reconstituted, the synbiotic and placebo preparations looked identical in colour and turbidity owing to the high maltodextrin content in both types of capsule. *Lactobacillus* species was confirmed by sequencing and genetic matching with Applied Biosystems Microseq sequence database at Silliker Laboratories (South Holland, Illinois, USA). Laxai USA handled all aspects of contract manufacturing, labelling, quality control during batch release, and maintenance of cold chain by shipment with coolant packs in India. Clinical trial materials (including placebo) was prepared by Indo Medix, India. Trial packages contained ten capsules of placebo/synbiotics, ten vials of dextrose saline, individually packed, sterile mixing containers, and syringes with needles, providing three extra doses to allow for accidental spillage or immediate vomiting by the infant. Colony counts of this preparation after exposure to defined temperatures revealed minimal loss in viability over four weeks at 37 °C and further drastic drop by tenth week. The *L. plantarum* strain was susceptible to penicillin, amikacin, cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, and gentamicin by disc diffusion method, showed resistance to vancomycin and ciprofloxacin, and did not harbour any plasmid.

Study management and procedures. The trial was built on an existing field research network. At the first level, a trained female CHV was assigned to each village and worked closely with a village level government worker, the Anganwadi worker, whose responsibilities include recording pregnancies, providing prenatal care, and preschool education. At the second level, each group of 8–10 CHVs had a mid-level field manager. At the third level, field work was monitored by 4–5 field supervisors reporting to a senior program officer in each revenue block (Extended Data Fig. 2). Study personnel, including field staff, had experience in conducting community-based research in neonatal health[50].

Written informed consent was obtained during the third trimester of pregnancy by the CHV. She identified the participating newborn after birth, collected maternal and newborn demographic data and prepared and administered synbiotics/placebo in the infant's home under the supervision of the field supervisor (who supplied the clinical trial material from the field office) on the first day of administration. After this, the CHV administered the remaining six daily doses (second to seventh dose). Infants were breastfed and burped before receiving the dose, and watched for 30 min by the CHV. In the case of vomiting, the dose was repeated an hour later, and the child was evaluated at home by a study physician before the next dosing. Infants were followed daily by the CHV until 60 days of age. CHVs and mid-level managers recorded signs or symptoms suggestive of sepsis daily following the pSBI criteria. In the event of suspect sepsis, infants were referred to study hospitals by field staff. Dedicated operators entered data into a web-based system. Data transmission to the University of Nebraska Medical Center server was done after data lock.

Along with focus group meetings, women of all ages (including mothers-in-law and grandmothers) were shown a documentary (available at: https://www.youtube.com/watch?v=73m9r1qgakw; https://www.youtube.com/watch?v=qofsiPjokAo) describing signs and symptoms of sepsis and the procedures to be followed when sick infants were transported to study hospitals. CHVs evaluated infants daily for 60 days for any of the 7 signs of pSBI, and all infants with suspected sepsis were transported to study hospitals for assessment by full-time study paediatricians, blood/cerebrospinal fluid collection, and clinical care. Care was taken not to change clinical practice by attending physicians, and no new procedures or interventions (except Bactec blood culture) were introduced during the study period.

Serious adverse events were defined as death, life-threatening illness, clinical sepsis, medical/surgical care to prevent permanent impairment or damage, persistent or significant disability or incapacity, any event considered serious by the paediatrician, and/or hospitalization for any cause. Adverse events were other non-serious complaints by parents and field staff for which the infants were evaluated by paediatricians and treated symptomatically on an outpatient basis without antibiotics. Serious adverse events were reported to the principal investigator via email or fax within 48 h, and adverse events within seven days. Failure to appropriately ascertain eligibility, late transmission of forms, wrong study ID sticker on forms, wrong recording of time, and/or failure to obtain appropriate samples for culture were regarded as protocol violations and reported within 48 h. The study protocol was approved by the University of Nebraska Medical Center IRB and local Indian IRB (Ethical Review Committee of Government of Odisha, Department of Health and Family Welfare). Further external scientific review was conducted by the Indian Council of Medical Research and approval obtained, followed by a final clearance by the Health Ministry Screening Committee of India.

The Drugs Controller General of India approved importation of the synbiotic preparation for the current clinical trial.

Microbiology. Reagents, chemicals and culture bottles were procured from BD–India (Becton Dickinson, Gurgaon, India). Sub-cultures were done following standard procedures. Bacterial species identification used conventional biochemical tests and confirmed by API strips. After identification of bacterial isolates, single colonies were stored at −80 °C in freezing medium at the two study hospitals. Each isolate was shipped on dry ice to the reference laboratory (All India Institute of Medical Sciences, New Delhi), for repeat analysis before final species designation was assigned. Micrococcus, Bacillus, Diptheroids, and coagulase-negative Staphylococci (CoNS) were considered contaminants.

Protocol violations. Any procedure not carried out properly, or any event not reported within the specified time period, constituted a protocol violation for which a separate form needed to be completed and entered into the database. Enrolling an ineligible infant, enrolling an infant out of the specified age window, a serious adverse event not reported within 48 h, an adverse event not reported within 7 days, wrong study ID in some of the study forms (incorrect barcode stickers on hospital or laboratory forms), and delayed intimation of congenital anomalies in the field were examples of violations reported in this study. If violations were consistently made by any study personnel, local re-training was performed.

Sample size estimation and statistical analysis. With a planned enrolment of 8,442 infants, the study was powered to detect, at two-sided $\alpha = 0.05$ and $\beta = 0.20$, a 20% reduction in an estimated cumulative incidence rate of 8% for the combined outcome of sepsis and death in the placebo arm. We compared the primary and secondary outcomes between the two groups by chi-square or Fisher's exact tests. Risk ratio (RR) and number needed to treat (NNT) to prevent one case of the primary outcome 95% confidence intervals were calculated using SAS version 9.4 (SAS Institute Inc.).

Data safety monitoring. The data safety and monitoring board (DSMB) for this study included experts in neonatology, probiotics, clinical trials, and biostatistics. Although the protocol was designed to enrol infants up to three days of life, during a field logistics feasibility piloting it was noted that a sizable proportion of mothers were unable to reach home by the third day after delivery in hospitals and auxiliary-nurse-midwife centres. Upon request by investigators and concurrence of IRBs, the DSMB reviewed this change and approved enrolment up to four days of life. This change was recommended by the DSMB at the first pre-enrolment meeting, approved by the IRBs and instituted before enrolment of the first infant in the current trial. At this pre-enrolment meeting, the DSMB prescribed yearly interim analyses and to meet in person every year to review patient enrolment, adverse events, and primary and secondary outcomes in a blinded fashion. Four meetings were held and at the fourth meeting, the DSMB unblinded the data after enrolment of 4,556 infants. The consensus among members was that the study had reached a significant endpoint using O'Brien–Fleming rules. The DSMB advised that recruitment of additional subjects would be highly unlikely to, statistically, reverse the findings, and hence advised the trial to be terminated early. This trial was registered at clinicaltrials.gov with the registration numbers NCT01214473 and NCT00518596. All primary and secondary outcomes were specified before enrolment of the first subject in this study. The Supplementary Information section contains additional details on the timing of the two trial registrations and further details on the NIH U0-1 and R0-1 funding that enabled the execution of the current clinical trial.

Role of the funding source. The current community-based trial under the R01 umbrella (HD R01 53719) was built on the U01 (HD UO1 40574) platform but managed independently by study investigators. The Eunice Kennedy Shriver National Institute of Child Health and Human Development had no role in study design, data collection, data analysis, interpretation, or writing of the report. The corresponding author had full access to all data and final responsibility for the decision to submit the manuscript for publication.

Data availability. The full study protocol and the datasets generated during and/or analysed during the current study have been deposited with Dryad digital repository and are publicly available at http://dx.doi.org/10.5061/dryad.275d4. The consort checklist for this trial is included in the Supplementary Information.

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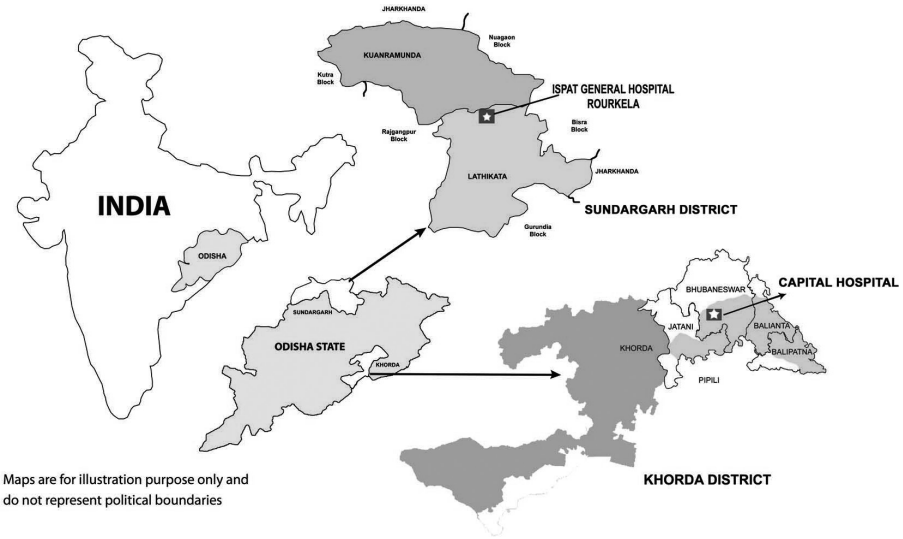
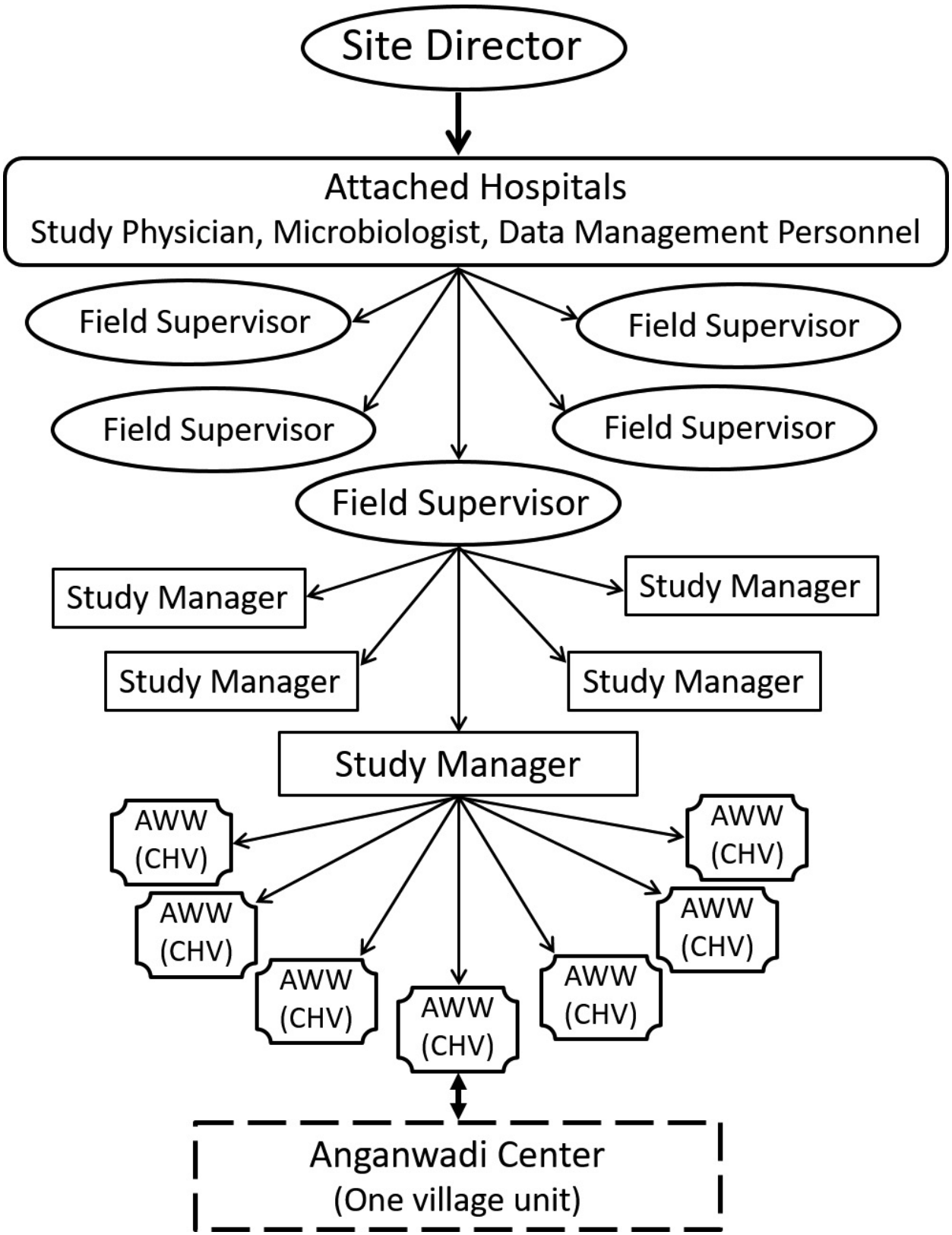


Extended Data Figure 1 | Site maps. Location of study sites in Odisha, India. Source: India and Odisha state maps, redrawn to show the geographic location of study sites. Reproduced with permission from ref. 51.



Extended Data Figure 2 | Site structure. Three-tier structure for field operations and implementation of the clinical trial in the community. Reproduced with permission from ref. 51.

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Experimental design

1. Sample size

Describe how sample size was determined.

Considering the neonatal sepsis rate to be 8%, we planned an enrollment of 8,442 infants. This was powered to detect, at two-sided α=0.05 and β=0.20, a 20% reduction in an estimated cumulative incidence rate (8%) for the combined primary outcome of sepsis and death in the placebo arm.

2. Data exclusions

Describe any data exclusions.

No data were excluded. An intent to treat analysis was done.

3. Replication

Describe whether the experimental findings were reliably reproduced.

N/A. This was a clinical trial not involving experiments that need replication.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

All eligible infants born in the 149 villages were individually randomized to either the placebo or the synbiotic group.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

This was a double blind study. Field personnel administering the synbiotic to infants, caregivers in the hospitals treating sick infants, and investigators were blinded to allocation.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☒	☐	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☒	☐	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	SAS 9.4

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	N/A. This clinical trial is now complete and closed.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	No antibodies were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	No eukaryotic cell lines were used.
b. Describe the method of cell line authentication used.	No eukaryotic cell lines were used.
c. Report whether the cell lines were tested for mycoplasma contamination.	No eukaryotic cell lines were used.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No eukaryotic cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	Male and female infants, born in eastern part of India in rural areas, were enrolled during 1-4 days of life and followed for sign/symptoms of sepsis until they were 60 days-old.