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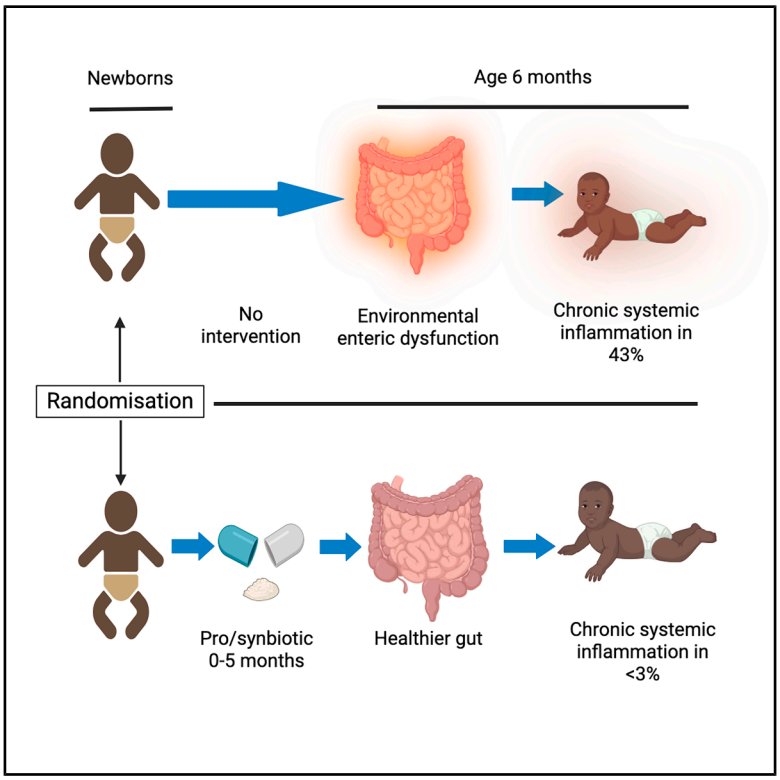
Pro/synbiotics reduce systemic inflammation and improve gut health in Kenyan infants: An open label, randomized, 4-arm, phase II trial

Graphical abstract



Highlights

- Live *Bifidobacterium* spp. and *Lactobacillaceae* from age 0–6 months markedly reduce CSI
- Pro/synbiotics improve gut health and growth hormones
- Beneficial effects persist to age 12 months
- Adverse events are similar in intervention and control infants

Authors

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In brief

In this open-label, randomized trial in 600 vulnerable infants in western Kenya, Otiti et al. find that chronic systemic inflammation occurs at age 6 months in 43% controls but in <3% of infants receiving one of 3 different multi-strain, live pro/synbiotics from 0 to 6 months. Gut health and growth hormones are also improved.

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Pro/synbiotics reduce systemic inflammation and improve gut health in Kenyan infants: An open label, randomized, 4-arm, phase II trial

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SUMMARY

Environmental enteric dysfunction, universal in young children exposed to poor sanitation and hygiene, impairs growth and development through malabsorption and as a driver of chronic systemic inflammation (CSI). In an open-label, randomized, four-arm, phase II clinical trial, infants with birthweight ≥2000 g in Homa Bay County, western Kenya receive live, multi-strain *Bifidobacterium* spp. and *Lactobacillaceae* pro/synbiotics from 0 to 6 months. CSI (plasma α_1-acid glycoprotein >1 g/L) at age 6 months (primary outcome) occurs in 60/138 (43%) controls versus 4/144 (3%; risk ratio [RR], 0.06; 95% confidence interval [CI], 0.02–0.17) infants in the Labinic synbiotic arm, 3/132 (2%; RR = 0.05; 95% CI, 0.02–0.16) in the Lab4b synbiotic arm, and 3/141 (2%; RR, 0.05; 95% CI, 0.02–0.15) in the Lab4b probiotic arm. Biomarkers of gut health and growth hormones also improve, and no serious adverse events are attributed to the interventions. Pro/synbiotics safely and markedly reduce CSI in a highly disadvantaged population, warranting further investigation of health impacts. The trial is registered at https://pactr.samrc.ac.za; identifier: PACTR202003893276712.

INTRODUCTION

Vulnerable infants in low- and middle-income countries (LMICs) are at risk of stunting, which affects about 1 in 2 children in Asia and 2 in 5 children in Africa.[1] Stunting is characterized by systemic inflammation,[2–6] with chronic systemic inflammation (CSI) contributing to stunting through growth hormone resistance[7,8] and, beyond growth, impaired organ development,[9] including cognitive development[9–11] and increased risk of longer-term non-communicable diseases (NCDs).[12]

Environmental enteric dysfunction (EED), an asymptomatic disorder manifesting as growth faltering, is characterized by small intestinal inflammation, villous atrophy, and increased mucosal permeability. EED impairs childhood growth and development through malabsorption and as a major driver of CSI.[2,13] EED results from enteropathogen colonization, and both EED and CSI occur as early as age 6–12 weeks of age, even in exclusively breast-fed infants.[3,6,14] Since the highest incidence of stunting onset occurs between 0 and 3 months, with few children catching up later in childhood, interventions are required during the first 6 months before complementary feeding starts.[15]

In breast-fed infants, prebiotic human milk oligosaccharides result in a dominance of *Bifidobacterium* in the gut, which drives microbial community assembly and has a key role in colonization resistance against enteropathogens, as well as in the development of gut, mucosal, and systemic immunity.[16–19] However, multiple adverse environmental factors, such as poor sanitation and hygiene, caesarean section delivery, faulty feeding practices, and antibiotic exposure,[17] result in "dysbiosis" (used here to mean a perturbation or delay in the development of the gut microbiota), which may compromise these functions[17,18] and occurs in childhood malnutrition.[20,21]

We aimed to assess whether administration of pro/synbiotics from 0 to 6 months reduced CSI through improved gut health compared with no intervention. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host."[22] A prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a

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health benefit."[23] A synbiotic is "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confer a health benefit on the host".[24] Pro/synbiotics may boost colonization resistance against enteropathogens, enhance gut development, and provide immune benefits.[25,26] In view of strain-specific effects of probiotic organisms[22] and the possible benefits of synbiotics versus probiotics,[24] we compared the effects of two different multi-strain synbiotics (Lab4B synbiotic and Labinic synbiotic) and a probiotic consortium without a prebiotic (Lab4B probiotic) versus no intervention, resulting in a 4-arm trial.

RESULTS

Study participants

Of 816 newborns screened between October 28, 2020, and January 13, 2022, 600 were enrolled, with 150 infants randomized to receive the Labinic synbiotic, 151 the Lab4b synbiotic, 150 the Lab4b probiotic, and 149 controls (Figure 1). Exclusions were due to parents or carers planning to leave the study area (N = 82), declining to participate (N = 79), or health staff concerns regarding the infant's health (N = 55). Baseline demographic and clinical variables for mothers and newborns were similar across study arms (Table 1). Most participants (64%) resided in rural parts of Homa Bay County. Most (78%) mothers were married, and 18% were HIV infected. Nearly all women had a vaginal delivery (96%) and delivered in hospital (91%). Overall, 7% of infants were low birthweight (<2500 g). Water, sanitation, and hygiene (WASH) practices were also similar across study arms and reflected a high level of disadvantage (Table S1).

Follow-up at 6 months was completed in 559 (93%) infants and was 89% or greater in each study arm (Figure 1). In total, 39 (7%) children withdrew, with similar frequencies and reasons for withdrawal across study arms (Table S2). Follow-up at 24 months was completed in January 2024 and included 546 (91%) of the children enrolled (Figure 1).

The number of infants receiving all 32 doses of pro/synbiotics was 81% or greater in each intervention arm, and 126 (85%) control infants completed all 32 visits (Table S3). Overall, 484/565 (86%) infants were exclusively breastfed at 3 months and 220/559 (39%) at 6 months, with similar frequencies across study arms (Table S4).

Pro/synbiotics markedly reduced CSI at age 6 months (primary outcome) and also at 3 and 12 months

At 6 months, the frequency of CSI (assessed by plasma α_1-acid glycoprotein (AGP) >1 g/L; primary outcome) was 60/138 (44%) in controls compared with 4/144 (3%; risk ratio [RR], 0.06; 95% confidence interval [CI], 0.02–0.17) infants in the Labinic synbiotic arm, 3/132 (2%; RR, 0.05; 95% CI, 0.02–0.16) in the Lab4b synbiotic arm and 3/141 (2%; RR, 0.05; 95% CI, 0.02–0.15) in the Lab4b arm. Results were similar in the adjusted analysis accounting for potential confounders (Figure 2). Similar differences in the frequency of CSI at age 6 months between intervention arms and controls were observed in sub-group analyses (Table S5), and also in the per-protocol (PP) population (Figure S1).

At age 6 weeks, no infants had CSI (Table 2; Figure S2). The frequency of CSI was markedly reduced in the intervention versus control arm at 3 and 12 months (Figure 2). Geometric mean AGP concentrations increased in all study arms after 6 weeks but to a greater extent in the control arm (Table 2).

Pro/synbiotics reduced acute systemic inflammation

Increased acute inflammation (plasma C-reactive protein (CRP) > 0.45 mg/dL) was observed in half or more of the infants in all arms and at all time points. Geometric mean CRP was significantly higher in controls versus each intervention arm at 3 and 12 months and in the Lab4b probiotic arm at 6 months (Table 2).

Pro/synbiotics reduced biomarkers of EED after 6 weeks

At 6 weeks, intestinal inflammation (fecal myeloperoxidase [MPO] concentration $\geq$0.2 mg/dL) was evident in one-third or more of infants in all study arms, and the geometric mean was significantly higher in the Labinic synbiotic arm than in controls. Levels rose after 6 weeks in all study arms but were significantly lower in all three intervention arms than the control arm at 6 months. Levels had increased markedly by 12 months and were similar across study arms (Table 2).

Intestinal permeability was increased (fecal α_1-antitrypsin [AAT] $\geq$26.8 mg/dL) in up to 1 in 4 infants and was similar across study arms at 6 weeks and 3 months. Geometric means were significantly lower in each intervention arm than controls at 6 months. Levels increased further at 12 months, with 1 in 2 or more infants in all study arms having raised levels and with similar levels across study arms (Table 2).

The concentration of plasma intestinal fatty acid binding protein (IFABP), used to assess mucosal integrity, was below the manufacturer's upper limit of normal (1.8 ng/mL) in the majority of infants in all study arms at all time points. Geometric means were significantly lower in each intervention arm than controls at 6 months (Table 2).

Stools tended to be more acidic in all study arms at 6 weeks (median pH 5.04–5.23), with pH rising gradually to 12 months (pH 5.47–5.68). Geometric mean stool pH was significantly lower at 6 weeks in the Lab4b synbiotic arm than in controls but was otherwise similar across study arms and time points (Table 2).

Pro/synbiotics increased growth hormone concentrations

Plasma insulin-like growth factor (IGF)-1 concentrations were within the normal range in most infants at most time points (Table 2; Figure S4A). Geometric mean IGF-1 was significantly higher in all 3 intervention arms versus controls at 6 months (Table 2). Compared with controls, IGFBP-3 concentrations were significantly higher in the Labinic synbiotic and Lab4b probiotic arms at 3 months and in all 3 intervention arms at 6 months (Table 2).

Biomarker values are also reported in boxplots to illustrate distributions according to study arm and time point, as well as normal ranges (Figures S2, S3, and S4).

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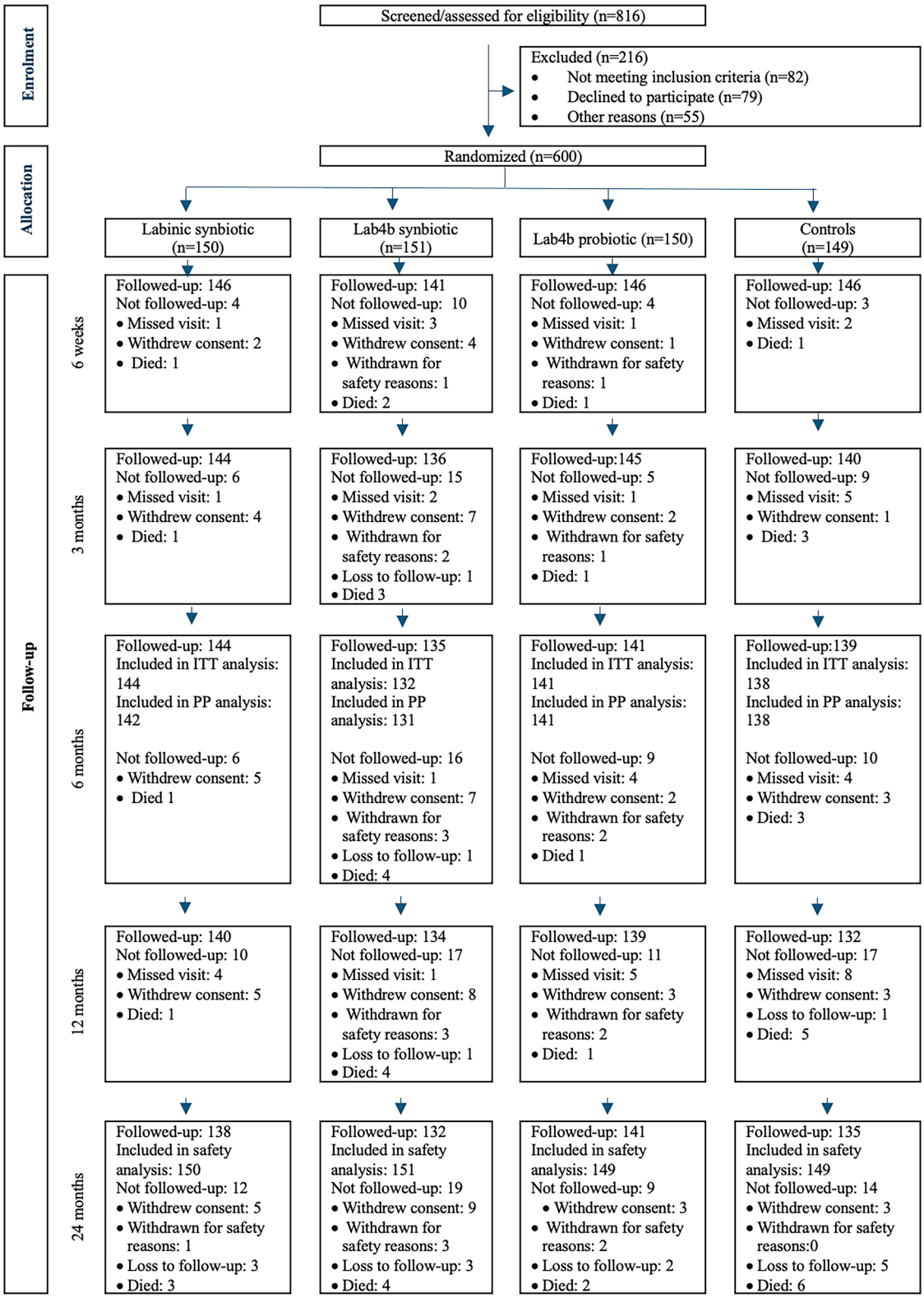

Figure 1. Trial profile

Numbers at follow-up time points include children who attended even if they missed previous follow-ups. Numbers of children withdrawn and who died at each time point are cumulative. See also Tables S2, S3, and S4

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Table 1. Baseline maternal, delivery, and infant demographic and clinical characteristics were similar across the trial arms

Variable	Labinic synbiotic *N* = 150	Lab4b synbiotic *N* = 151	Lab4b probiotic *N* = 150	Control *N* = 149
Residence type[a]				
● Rural	92 (61%)	97 (65%)	97 (65%)	97 (65%)
● Peri-urban	44 (29%)	44 (29%)	42 (28%)	43 (29%)
● Urban	14 (9%)	9 (6%)	11 (7%)	9 (6%)
Water, sanitation, and hygiene				
● Drinking water	79 (53%)	90 (60%)	83 (55%)	88 (59%)
○protected				
○unprotected	71 (47%)	60 (40%)	67 (45%)	61 (41%)
● Household lavatory	–	–	–	–
○improved	103 (69%)	106 (71%)	106 (71%)	106 (71%)
○unimproved	47 (31%)	44 (29%)	44 (29%)	43 (29%)
● Disposal of child stools	–	–	–	–
○with garbage	137 (91%)	135 (90%)	134 (89%)	136 (91%)
○other	13 (9%)	15 (10%)	16 (11%)	13 (9%)
Mother				
● Age (yrs)	26 (6.3)	26 (6.7)	25 (5.7)	25 (6.4)
● Height (cm)	161 (6.1)	162 (5.4)	161 (4.9)	161 (6.0)
● Weight (kg)	66 (10.2)	66 (14.9)	65 (9.9)	66 (12.7)
● Marital status	–	–	–	–
○Single	29 (19%)	32 (21%)	29 (19%)	30 (20%)
○Married	119 (79%)	114 (75%)	115 (77%)	118 (79%)
○Separated/divorced	2 (1%)	3 (2%)	5 (3%)	1 (1%)
○Widowed	0 (0%)	2 (1%)	1 (1%)	0 (0%)
● Previous number of live births	1.8 (1.76)	1.8 (1.79)	1.5 (1.50)	1.6 (1.68)
● HIV positive	27 (18%)	27 (18%)	27 (18%)	26 (17%)
● Well during pregnancy	135 (90%)	126 (83%)	132 (88%)	136 (91%)
● Antibiotics in 7 days before delivery	3 (2%)	4 (3%)	1 (1%)	2 (1%)
Delivery				
● Rainy season[b]	79 (53%)	82 (54%)	79 (53%)	83 (56%)
● Place	–	–	–	–
○Hospital	140 (93%)	133 (88%)	138 (92%)	136 (91%)
○Health Center	1 (1%)	7 (5%)	5 (3%)	9 (6%)
○Home	8 (5%)	10 (7%)	5 (3%)	4 (3%)
○On the way to facility	1 (1%)	1 (1%)	2 (1%)	0/(0%)
● Mode	–	–	–	–
○Spontaneous vaginal	144 (96%)	148 (98%)	142 (95%)	139 (93%)
○Cesarean Section	6 (4%)	3 (2%)	8 (5%)	10 (7%)
● Complications	15 (10%)	24 (16%)	29 (19%)	15 (10%)
● Delayed cord clamping	137 (91%)	140 (93%)	133 (89%)	133 (89%)
Infant				
● Male sex	68 (45%)	71 (47%)	74 (49%)	70 (47%)
● Gestational age by Ballard score (weeks)	38 (2.0)	38 (1.9)	38 (1.6)	38 (1.8)
● Birth weight (kg)	3.1 (0.40)	3.1 (0.46)	3.1 (0.41)	3.1 (0.41)
● Low birth weight (<2500g)	6 (4%)	14 (9%)	8 (5%)	13 (9%)
● Time of first feed (hours)	1.4 (0.77)	1.5 (0.88)	1.6 (1.21)	1.7 (1.96)

https://climateknowledgeportal.worldbank.org/country/kenya/climate-data-historical. Data are *n* (%) or mean (standard deviation). See also Table S1.

[a]Residence type was missing for one family in the Lab4b synbiotic arm.

[b]Rainy season: Oct-–Dec and March–May; dry season: Jan–Feb and June–Sept.

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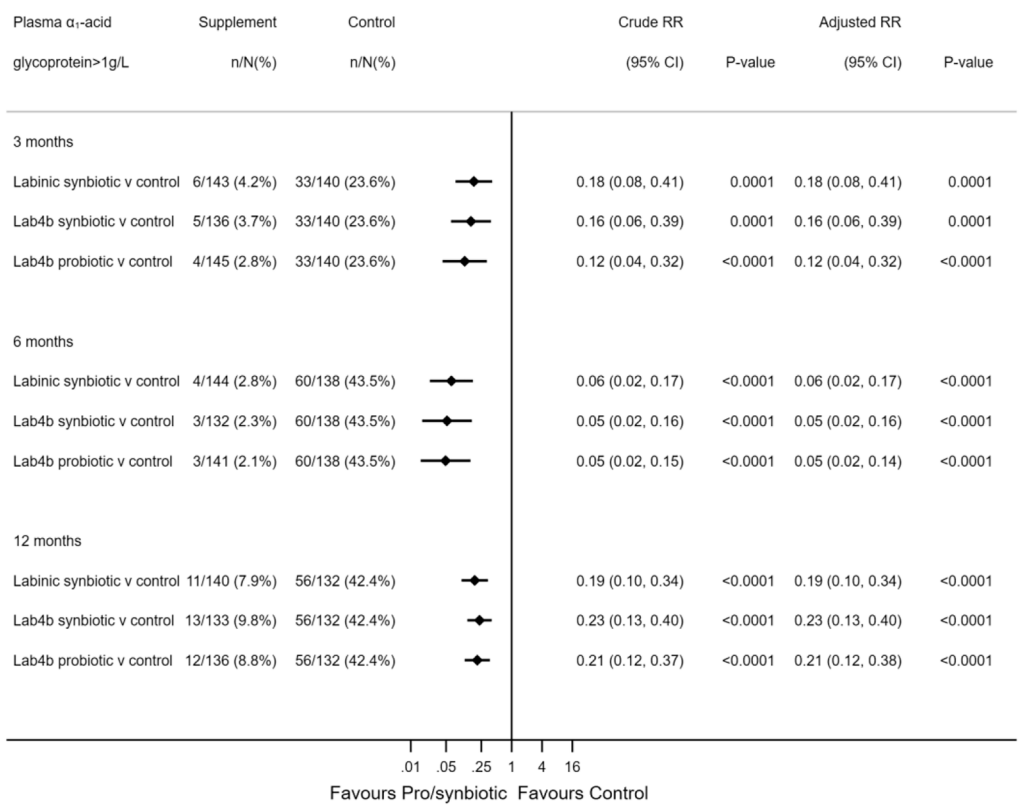

Figure 2. Forest plot shows markedly reduced relative risk of raised AGP in intervention arms vs. controls in the ITT population

In crude analysis, treatment was the only predictor. Adjusted analysis accounted for season of birth (dry vs. rainy), HIV exposure, mode of delivery (vaginal vs. cesarean section), sex, low birth weight, urban or semi-urban vs. rural residence, drinking water source (protected vs. unprotected), household lavatory (improved vs. unimproved), disposal of child stools (with garbage vs. other), and previous number of live births as covariates. Maternal antibiotic exposure was not included because raised AGP did not occur in infants of the few mothers exposed to antibiotics prior to delivery. Mode of feeding was not included because, consistent with the inclusion criteria, all infants were initially breast-fed. The 6-week time point is not reported as all infants had AGP within the normal range. All samples were tested in duplicate, and control samples were included. ITT, intention to treat; RR, risk ratio; CI, confidence interval; n/N, number with raised plasma AGP/total number in whom plasma AGP was measured in each study arm and time point; the error bars show the 95% CI for the crude risk ratio. See also Table S5 and Figure S1.

Pro/synbiotics were not associated with adverse events

Over 0–24 months, 102 serious adverse events (SAEs) occurred in 88 (15%) children (Table 3). Clinicians made more than one diagnosis for many SAEs, with a total of 151 diagnoses; the most common diagnoses were pneumonia (31; 20%), malaria (20; 13%), and gastroenteritis (16; 11%). Overall, 17 (3%) children died. The number of SAEs, diagnoses, and deaths were similar across study arms, and none were attributed to the interventions (Table 3; Table S6).

DISCUSSION

Administration in early life of live, multistrain *Bifidobacterium* spp. and *Lactobacillaceae* pro/synbiotics markedly reduced CSI in highly disadvantaged infants exposed to poor sanitation and hygiene and was not associated with adverse effects. The magnitude of differences in AGP concentrations between the control arm and intervention arms from age 3 months (Table 2) is likely clinically meaningful, as they are greater than those between non-stunted and stunted children at similar ages in Zimbabwe (mean differences ranging from 0.06–0.13 g/L)[6] and between non-stunted and moderately stunted (difference in medians 0.10 g/L) and severely stunted (0.11 g/L) 6-month-old infants in rural Uganda.[27]

There are multiple adverse effects of CSI in infancy. Increased plasma AGP was significantly associated with impaired linear growth in Pakistan,[4,28] Uganda,[27] Tanzania,[29] and the multicountry MAL-ED[30] and BRINDA[5] studies, with reduced plasma IGF-1 in studies in Pakistan,[28] Mali,[31] Malawi,[32] and Zimbabwe[6] and with reduced plasma IGFBP-3, an indicator of adequate growth hormone signaling,[8] in children in Zimbabwe.[6] Our findings suggest that pro/synbiotics may be an appropriate intervention for growth hormone resistance in undernutrition.[7,8] Systemic inflammation during the first year of life also impairs organ

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Table 2. Biomarkers of systemic inflammation, EED, and growth hormones were improved in the intervention arms in the ITT population

Study arm[a]					Adjusted geometric mean ratio (95%CI) and *p* value[b]		
Visit	**Labinic synbiotic**	**Lab4b synbiotic**	**Lab4b probiotic**	**Control**	**Labinic synbiotic vs. control**	**Lab4b synbiotic vs. control**	**Lab4b probiotic vs. control**
Plasma AGP (g/L)							
6 weeks	145 0.26 (0.48)	141 0.26 (0.51)	146 0.25 (0.49)	146 0.28 (0.43)	0.95 (0.83,1.09) 0.46	0.91 (0.79,1.05) 0.19	0.90 (0.78,1.03) 0.12
3 months	143 0.36 (0.79)	136 0.32 (0.79)	144 0.31 (0.71)	140 0.61 (0.64)	0.58 (0.51,0.67) <0.0001	0.52 (0.45,0.59) <0.0001	0.52 (0.45,0.60) <0.0001
6 months	144 0.36 (0.86)	132 0.31 (0.89)	140 0.31 (0.71)	138 0.73 (0.70)	0.49 (0.42,0.56) <0.0001	0.42 (0.37,0.49) <0.0001	0.43 (0.37,0.49) <0.0001
12 months	140 0.36 (0.60)	133 0.37 (0.58)	135 0.36 (0.54)	132 0.60 (0.76)	0.62 (0.53,0.71) <0.0001	0.62 (0.54,0.72) <0.0001	0.62 (0.54,0.72) <0.0001
Plasma CRP (mg/dL)							
6 weeks	146 0.48 (1.28)	141 0.41 (1.33)	145 0.44 (1.06)	145 0.51 (1.43)	0.91 (0.73,1.15) 0.44	0.79 (0.63,1.00) 0.051	0.86 (0.68,1.08) 0.18
3 months	142 0.63 (1.41)	136 0.59 (1.23)	144 0.55 (1.27)	139 1.14 (1.28)	0.54 (0.43,0.68) <0.0001	0.51 (0.40,0.65) <0.0001	0.48 (0.38,0.60) <0.0001
6 months	144 0.64 (1.39)	132 0.50 (1.56)	140 0.48 (1.20)	138 0.63 (1.18)	1.00 (0.79,1.26) 0.98	0.80 (0.63,1.02) 0.070	0.77 (0.61,0.97) 0.026
12 months	140 0.55 (1.13)	133 0.56 (1.42)	138 0.52 (1.15)	132 0.82 (1.25)	0.65 (0.51,0.82) 0.0003	0.66 (0.52,0.84) 0.0007	0.62 (0.49,0.79) <0.0001
Fecal MPO (mg/dL)							
6 weeks	144 0.19 (0.79)	139 0.18 (0.73)	142 0.18 (0.67)	143 0.15 (0.70)	1.28 (1.08,1.52) 0.0039	1.19 (1.00,1.41) 0.050	1.17 (0.99,1.39) 0.070
3 months	143 0.23 (0.99)	136 0.24 (0.95)	140 0.22 (0.82)	136 0.26 (0.97)	0.88 (0.74,1.04) 0.14	0.89 (0.75,1.06) 0.20	0.84 (0.71,1.00) 0.046
6 months	144 0.22 (0.93)	133 0.24 (0.82)	139 0.22 (0.86)	130 0.31 (0.89)	0.71 (0.60,0.84) <0.0001	0.77 (0.65,0.92) 0.0043	0.71 (0.60,0.85) 0.0001
12 months	140 0.62 (0.76)	132 0.62 (0.93)	132 0.56 (0.78)	131 0.65 (0.76)	0.96 (0.80,1.14) 0.60	0.94 (0.79,1.12) 0.49	0.86 (0.72,1.02) 0.087
Fecal AAT (mg/L)							
6 weeks	147 17.71 (0.67)	141 16.41 (0.81)	144 17.15 (0.74)	143 16.95 (0.79)	1.03 (0.89,1.20) 0.65	0.96 (0.83,1.11) 0.59	1.02 (0.88,1.18) 0.81
3 months	144 16.57 (0.80)	136 17.06 (0.61)	141 16.52 (0.67)	136 17.43 (0.79)	0.94 (0.81,1.10) 0.44	0.97 (0.83,1.13) 0.70	0.94 (0.81,1.09) 0.39
6 months	144 14.94 (0.69)	133 14.86 (0.67)	139 15.21 (0.74)	131 20.56 (0.98)	0.71 (0.61,0.83) <0.0001	0.72 (0.61,0.84) <0.0001	0.73 (0.63,0.85) <0.0001
12 months	140 26.25 (0.54)	132 24.95 (0.59)	132 23.66 (0.64)	131 27.04 (0.63)	0.97 (0.83,1.13) 0.67	0.92 (0.79,1.07) 0.29	0.87 (0.74,1.01) 0.075
Plasma IFABP (ng/mL)							
6 weeks	146 0.91 (0.88)	141 0.92 (1.44)	146 0.90 (1.32)	146 1.05 (1.14)	0.87 (0.71,1.07) 0.18	0.87 (0.71,1.07) 0.19	0.86 (0.71,1.06) 0.15

(*Continued on next page*)

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Table 2. ***Continued***

Study arm[a]					Adjusted geometric mean ratio (95%CI) and *p* value[b]		
3 months	143 0.69 (0.91)	136 0.67 (1.05)	144 0.59 (0.88)	140 0.73 (0.85)	0.93 (0.76,1.15) 0.51	0.91 (0.74,1.12) 0.39	0.83 (0.67,1.01) 0.063
6 months	144 0.71 (1.04)	132 0.62 (1.14)	140 0.63 (1.06)	136 0.92 (1.28)	0.76 (0.62,0.93) 0.0074	0.66 (0.54,0.82) 0.0001	0.68 (0.56,0.84) 0.0003
12 months	140 0.88 (1.08)	133 0.83 (0.94)	135 0.82 (0.97)	132 1.08 (1.20)	0.82 (0.66,1.00) 0.055	0.77 (0.63,0.95) 0.016	0.77 (0.62,0.94) 0.012
Stool pH							
6 weeks	147 5.17 (0.12)	141 5.10 (0.12)	145 5.16 (0.12)	143 5.29 (0.10)	0.98 (0.95,1.01) 0.15	0.96 (0.93,0.99) 0.023	0.97 (0.94,1.01) 0.11
3 months	144 5.26 (0.14)	136 5.30 (0.15)	141 5.26 (0.14)	136 5.24 (0.13)	1.00 (0.97,1.04) 0.78	1.01 (0.98,1.05) 0.42	1.01 (0.97,1.04) 0.70
6 months	144 5.50 (0.17)	133 5.42 (0.16)	139 5.32 (0.15)	132 5.47 (0.17)	1.00 (0.97,1.03) 0.95	0.99 (0.96,1.02) 0.54	0.97 (0.94,1.00) 0.074
12 months	140 5.73 (0.13)	132 5.77 (0.16)	132 5.79 (0.14)	131 5.67 (0.13)	1.01 (0.98,1.04) 0.61	1.01 (0.98,1.05) 0.43	1.02 (0.99,1.06) 0.16
Plasma IGF-1 (ng/mL)							
6 weeks	146 55 (4.12)	141 56 (2.20)	146 55 (2.41)	146 62 (2.08)	0.91 (0.68,1.21) 0.50	0.93 (0.70,1.24) 0.61	0.92 (0.69,1.22) 0.55
3 months	143 55 (2.36)	136 60 (1.78)	144 66 (2.06)	138 47 (1.82)	1.15 (0.86,1.54) 0.34	1.29 (0.97,1.73) 0.085	1.40 (1.05,1.87) 0.021
6 months	144 67 (3.05)	132 82 (1.90)	140 75 (1.88)	137 40 (1.99)	1.68 (1.26,2.25) 0.0004	2.08 (1.55,2.80) <0.0001	1.89 (1.41,2.53) <0.0001
12 months	140 103 (0.79)	133 105 (1.02)	138 101 (0.95)	132 85 (1.17)	1.23 (0.92,1.64) 0.17	1.32 (0.98,1.78) 0.063	1.22 (0.91,1.63) 0.19
Plasma IGFBP3 (mg/L)							
6 weeks	146 1.22 (0.95)	141 1.16 (1.27)	146 1.30 (1.11)	146 1.23 (1.20)	1.01 (0.81,1.27) 0.90	0.96 (0.77,1.20) 0.72	1.06 (0.85,1.33) 0.59
3 months	143 1.39 (0.96)	136 1.23 (1.03)	144 1.63 (1.40)	138 1.04 (1.05)	1.37 (1.09,1.71) 0.0070	1.21 (0.96,1.52) 0.10	1.58 (1.26,1.98) <0.0001
6 months	144 1.27 (1.35)	132 1.22 (1.31)	139 1.34 (1.46)	137 0.87 (1.17)	1.49 (1.19,1.87) 0.0006	1.43 (1.13,1.80) 0.0027	1.54 (1.23,1.94) 0.0002
12 months	140 0.89 (1.37)	133 1.03 (1.42)	135 1.11 (1.42)	132 0.89 (1.45)	1.03 (0.81,1.29) 0.83	1.18 (0.93,1.49) 0.17	1.27 (1.01,1.61) 0.042

[a]Data are number, geometric mean (geometric coefficient of variation) for log-transformed data.

[b]Geometric mean ratios (95% CI) and *p* value; mixed model for repeated measures analysis of log-transformed data (ITT population) adjusted for covariates: season of birth (dry vs. rainy), HIV exposure, mode of delivery (vaginal vs. cesarean section), sex, low birth weight, urban or semi-urban vs. rural residence, drinking water source (protected vs. unprotected), household lavatory (improved vs. unimproved), disposal of child stools (with garbage vs. other), and previous number of live births as covariates. Maternal antibiotic exposure was not included because raised AGP did not occur in infants of the few mothers exposed to antibiotics prior to delivery. Mode of feeding was not included because, consistent with the inclusion criteria, all infants were initially breast-fed. Abbreviations are as follows: EED, environmental enteric dysfunction; ITT, intention to treat; CI, confidence interval; AGP, α1-acid glycoprotein; CRP, C-reactive protein; MPO, myeloperoxidase; AAT, α_1-antitrypsin; IFABP, intestinal fatty acid binding protein; IGF-1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor binding protein 3. See also Table S9; Figures S2 and S3 and S4.

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Table 3. Serious adverse events and deaths were similar across the study arms

Age group	Study arm				
	Labinic synbiotic *N* = 150	Lab4b synbiotic *N* = 151	Lab4b probiotic *N* = 149	Controls *N* = 149	Total *N* = 599
Serious adverse events					
0–6 months	8 (5)	11 (7)	5 (3)	9 (6)	33 (6)
7–24 months	13 (9)	11 (7)	17 (11)	14 (9)	55 (9)
Total	21 (14)	22 (15)	22 (15)	23 (15)	88 (15)
***p* value**[a]	0.85	0.96	0.98	–	–
Deaths					
0–6 months	3 (2)	1 (1)	1 (1)	3 (2)	8 (1)
7–24 months	3 (2)	2 (1)	1 (1)	3 (2)	9 (2)
Total	6 (4)	3 (2)	2 (1)	6 (4)	17 (3)
***p* value**[a]	0.34	0.54	0.17	–	–

Data are number (%) of enrolled children with event in the safety population. Only the first SAE is reported here; 9 infants experienced 2 SAEs, 1 experienced 3 SAEs, and 1 experienced 4 SAEs (total of 102 SAEs occurring in 88 children).

[a]χ^2 or Fisher's exact test comparing total number of children with one or more serious adverse events in each intervention arm vs. the control arm. See also Table S8.

development,[9] including cognitive development,[10,11] and increases the risk of longer-term NCDs.[12] Therefore, reduced CSI in infancy through pro/synbiotic administration is directly relevant to LMICs where 250 million (43%) children fail to reach their developmental potential[33] and where the burden of NCDs is greatest.[34]

The raised plasma CRP concentrations in half or more infants at all time points and in all study arms likely reflect frequent acute infections in this vulnerable population, as well as a consequence of intestinal inflammation. The reduced CRP concentrations in all intervention arms at 3 and 12 months and in the Lab4b arm at 3 months compared with controls may reflect less severe gastrointestinal infections or modulation of the inflammatory or immune response to infection.[25]

There was no consistent effect on inflammatory biomarkers of pre-, pro-, or synbiotics in a recent scoping review that identified 29 clinical trials in children.[35] However, the trials were undertaken in different geographical regions, included both newborns and older children who were healthy or had a wide range of different diseases, evaluated many different pre-, pro-, and synbiotic preparations, and measured many different pro- and anti-inflammatory biomarkers. Further research is needed to identify optimal pre-, pro-, or synbiotic preparations for countering inflammation occurring in specific populations and disease contexts.

From 3 to 12 months, pro/synbiotics improved biomarkers that reflect different elements of EED that have been measured in many other studies and are associated with impaired linear growth.[2,13,36] The statistically significant, though marginal, increase in intestinal inflammation in the Labinic synbiotic arm vs. controls at age 6 weeks was unexpected and merits further research.

Pro/synbiotics may improve gut health through multiple mechanisms, including boosting intestinal colonization against pathogens, modulating innate and adaptive immunity, and reducing intestinal permeability.[25,26] The positive findings in PROSYNK support the critical roles for *Bifidobacterium* spp. and *Lactobacillaceae* in early life, including the ability of pro/synbiotics to modulate the gut microbiota before it is well established in the specific context of countering exposure to poor sanitation and hygiene.[17,19]

We consider that the administration of an oral product in exclusively breast-fed infants is justified given evidence of intestinal inflammation, increased intestinal permeability, and enterocyte damage in many infants as early as 6 weeks of age despite exclusive breast-feeding in PROSYNK and other studies[6,14] and that the onset of stunting is highest in the first 3 months of life.[15] *Lactobacillus* GG administered to children aged 3–5 years at risk of EED in Malawi did not affect intestinal integrity assessed by a site-specific sugar-absorption test[37]; the discrepancy with our findings might be due to the use of a single probiotic and/or the older age of the participants in the Malawi study.

Although we did not evaluate the three pro/synbiotics head-to-head, the overall similar effects for each of the interventions suggest either that different strains of *Bifidobacterium* spp. and *Lactobacillaceae* share beneficial "core" properties or that strain-specific differences[23] can be overcome by using multi-strain products. Although the similar biomarker effects in the Lab4b probiotic and Lab4b synbiotic arms suggest no benefit of the addition of the prebiotic fructooligosaccharide (FOS), this needs to be evaluated in further research, especially in non-breast-fed infants who do not receive prebiotics in the form of human milk oligosaccharides.[38]

The reduced acute and CSI and enterocyte damage in all three intervention arms were still evident at 12 months, even though pro/synbiotic administration stopped at 6 months. Further research is needed to determine if beneficial modulation of the gut microbiota, once established, persists long term and whether this is due to long-term engraftment of probiotic organisms. Alternatively, improved early gut health and/or immunity may provide longer-term resilience against adverse environmental factors. Non-food pro/synbiotics may reduce mortality

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in preterm infants[39] and adults in the USA,[40] but further research is needed to assess effects on mortality in LMIC settings and other specific populations.

Qualitative research during PROSYNK revealed that mothers, carers, and health professionals were highly supportive of the pro/synbiotic intervention,[41] and an economic analysis indicated that this intervention may be affordable, especially if probiotics that do not require a cold chain are used.[42]

Strengths of our study were that randomization resulted in similar baseline demographic and clinical variables across study arms. We maintained the same level of contact and general support for the control infants as occurred in the intervention arms so that differences between study arms could be attributed to the interventions. We also supervised dosing of the pro/synbiotics and confirmed probiotic viability at point of use.

Limitations of the study

Although laboratory analyses were undertaken by staff blinded to treatment allocation, a limitation was that assessment of morbidity may be biased in an open-label study. In addition, the findings regarding secondary outcomes should be interpreted with caution given the large number of analyses. Although the pro/synbiotics administered in early life in this trial may counter the multiple adverse outcomes associated with undernutrition and EED in vulnerable children in LMICs, the early onset of intestinal inflammation, marked increase in intestinal inflammation at 12 months, and increased CSI at 12 months in some infants in the intervention arms indicate that further research is needed to optimize the pro/synbiotics and the administration regimen. This could include alternative formulations, more frequent administration, and continuing administration beyond 6 months. Selection of specific probiotic strains, including microbes isolated from healthy Kenyan infants, could be guided by genomic and *in vitro* analyses to identify important functional pathways such as the ability to metabolize human milk oligosaccharides and production of bacteriocins.[16,25] In addition, further research is needed to assess the effects of reduced CSI in early life on growth, as well as other key clinical outcomes such as organ development, including cognitive development, and risk of longer-term NCDs.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Stephen Allen (stephen.allen@lstmed.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- For the probiotic organisms, raw sequence reads (FASTQ) are available on the NCBI SRA as BioProject: PRJNA1165510 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1165510/). DNA sequences of HMO gene clusters can be accessed via https://github.com/raymondkiu/databases/tree/main/HMO.
- This paper does not report any original custom computer code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

S.A., conceptualization and secured funding; M.I.O., M.J., L.H., D.W., S.K., F.O.t.K., and S.A.; study design; M.I.O. and M.J., administration and supervision of data collection; A.K., data curation; M.C. and K.O., laboratory analyses; R.K. and L.H., advice on quantitative culture of probiotic organisms, genome-wide sequencing, and bioinformatics analysis of probiotic organisms; M.I.O., A.K., J.D., Y.L., and D.W., formal analysis; M.I.O., A.K., and J.D., validation; all authors had permission to access all the data in the study if they wished; M.I.O. and S.A., first draft; A.K. and J.D., figures; all authors reviewed and edited the manuscript and had final responsibility for the decision to submit for publication.

DECLARATION OF INTERESTS

All authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2026.102834.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Labinic Synbiotic	Biofloratech Ltd, Surrey, UK	N/A
Lactobacillus acidophilus	–	NCFM
Bifidobacterium infantis	–	Bi-26
Bifidobacterium bifidum	–	Bb-06
Lab4b probiotic/synbiotic	Cultech Ltd., Port Talbot, UK	N/A
Ligilactobacillus salivarius	–	CUL61; NCIMB 30153
Lacticaseibacillus paracasei	–	CUL08; NCIMB 30154
Bifidobacterium animalis subsp. *lactis*	–	CUL34; NCIMB 30172
Bifidobacterium bifidum	–	CUL20; NCIMB 30153
Biological samples		
Blood samples	Human	N/A
Stool samples	Human	N/A
Critical commercial assays		
Human α1-Acid Glycoprotein Quantikine ELISA, R&D Systems, Inc., Minneapolis, USA	R&D Systems, Inc., Minneapolis, USA	Cat#DAGP00
Human C-reactive protein/CRP Quantikine ELISA, R&D Systems, Inc., Minneapolis, USA)	R&D Systems, Inc., Minneapolis, USA	Cat#DCRP00
Human Myeloperoxidase Quantikine ELISA; R&D Systems, Inc., Minneapolis, USA	IBL International GmbH; Hamburg, Germany	Cat#ID59331
Fecal α_1-antitrypsin	ImmuChrom GmbH, Heppenheim, Germany	Cat#IC6200
Human FABP2/IFABP Quantikine ELISA; R&D Systems, Inc., Minneapolis, USA)	R&D Systems, Inc., Minneapolis, USA	Cat#DFBP20
Human IGF-I Quantikine ELISA; R&D Systems, Inc., Minneapolis, USA	R&D Systems, Inc., Minneapolis, USA	Cat#DG100
Human IGFBP3-I Quantikine ELISA; R&D Systems, Inc., Minneapolis, USA	R&D Systems, Inc., Minneapolis, USA	Cat#DGB300
Software and algorithms		
STATA v.17	StataCorp LLC, Texas, USA	https://www.stata.com/
SAS v9.4	SAS Institute	https://www.sas.com
Other		
Labinic synbiotic: BENEO Orafti Synergy1	BENEO GmbH, Mannheim, Germany	N/A
Lab4b synbiotic: long-chain fructo-oligosaccharide	Cultech Ltd., Port Talbot, UK	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Enrolled participants

Ethical approval was provided by the Kenya Medical Research Institute - Scientific and Ethics Review Unit (KEMRI/SERU/CGHR/320/3917), Kenyan Pharmacy & Poison Board, Kenya (PPB/ECCT/20/04/02/2020(063)), National Commission for Science Technology and Innovation, Kenya (PPB/ECCT/20/04/02/2020(063) and the Liverpool School of Tropical Medicine (19–048). The parents/carers of all participants gave informed and written consent prior to enrollment. The protocol has been published,[43] and the full protocol is available (Data S1 Trial protocol).

600 newborns aged 0–3 days who were well, had taken a breast feed well and had no acute illness or conditions that may limit growth or survival were enrolled in the study. Overall,

283 males and 317 female newborns were recruited. Demographics of enrolled patients is included in results above and Table 1.

METHOD DETAILS

Study design

The PRObiotics and SYNbiotics in infants in Kenya (PROSYNK) study was a prospective, open-label, individually randomized, 4-arm, phase II trial in Homa Bay County, western Kenya where approximately 13% of under 5s are stunted (height-for-age *Z* score <-2).[44] After securing signed and dated informed consent from parents/carers, singleton, term infants aged 0–3 days, with a birth weight of ≥2000g, who were breastfed, had taken a breastfeed well and lived in the hospital catchment area were enrolled by research clinicians at Homa Bay County Teaching and Referral Hospital, a 280-bed government facility. Exclusion criteria were suspicion or presence of any acute illness, congenital anomalies that might be life-threatening or impair growth, potential contraindication to pro/synbiotics (e.g., suspected immune suppression; cardiac abnormality), mother unlikely to stay in study area for the duration of the study and any health or research staff concerns regarding safety to participate in the trial.

Newborns were allocated according to a computer-generated random sequence with blocks of random size and stratified by HIV exposure, 1:1:1:1 to one of four study arms by the research clinicians opening a sealed opaque envelope. The random sequence was generated by the research statistician and withheld from all other research staff until databases were cleaned and locked.

Interventions

Study staff visited infants' homes to administer under supervision Labinic synbiotic (recently named ''Labisyn'' by the supplier; BENEO Orafti Synergy1; 50 oligofructose:50 FOS; 200mg + *Lactobacillus (L.) acidophilus* NCFM, *Bifidobacterium (B.) longum* subsp. *infantis* Bi-26 and *B. bifidum* Bb-06; total of 5×10^9 organisms/day, Biofloratech Ltd., Walton-on-Thames, UK); Lab4b probiotic (*Ligilactobacillus salivarius* CUL6 (NCIMB30211), *Lacticaseibacillus paracasei* CUL08 (NCIMB 30154), *B. bifidum* CUL20 (NCIMB 30153), and *B. animalis* subsp. *lactis* CUL34 (NCIMB 30172); total of 10^{10} organisms/day), Lab4b synbiotic (Lab4b probiotic plus long-chain FOS 150mg/day, Cultech Ltd., Port Talbot, UK) or no pro/synbiotic (controls). The *Bifidobacterium* possessed partial or complete human milk oligosaccharide (HMO) gene clusters signifying ability to metabolize and utilize HMOs (see below). The interventions were selected based on production in facilities approved by appropriate regulatory authorities including quality control procedures, evidence of safe use of the probiotic strains in preterm infants (Labinic)[45] and newborns (Lab4b),[46] and the potential additional benefits from inclusion of prebiotics.[23,24] The treatment regimens were pragmatic and guided by the potential advantages of multi-strain preparations with a higher number of organisms.[22]

The pro/synbiotics were presented as powder in capsules of a different color for each product. The capsules were opened, and the powder sprinkled directly into the infant's open mouth before feeding or mixed in a clean container with sterile water. Administration was repeated once if the infant vomited within 30 min. Administration occurred daily for the first 10 days and then weekly to age 6 months (total of 32 doses). These live products were shipped, stored and delivered to homes using a cold chain. Quantitative culture confirmed viability of the pro/synbiotics at point-of-use (see below). Control infants received the same home visits and contact with the research team as those in the intervention arms, except that some scheduled visits were done by mobile phone during COVID-19 restrictions. During all home visits/phone calls, research staff gave general advice on infant feeding, hygiene and care to all mothers/carers and facilitated referral for clinical review if the infant was unwell.

Data collection and sampling

A research clinician reviewed clinical records for information regarding the mother, pregnancy and delivery, collected baseline demographic and clinical data and examined newborns including to determine gender. Parents/carers informed staff of illnesses by mobile phone and serious adverse events (SAEs) and mortality were recorded to age 24 months during home visits. Medical records were reviewed for infants admitted to hospital.

Outcomes

The primary outcome was CSI at age 6 months defined as plasma AGP >1 g/L based on previous studies in young children in LMIC settings.[3,4,27,29] Secondary outcomes were plasma AGP at other timepoints, plasma CRP to assess acute inflammation, biomarkers of intestinal inflammation (fecal MPO), intestinal permeability (fecal AAT), mucosal integrity (plasma IFABP), stool pH and growth hormones plasma IGF-1 and IGFBP-3 at 6 weeks and 3, 6 and 12 months. We selected these biomarkers as they reflect different aspects of the intestinal pathology attributed to EED and have been used in many other studies.[13] Other outcomes were SAEs and deaths.

Laboratory methods

Analyses of stool and blood samples

Biomarkers of systemic inflammation, EED and growth were measured by ELISA in capillary blood (up to 1 mL) and stool samples collected either at the research clinic or from homes using a cool box (temperature range 9-11°C) at ages 6 weeks, 3, 6 and 12 months[43] ELISAs were carried out in duplicate and included control samples, according to the manufacturer's instructions and

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analyzed sequentially so that samples from all study arms were likely to be analyzed on each ELISA plate. Laboratory staff were blinded to the study arms. Stool pH was measured using a pH meter.

DNA extraction and Whole Genome Sequencing of probiotic organisms

The powder from the Labinic synbiotic and Lab4b probiotic capsules was serially diluted and spread plated on MRS agars to obtain pure colonies, followed by culturing and genomic DNA extraction using FastDNA Spin Kit for Soil (MP Biomedicals) according to manufacturer's instructions. Genomic DNA were whole-genome sequenced on NextSeq 500 to generate 150-bp paired-end reads at 80X coverage.

Raw sequence reads (FASTQ) were quality-filtered with fastp v0.20.0[47] (-q 20) prior to *de novo* genome assembly via unicycler v0.5.0[48] which functioned as an optimizer for assembler SPAdes v3.15.4.[49] at default parameters. Contigs <500 bp were subsequently discarded in each genome assembly. Genome assembly statistics were generated via sequence-stats v1.0[50] (see Table S7). Prior to further analyses, all genome assemblies were subjected to contamination check via checkm v1.1.3[51] and all genomes were >97% completeness, <0.2% contamination. Next, species identification for each isolate genome was performed using gtdb-tk v2.3.2[52] with gtdbtk database release214. Construction of neighbor-joining tree was performed via mashtree v1.2.0[53] (–reps 100) based on all 17 probiotic genomes and 6 bacterial type strains including *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Ligilactobacillus salivarius*, *Bifidobacterium bifidum* and *Bifidobacterium animalis* (both subsp. *lactis* and subsp. *animalis*) retrieved from NCBI Nucleotide database for comparison and validation. The exception was Labinic probiotic strain *Bifidobacterium longum* subsp. *infantis* Bi-26 which had been sequenced previously[54] and was retrieved from NCBI RefSeq Assembly database (GCF_004919065.2). Gene search of Human Milk Oligosaccharide (HMO) gene clusters was performed via ABRicate v1.0.1[55] (–mincov = 90, –minid = 70) based on known HMO gene clusters.[56] Tree and HMO profile visualization was performed via iTOL v6.9.1.[57]

There was a total of 6 bacterial species identified in these 18 probiotic genomes including *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Ligilactobacillus salivarius*, *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium animalis* (subsp. *lactis* and subsp. *animalis*). The *Bifidobacterium* possessed partial or complete HMO gene clusters signifying ability to metabolize and utilize HMOs. Genera *Lactobacillus* and *Ligilactobacillus* did not harbor any of the known HMO gene clusters (see Figure S5).

Raw sequence reads (fastq) are made available on NCBI SRA under bioproject PRJNA1165510. DNA sequences of HMO gene clusters can be accessed via https://github.com/raymondkiu/databases/tree/main/HMO. Type strains including *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Ligilactobacillus salivarius*, *Bifidobacterium bifidum* and *Bifidobacterium animalis* (both subsp. *lactis* and *subsp. animalis*) were retrieved from NCBI Assembly database (downloaded on 25 September 2024). Labinic probiotic strain *Bifidobacterium longum* subsp. *infantis* Bi-26 was also retrieved from NCBI RefSeq Assembly database (GCF_004919065.2). Quantitative culture of the probiotic microorganisms at point of use in Kenya.

Twenty-four probiotic/synbiotic capsules were selected from the first and 7 capsules from the second shipment received for the trial. A cold chain within 2°C–8°C was maintained during shipment and storage.

Preparation of samples for evaluation

The powder from pro/synbiotic capsules was diluted in a 1:10 ratio by adding 1.00 g ±0.01 g of testing sample to 9 mL maximum recovery diluent (MRD). Once the initial dilution was made, a decimal dilution series (1:10 dilutions) MRD was set up to enable colony numbers of between 10 and 120 cfu/plate to be counted. The prepared samples were then placed on a roller mixer or magnetic stirrer for maximum of 15 min to achieve homogeneity. All weights of samples were then recorded in the laboratory record book. The modified Miles and Misra plate count technique was used for this evaluation as described below.

Modified Miles and Misra plate count technique

Plating Out Method. Once the dilution series was made, the sample was placed onto the pre-labelled, dried selective agar plates. To plate out the sample, we started with the highest dilution and worked to the lowest (i.e.,10^{-5} is higher than 10^{-1}). We then used an electronic pipette to dispense 10 × 10 μL drops onto the surface of the agar (100 μL total per plate), ensuring that the drops were evenly spread. We then allowed the plates to dry. If a drop bounced when it was placed onto the agar plate, it was marked with a star to identify that colonies could be outside of the sample dropped areas.

Detection of Lactobacillus spp

MRS Agar was used for *Lactobacillus, Ligilactobacillus,* and *Lacticaseibacillus* spp. and the surface inoculated with 100 μL of suspension using the modified Miles and Misra plate count technique described above.

Detection of Bifidobacterium spp

MRS-X agar was used for *Bifidobacterium* spp. and the surface inoculated with 100 μL of suspension using the modified Miles and Misra plate count technique. Each dilution was plated onto a separate plate as follows: (A) 100μL of the 10^{-1} dilution, (B) 100μL of the 10^{-2} dilution, (C) 100μL of the 10^{-3} dilution, (D) 100μL of the 10^{-4} dilution, (E) 100μL of the 10^{-5} dilution, (F) 100μL of the 10^{-6} dilution and (G) 100μL of the 10^{-7} dilution The plates were then incubated anaerobically at 37°C ± 1°C for 72 h ± 10 h.

Reporting of the probiotic count

After incubation, the plates were analyzed by counting colony numbers. Only plates that fitted within the 30–300 colonies range were used. The results of the selected plate were recorded in the processing logbook. In cases where there were no colonies on the plate, we recorded the count as zero against the lowest dilution tested, enabling the limit of detection

to be calculated. When more than one colony type was present on one MRS agar plate, a brief description was also be recorded such as: size of colony (e.g., L = large, M = medium, S = small) and color of colony (e.g., W = white, G = gray, C = cream, Y = yellow).

Calculation and reporting the number of organisms

The number of colony-forming units (cfu) was used to calculate the total number of microorganisms in a product. This was done by counting the number of colonies on the plate then multiplying the total number by 10 to give cfu/ml in plating dilution. To calculate the cfu/g in an undiluted sample, we multiplied by the dilution factor of the selected plate. For example; to convert 10^{-2} to 10^{-1}, we multiplied by 10 and to convert 10^{-3} to 10^{-1} we multiplied by 100. All the results were expressed as a number between 1.00 and 9.99 multiplied by 10^{n}, where n was the appropriate power of 10 and expressed in two decimal places.

(colony count x dilution factor (the inverse of the dilutions) = CFU/ml of original culture):

e.g., 52 CFU $\times$ 106 $\times$ 10^{1} = 5.2 $\times$ 10^{8} CFU/mL.

The number of viable organisms per pro/synbiotic capsule was calculated according to the weight of probiotic product in each capsule as follows.

- Labinic synbiotic: 313.9mg
- Lab4b synbiotic: 255mg
- Lab4b probiotic: 280mg

Results

All the capsules that were being used by participants and maintained under a cold chain within 2°C–8°C had viable organisms. The colony-forming units (CFUs) for each product at different dilutions is summarized in Table S8.

QUANTIFICATION AND STATISTICAL ANALYSIS

A total of 524 infants (131 per am) would allow the detection of a 50% reduction (risk ratio = 0.50) in the prevalence of CSI at age 6 months from 35% in the control arm[5] to 17.5% in any of the intervention arms with 80% power and with $\alpha = 0.0167$ to allow for 3 primary comparisons against the control arm based on the intention to treat population. In the absence of data to help predict the reduction of the frequency of CSI in the intervention arms, we considered that a 50% fall would be sufficient to justify further research to assess the feasibility and affordability of pro/synbiotics as a public health intervention in LMIC settings. We aimed to recruit 150 infants/arm to allow for 13% dropouts.

Primary data analyses were based on the intention-to-treat (ITT) population and supportive analyses were on the per-protocol (PP) population. The primary outcome (infants with CSI (plasma AGP >1 g/L) at age 6 months) was analyzed using generalized linear model (GLM) with binomial distribution and log link function, and treatment as the only predictor, generating the risk ratio (RR) as measurement of treatment effect and their 95% confidence intervals (CI). Covariate adjusted analysis of the primary outcome included variables pre-specified at baseline which may affect the occurrence of the primary outcome based on empirical studies and clinical judgment: season of birth, HIV exposure, mode of delivery, gender, low birthweight, urban/semi-urban vs. rural residence, drinking water source, household, disposal of child stools, and the previous number of live births. Covariates were included as categorical independent variables (with the exception of number of live births). Because of convergence difficulties with a log-binary model, Poisson models with log link and robust standard errors were used for the covariate adjusted analysis. A correlation matrix of covariates was computed using Stata showed no values with $r > 0.3$. For a regression model with a continuous outcome (plasma AGP concentration), mean variance inflation factor (VIF) of adjusted model with covariates was 1.10, and no individual VIF was >1.4.

Subgroup analysis of primary outcome was performed on these covariates. No interaction effect between study arm and covariates was observed (see Table S5).

Binary secondary outcomes were analyzed using GLM as for the primary outcome analysis. For continuous secondary outcomes with repeated measurements, generalized linear mixed models (GLMMIX) with a normal distribution and identify link function were employed. The GLMMIX models had treatment (Labinic synbiotic, Lab4b synbiotic, Lab4b probiotic, and Control), visit (3 months, 6 months, and 12 months), interaction between treatment and visit as fixed effects, baseline measurement of an outcome as covariate, and subject as random effect to correct for multiple observations in the same subjects over time. Time was categorical, because of the different periods of time between visits, and the limited number of timepoints at which observations were reported. By treating visit as categorical, we allowed the model to capture visit-specific effects and their interactions with study intervention without assuming a specific functional form, implicitly accommodating potential nonlinear trends across visits. Continuous outcomes were log transformed and histograms for the residuals from GLMMIX models plotted to assess normality of the residuals from GLMMIX model analyses of the log transformed values. Inspection of histograms for the residuals showed transformed data to be much closer to normal (see Figure S6). The treatment effect measured as geometric mean ratio between two treatment arms at each visit together with its 95% CI was derived. Missing data were treated as missing at random in the GLMMIX model analysis and no imputation of primary endpoint was made. The amount of missing data was small. For the

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primary outcome (plasma AGP), missing data ranged from 4% at the 6-week follow-up visit to 10% at the 12-month visit; and by 4–10% between arms. Little's test for missing completely at random was performed with intervention arm, visit, and covariates as explanatory variables, $p < 0.001$. The variation in missingness by arm and visit suggests missingness is related to observed variables and a reasonable assumption given arm, visit and covariates were included in the model. Missingness patterns by visit are shown in Table S9.

Statistical analyses were performed using STATA v.17 (StataCorp LLC, Texas, USA) and SAS v9.4 (SAS Institute). In this exploratory analysis, the level of statistical significance was $p < 0.05$ to avoid a type II error.

Additional resources

None