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ABSTRACT

Introduction Children with moderate or severe wasting are at particularly high risk of recurrent or persistent diarrhoea, nutritional deterioration and death following a diarrhoeal episode. Lactoferrin and lysozyme are nutritional supplements that may reduce the risk of recurrent diarrhoeal episodes and accelerate nutritional recovery by treating or preventing underlying enteric infections and/or improving enteric function.

Methods and analysis In this factorial, blinded, placebo-controlled randomised trial, we aim to determine the efficacy of lactoferrin and lysozyme supplementation in decreasing diarrhoea incidence and improving nutritional recovery in Kenyan children convalescing from comorbid diarrhoea and wasting. Six hundred children aged 6–24 months with mid-upper arm circumference <12.5 cm who are returning home after an outpatient visit or inpatient hospital stay for diarrhoea will be enrolled. Children will be randomised to 16 weeks of lactoferrin, lysozyme, a combination of the two, or placebo and followed for 24 weeks, with biweekly home visits by community health workers and clinic visits at 4, 10, 16 and 24 weeks. The primary analysis will compare the incidence of moderate-to-severe diarrhoea and time to nutritional recovery between each intervention arm and placebo. The trial will also test whether these interventions reduce enteric pathogen carriage, decrease enteric permeability and/or increase haemoglobin concentration in enrolled children. Finally, we will evaluate the acceptability, adherence and cost-effectiveness of lactoferrin and/or lysozyme.

Ethics and dissemination The trial has been approved by the institutional review boards of the Kenya Medical Research Institute, the University of Washington, the Kenyan Pharmacy and Poisons Board, and the Kenyan National Commission on Science, Technology and Innovation. The results of this trial will be shared with local and international stakeholders and published in peer-reviewed journals, and the key findings will be presented at relevant conferences.

Trial registration number NCT05519254, PACTR202108480098476.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This is a randomised, placebo-controlled, blinded, factorial trial with an intention-to-treat analysis.
⇒ Comprehensive data collected includes detailed clinical and laboratory data to assess mechanisms related to diarrhoea and nutritional recovery, as well as qualitative data collected from caregivers and health workers to provide information on adherence, acceptability and cost-effectiveness of the interventions.
⇒ Random assignment to treatment arm by study statistician and daily intervention-containing sachets labelled with the Participant Identification Number promote allocation concealment and blinding, but complete blinding may not be possible due to light pink hue of lactoferrin versus other white-appearing powders.
⇒ Relatively small per-arm sample sizes may limit power to detect modest effect sizes and intervention effects; however, factorial design allows for gained efficiency by combining arms.

BACKGROUND

Five hundred thousand children die each year from diarrhoea, mostly in low-income and middle-income countries (LMICs).[1]

Children who survive diarrhoea are at increased risk of enteric dysfunction, recurrent diarrhoea, wasting and stunting.[2 3] Wasting and stunting further increase a child's vulnerability to infection and are associated with delayed cognitive development.[4–6] Current diarrhoea management strategies in LMICs (oral rehydration solution (ORS), ReSoMal and zinc) focus primarily on the management of dehydration and micronutrient replacement and appear to have negligible impact in preventing future diarrhoeal episodes or improving long-term nutritional outcomes such as length for age z-score (LAZ).[7–11] Empiric antibiotic management of diarrhoea does not appear to alter clinical outcomes but has shown some effect on growth.[12] However, substantial concerns exist regarding rising antibiotic resistance in these settings and may prevent incorporation of antibiotics into diarrhoea management.[12] Novel non-antibiotic interventions for young children recovering from moderate-to-severe diarrhoea in LMICs are needed.

Lactoferrin and lysozyme are antimicrobial proteins contained in human and animal milk that may reduce the risk of recurrent diarrhoeal episodes and accelerate nutritional recovery by treating or preventing enteric infections and improving enteric function.[13–17] These proteins are not currently used in paediatric clinical practice but they are known to be safe, inexpensive, well tolerated and easily incorporated into food, making them a potentially cost-effective and scalable intervention to improve child health and survival.[13 15 17–30] In clinically healthy Malawian children, coadministration of lactoferrin and lysozyme improved enteric barrier dysfunction, a key mechanism underpinning poor longer term diarrhoea outcomes, within 8 weeks of enrolment.[21] In clinically healthy Peruvian and Chinese children, lactoferrin reduced the cumulative prevalence of diarrhoea episodes, including severe episodes.[22 31] However, only one small trial (in Peru) has tested these products as a therapeutic intervention for diarrhoea, reporting a 2 days faster recovery from diarrhoea with the use of lactoferrin and lysozyme-supplemented ORS.[25] In addition, lactoferrin may promote recovery from childhood anaemia due to its ability to bind iron in the gut thereby enhancing enteric iron uptake.[32] Children with moderate or severe wasting are at particularly high risk of death, diarrhoea recurrence and nutritional deterioration following a diarrhoeal episode.[3 4 33–35] Interventions targeting enteric bacterial infections and improved enteric barrier function may substantially benefit children with diarrhoea and wasting.

OBJECTIVE

The primary objective of this factorial, blinded, placebo-controlled, randomised controlled trial is to determine whether a 16-week course of lactoferrin, lysozyme or a combination of these products reduces the incidence of moderate-to-severe diarrhoea and shortens time to recovery from wasting (mid upper arm circumference (MUAC) ≥ 12.5 cm) during the 6 months following presentation to a health facility with diarrhoea and childhood wasting (MUAC <12.5 cm). The secondary objectives are (1) to explore whether a 16-week course of lactoferrin, lysozyme or combination therapy improves secondary clinical, nutritional, enteric pathogen and enteric permeability outcomes; and (2) to evaluate acceptability, adherence and cost-effectiveness of lactoferrin and lysozyme in Kenya.

Methods

Reporting of this study protocol has been verified in accordance with the Standard Protocol Items for Randomized Trials recommendations.[36]

Eligibility

Children aged 6–24 months, managed as an outpatient or inpatient for diarrhoea (three or more abnormally loose or watery stools per 24 hours) at one of the seven recruiting sites who are ready to return home will be screened for eligibility in the trial. The average of two MUAC measurements will be taken by a trained study staff to determine the eligibility based on MUAC. If the difference between the two measurements is >0.5 cm, a third measurement will be obtained and the median of the three measurements will be used to determine eligibility. A child will be excluded if any of the following criteria are met: MUAC at the time of screening is greater than or equal to 12.5 cm; the accompanying caregiver does not provide consent to study participation; the caregiver reports that the child will not stay within the study area for the next 6 months or longer; the child has a history of two or more blood transfusions in the past 12 months; the child has a history of allergy to dairy products; the child is enrolled in another study; the child is exclusively breastfeeding at the time of enrolment, the child is not ready to return home (is not yet discharged); the caregiver is unwilling to have their child participate in the dual sugar permeability substudy if selected; the child was discharged against medical advice; or the child has a history of congenital defect or syndrome that prevents age-appropriate feeding (eg, cleft palate).

Recruitment

Children will be recruited from outpatient and inpatient departments at county and subcounty hospitals in Homa Bay County (Homa Bay County Referral Hospital), Kisii County (Kisii Teaching and Referral Hospital) and Migori County (Rongo Sub-County and Isebania Sub-County Hospitals). Prior to the screening process, potential participants will be prescreened for eligibility by the study staff. The study staff will obtain verbal consent from the caregiver for prescreening of medical records and screen the child for eligibility once the child is ready to return home after being discharged (from inpatient or outpatient facilities). If the child is eligible, the caregiver will undergo written informed consent in their preferred language (English, Kiswahili, Kisii, Kuria or Luo). If the

accompanying caregiver is illiterate, they will be read the informed consent form and provide their fingerprint in the presence of an impartial witness (independent from the study staff) (see online supplemental file). Eligible children of consenting caregivers will be enrolled in the study.

Enrolment

At enrolment, primary caregivers will be interviewed to assess the child's demographic information, medical history and contact information. Clinical and laboratory information will be abstracted from the medical record including antibiotic use and any other medical management received in the hospital and the information on the inclusion in a community-based management of acute malnutrition (CMAM) programme. The CMAM programmes for children with wasting identified at a sick visit at the recruiting facilities include biweekly home visits, provision of therapeutic or supplementary foods and counselling around feeding until the child's nutritional status has recovered. All enrolled participants will undergo a physical examination performed by the study clinician. Study staff trained in anthropometry will collect dual measurement of length and weight, with a third measurement if there is >0.5 cm (length) or >0.1 kg (weight) difference between the two measures.

Specimen collection

Two flocked rectal swabs, a whole stool sample (if passed prior to the child being ready to leave the facility), and venous blood (< 5 mL) will be collected from children during enrolment prior to administration of the investigational product (IP). If the child is unable to produce whole stool within 1 hour of enrolment, the caregiver will be provided with instructions and materials to collect a whole stool sample from the child at home, which will be retrieved by a community health worker (CHWs) within the end of the next working day after enrolment, and brought back to the recruitment facility for processing and storage. Blood will be used for dried blood spots and remaining blood will be immediately centrifuged for plasma and serum storage at −80°C.

Randomisation

Each subject will be assigned a unique patient identification number (PID) which will be randomised to one of the four treatment arms. Randomisation will be stratified by hospital admission status (ie, inpatient vs outpatient) to account for the differences in severity of wasting and dehydration between hospitalised children and those seen as outpatients. Block randomisation (1:1:1:1) in random sized blocks of no more than 12 within each stratum will be used to assign treatment groups at study enrolment. A file containing the PIDs assigned to treatment arms will be password protected and will be accessible only to the study statistician conducting the randomisation, the Kenya Medical Research Institute (KEMRI) pharmacist and the IP packaging team. Treatment allocation (once assigned) will remain blinded to the participant, the study staff, the hospital clinicians and the investigators during all data collection and analysis phases of the study. Participants, all investigators and outcome assessors will be blinded to allocation arm. However, subtle difference in the colour of powders containing Lactoferrin may allow study staff to guess randomisation arm if comparing two products side-by-side (see online supplemental figure 1). We therefore conservatively classify this trial as a single-blind study.

Intervention

Children will receive a 16-week course of lactoferrin, lysozyme, combination therapy or near identically placebo (collectively deemed the IP). The products will be in powder form provided in daily sachets (containing 41.5 g of the IP) labelled with the PID, expiration date and study contact information for cases of suspected adverse events (see online supplemental figure 2). Two weeks and 1 day (15 days) of IP will be supplied at enrolment and replenished during follow-up visits. Pictorial instructions and a 2-week adherence log (see online supplemental figure 3) will be provided to participants at enrolment and during each 2-week IP replenishment. Caregivers of children in each arm will be instructed to provide one sachet daily which will contain a mixture of lactoferrin and/or lysozyme and/or unmodified rice powder. The powder will be mixed with 125 mL of the child's morning porridge, as was done in other lactoferrin/lysozyme studies.[21 37] The IP for each arm will be prepared and packaged by an independent company, Biodeal Laboratories, Nairobi, Kenya.

The Kenya Ministry of Health guidelines recommend introduction of complementary foods, such as porridge, starting at 6 months of age.[38] In Kenya, Uji (porridge) made from dried maize or sorghum flour is a common complementary food given to infants. Uji is also commonly given to children and adults who are unwell, which may increase its acceptability as delivery mechanism for the study IP. Uji will be provided to study participants at enrolment. For children with moderate acute malnutrition, the IP will be mixed with their ready-to-use supplementary food (RUSF), instead of Uji. For children who are severely malnourished, the IP will be mixed with Uji while the children continue to take their ready-to-use therapeutic food (RUTF).

The first dose of the IP will be administered by the caregiver during the enrolment visit (fresh porridge will be provided by the study) and therefore directly observed. During this time, the caregiver will be counselled on mixing the IP with porridge/RUSF, adding the IP to the porridge only after the porridge has cooled to an edible temperature so as to avoid any alterations in the IP from cooking or boiling, and the importance of daily administration, not giving the IP to other household members (including other children), and leaving any unused IP in the containers for returning to the health facility at monthly visits.

Study CHWs will visit all participants in their homes daily for the first 2 days (D2 and D3) of study participation. During this visit, the CHW will assist the family in preparing and administering the IP/porridge mixture and completing the daily adherence log. If the child is not hungry during the visit, the IP/porridge may be stored for up to 8 hours until the participant is ready to take it. During this visit, the CHW will talk with the caregiver (or directly observe) about their experience preparing and feeding the IP and administer a short questionnaire to determine if the child experienced any potential adverse events. If the child has a suspected severe adverse event (SAE), the CHW will immediately contact the study clinician. If the clinician decides that the child needs to be seen at the health facility, the CHW will arrange transportation for the child and caregiver to be seen by a study clinician at the nearest study site. The CHW will also ensure the IP is being stored in a cool, dry location within the house. The CHW will aim to observe the child eating the IP/porridge. If the child is not hungry and unable to consume the IP/porridge mixture during the CHWs visit on the first 2 days, the caregiver will be instructed to offer the IP/porridge to the child as soon as he/her is hungry (within an 8-hour window). A daily diarrhoea diary card and associated counselling on card completion will be provided to the caregivers at enrolment to record the child's diarrhoea episodes throughout the study period. Study CHWs will help the caregivers complete the diary card during all follow-up visits, including the first 2 days.

Follow-up procedures, specimen collection and storage

Study visits

The study follow-up visits (see online supplemental figure 4) will occur in addition to the existing CMAM programme for children with wasting identified at a sick visit. The CMAM programme includes fortnightly nutritional clinic visits, provision of food and counselling around feeding until the child's nutritional status has recovered (MUAC ≥12.5).

Home study visits

The CHW will conduct daily home visits, for the first 2 days after enrolment and then further home follow-up at W2, W6, W8, W12, W14, W18, W20 and W22. At these visits, the CHW will collect basic health information from the caregiver about the child, including appetite, fever, vomiting, respiratory symptoms, concomitant medication use (including antibiotics) and any new clinic visits or hospitalisations using a short-standardised questionnaire. CHWs will collect the diarrhoea diary and use it to facilitate the caregivers' answering of questions around diarrhoea and diarrhoea severity in the last 2 weeks. They will also collect caregiver pictorial adherence logs and distribute new logs and stickers to the caregivers (W2, W6, W8, W12 and W14 only) and a short, standardised acceptability questionnaire will be administered to caregivers.

Facility study visits

All enrolled children will be scheduled to return to the study hospital at 4, 10, 16 and 24 weeks after enrolment for a study visit. Caregivers will be encouraged to take their child to the health facility where enrolment occurred for assessment of any medical conditions that arise outside of the standard visit schedule, including diarrhoea. Transportation and time reimbursement will be paid by the study for scheduled clinic visits (500 Kenyan shillings (KSH), equivalent to US$3.90 as of 10 March 2023) and unscheduled clinic visits (300 KSH) due to illness. During scheduled and unscheduled facility-based follow-up visits, clinicians will perform a brief physical exam and will assess signs and symptoms related to the child's health including diarrhoea and diarrhoea severity (leveraging the returned diarrhoea diary), appetite, fever and abdominal pain and will manage any illness as per standard of care. Study staff will complete the standardised questionnaire including ascertainment of medication use, recent healthcare seeking and any other relevant health information. At all scheduled facility visits, two independent study staff will perform anthropometry measurements (length, weight and MUAC).

Whole stool samples, two rectal swabs and whole blood will also be collected. All biological samples will be collected by staff trained in biosafety and Good Clinical Laboratory Practice. If the child is unable to produce whole stool within 1 hour of the visit, the caregiver will be advised on how to collect whole stool on returning home and a CHW will arrange to pick up the stool at the child's household within the end of the next working day of the child passing stool (within 7 days of the scheduled follow-up visit). Faecal samples will be stored for eventual pathogen and enteric dysfunction biomarker testing. Whole stool will be aliquoted and stored in −80°C freezer at each recruitment site within 1 hour of collection (within the end of the next working day of the child passing stool for sample collected at home). Rectal swabs will be stored at −80°C. Blood (<5 mL) will be collected at facility-based schedule hospital visits (week-4 (W4), W16 and W24) for obtaining plasma, serum and dried blood spots. The W16 blood sample will be tested for haemoglobin and the results will be made available to the caregiver, hospital and study clinicians. Any remaining blood collected at each visit will be immediately processed and stored/archived at −80°C for future mechanistic studies. Dry ice shipments transferring the −80°C stored samples to a larger storage facility at KEMRI in Nairobi will occur monthly. At the W4, W10 and W16 facility follow-up visit, a short, standardised acceptability questionnaire will be administered to caregivers to record any challenges or discomfort they felt administering the IP. Caregiver pictorial adherence logs will be collected at these visits and caregivers will be provided new logs at each visit (W4, W10 and W16 only).

Dual sugar permeability test

In a subset of 50 children per arm (200 children total), the lactulose (L): rhamnose (R) ratio dual sugar test will be performed at all follow-up facility visits (W4, W10, W16 and W24) as a functional assessment of enteric integrity. Random allocation to the L:R substudy will occur during the treatment arm randomisation assignment and L:R assignment (yes/no) indicated in a sealed PID-labelled envelope opened at the time of PID assignment. Children selected for the L:R substudy who have diarrhoea on the day of a scheduled test will be asked to return after 72 hours, as the administration of lactulose is known to cause mild diarrhoea among some participants. For similar reasons, the dual sugar test will not be administered at enrolment to avoid exacerbating the presenting diarrhoea. We will employ a standardised L:R testing regimen that was recently validated in Peru, Zambia and the USA, and recently conducted in Migori County.[39 40] Caregivers will be asked to fast (food, drink and breastmilk) their child for 1 hour. At the beginning of this hour, a urine bag will be placed to obtain a pretest urine sample. A 10 mL oral L:R solution containing 1500 mg lactulose and 300 mg L-rhamnose will be administered at the end of the fasting hour and a new urine bag placed. Postadministration collection lasts for 2 hours. The caregiver will be encouraged to breastfeed the child during these 2 hours or give water. If the bag becomes full, staff will replace the bag in a quick but careful fashion ensuring no urine is missed or spilled. All urine passed during the 2 hours will be collected. Urine from each time period during collection will be aliquoted into 1000 µL cryovial and stored at −80°C. If the dual sugar test fails (no urine is passed during the collection period, or the sample is spoiled by stool contamination), the test will be repeated 48 hours later. The subset of children receiving the L:R test will receive an additional 300 KSH for the additional time required to complete the test.

Adherence to the IP

Adherence will be assessed on all study participants with each course (14 days) of IP by counting the number of returned sachets at the home (W2, W6, W8, W12 and W14) and clinic visits (W4, W10 and W16) as well as evaluating the caregiver-reported daily IP administration in the pictorial adherence logs provided to caregivers. The caregivers of participants will be reimbursed 100 KSH for their return of their 15 IP sachets (used or unused), which will also provide an IP adherence measure. The logs will have spaces for caregivers to mark when they administer the IP each day, with the goal of supporting high adherence among all participants. Use of pictorial logs ensures that literacy is not a barrier to use.

Acceptability of the IP

After the completion of each 14-day course of the IP, a standardised acceptability questionnaire will be administered to caregivers. The questionnaire will include items regarding acceptability of the IP, and facilitators and barriers of IP administration and use. This will take place at the home (W2, W6, W8, W12 and W14) and clinic follow-up visits (W4, W10 and W16A). Responses to the acceptability items administered by lay health workers at home may be less biased as compared with questionnaire administration by study personnel in hospitals. Determinants of adherence and acceptability will also be investigated in focus groups with caregivers and health workers.

Focus groups

Focus group discussions (FGDs) will be conducted with caregivers and healthcare workers to determine acceptability of the IP and administration method. Between 24 and 32 caregivers of enrolled participants will be invited to participate in 2-hour FGDs comprising six to eight people per group. Participants will be purposively selected using stratified random sampling and invited to attend two FGDs with caregivers of children with high self-reported adherence (≥95% of doses consumed) and two FGDs with caregivers of children with moderate to low self-reported adherence (<85% of doses consumed). Caregivers will be invited to participate in FGDs after completion of the intervention. Semistructured FGD question guides will be informed by the Theory of Planned Behavior to understand individual behaviours and behavioural intent affecting IP acceptability and use, including perceptions of diarrhoeal risk, malnutrition and community healthcare-seeking norms.[41] FGD guides will be piloted at the beginning of the study and updated as needed (see online supplemental file). Additional areas of inquiry include the caregivers experience administering the IP and possible reformulations or delivery methods of the IP that might influence future acceptability or adherence. Healthcare workers at recruiting sites will also engage in FGDs, with six to nine healthcare workers participating in FGDs at each site. A minimum of four and a maximum of six healthcare worker FGDs will be conducted in total. Healthcare workers will be purposively selected to participate in FGDs if they are engaged in paediatric and/or nutritional care. They will be invited to participate in FGDs after at least 50% of participants (n=300) have been enrolled. Semistructured question guides will be used to determine healthcare worker perceptions of the IP and administration method, perceived facilitators and barriers to uptake and adherence, and opportunities to optimise IP administration in future demonstration projects. FGDs will be conducted during the lunch hour or after work to minimise disruption to health facility operations, and refreshments will be provided to all participating health workers. FGD participants will be compensated 600 KSH for their time.

Laboratory procedures

For molecular faecal assays (qPCR), total nucleic acid will be extracted from faecal samples using the QIAamp Fast DNA stool Mini kit with pretreatment.[42] Rectal swabs will be used for qPCR testing because swabs contain ample

Table 1 Sample storage and laboratory processing methods

Specimen	Purpose	Tests performed
Flocked rectal swabs	Quantitative PCR (qPCR)	Presence and quantity of enteric pathogens will be determined using qPCR with prespecified primers and probes. qPCR will be performed on swabs from all participants at W4 and a subset of 200 participants (50/arm) again at W16 and W24. This work will be performed at KEMRI-Wellcome in Kilifi, Kenya
	Storage	Rectal swabs from all clinical timepoints will be stored at −80°C for potential sequencing.
Whole stool	Storage	Whole stool from all clinical timepoints will be stored at −80°C for potential sequencing.
	Enteric dysfunction biomarkers	Commercially available ELISA, such as alpha-1-antitriypsin (A1AT), myeloperoxidase and calprotectin or similar tests will be used to measure enteric inflammation if funding becomes available. These markers will be assessed in all participants with whole stool available at the following timepoints: D0 (enrolment), W4, W10, W16 and W24. This work will be performed at the KEMRI Centre for Microbiology Research in Nairobi, Kenya.
Urine	Intestinal permeability	Concentrations of lactulose (L) and rhamnose (R) will be determined using high-performance liquid chromatography-mass spectrometry. This work will be conducted at the internationally recognised reference L:R laboratory at the Mayo Clinic (Rochester, Minnesota) or a similar institution that can provide high-performance liquid chromatography. L:R will be determined at W4, W10, W16 and W24 in a subset of children.
Blood	Haemoglobin	Haemoglobin concentrations will be performed at local clinical laboratory as part of a complete blood count, which has previously conducted testing for international paediatric research studies. Haemoglobin will be measured at W16 only. All children with moderate/severe anaemia will be referred for treatment at this timepoint according to national guidelines.
	Storage	Plasma and serum will be centrifuged from whole blood and stored at −80°C within 30 min of collection for potential inflammatory assays, such as C reactive protein. Additionally, dried blood spots will be created from remaining whole blood and stored for future research. Blood is collected at all health facility visits (enrolment, W4, W10, W16 and W24).

faecal material for testing. The laboratory testing outline is summarised in table 1.

Data management and confidentiality

All study staff will be trained in data protection and privacy. Participant questionnaires, laboratory samples and databases will be identified with a unique PID. A participant identifier link log will be maintained at the study sites in a locked cabinet in the study office and will be the only link between participant identification numbers and identifiable data. Study data captured using deidentified case report forms (CRFs) will be stored at the research site in a locked cabinet in a locked and secure study office that is accessible only by study staff. Data will be entered from the CRF into a comprehensive and secure web-based database, such as REDCap. The database will be password protected, will not contain any identifiers and data storage and handling will follow Good Clinical Practices at every stage. Data reports will be disseminated to the study team and to study coinvestigators and data monitors on a regular basis. Ongoing data cleaning will be performed by querying the data regularly.

Data analysis

Primary endpoints: the primary study endpoints are moderate-to-severe diarrhoea and nutritional recovery.

1. The incidence of moderate-to-severe diarrhoea will be defined as total number of new diarrhoea episodes (>48 hours after a diarrhoea-free period) deemed moderate to severe, divided by the child time at risk during the 6-month follow-up period. Time at risk will be censored at the date of last follow-up for children who have died or are lost to follow-up. Also, child time at risk during a diarrhoeal episode, including during the index diarrhoea, and the 48 hours after, will not be included in the denominator. Moderate-to-severe diarrhoea (instead of all diarrhoea) will be used because it is associated with poorer outcomes and incurs costs to healthcare systems and families, making it the most important to prevent. Moderate-to-severe diarrhoea

will be defined as ≥3 using the CODA (Community DiarrhoeA) diarrhoea severity score or dysentery (evidence or reported visible blood in stool).[43] The CODA diarrhoea severity score was developed to assess diarrhoea severity in the context of community-based studies and is validated against outcomes of weight and length. A cut-off of ≥3 (instead of CODAs original ≥1) will be used to add specificity to this severity indicator, as caregiver report of anorexia (defined as the child not willing to eat as usual) that is included in the CODA score has been found to lead to heterogeneity in severity scoring.[44] Diarrhoea information during the follow-up period will be ascertained through follow-up visit questionnaires administered every 2 weeks and enhanced by caregiver-completed daily diarrhoea diary entries. If care is sought at a study facility, diarrhoea information will also be ascertained through physical examination and caregiver interview at the time of care seeking.

2. Time to nutritional recovery will be defined as the number of days since enrolment to the date of the second of two consecutive MUAC measurements ≥12.5 cm. For participants who do not reach nutritional recovery, due to death, loss to follow-up or completion of the study prior to reaching recovery, they will be censored at the date of their last visit. Recovery by MUAC and weight-for-length z-score (WLZ) (depending on which is used to identify acute malnutrition by the nutrition programme) is the definition used by WHO to inform discharge from malnutrition programmes making it a policy-relevant outcome. We focused on MUAC instead of WLZ because MUAC is less sensitive to hydration status than weight.

Secondary endpoints include the following:

1. Diarrhoea (any severity) will be defined as diarrhoea (three or more abnormally loose or watery stool) during follow-up, irrespective of severity, ascertained through follow-up visit questionnaires and a diarrhoea diary. Episodes will be defined as per the primary outcome.
2. Severe diarrhoea will be defined by the CODA diarrhoea severity score of 7 or more or dysentery. Episodes will be defined as per the primary outcome.
3. Dysentery will be defined as evidence or reported visible blood in stool. Episodes will be defined as per the primary outcome.
4. Medically attended diarrhoea will be defined as diarrhoea that led to an outpatient or inpatient visit at a health facility or hospital that is typically attended by a nurse, clinical officer and/or physician. Episodes will be defined as per the primary outcome.
5. Cumulative duration of diarrhoea will be defined as cumulative days of diarrhoea ascertained from follow-up visit questionnaires and a diarrhoea diary, irrespective of episodes.
6. Incidence of hospitalisation will be defined as any inpatient admission that results in an overnight stay (irrespective of diagnosis) in a health facility and time to hospitalisation or death analysed as a combined outcome.
7. Haemoglobin concentration will be determined at week 16.
8. Growth that includes length and weight measurements at each time point will be used to create age-standardised z-scores, calculated using WHO-established reference standards and the WHO ANTHRO software. Linear growth will be defined as change (Δ) in LAZ. Ponderal growth will be defined as change (Δ) in WLZ and Δ MUAC.
9. Concentrations of specific markers of enteric function will include faecal alpha antitrypsin, myeloperoxidase, calprotectin at baseline, 4, 16 and 24 weeks; and the lactulose:rhamnose ratio at 16 and 24 weeks. Baseline samples that were collected after administration of the IP (eg, if whole stool could not be collected within 1 hour of enrolment) will be excluded in sensitivity analyses. Enteric dysfunction biomarkers have been selected based on the best currently available evidence. However, in this rapidly evolving field, other biomarkers of greater clinical significance may emerge during the study. Therefore, collected stool samples will be stored until the latter half of the study, and these named biomarkers may be substituted for the gold standard tests as determined by the best available evidence at the time of testing.
10. Prevalence of enteric infections will be determined by qPCR at or above the minimum limit of detection (ie, below cycle thresholds (CT) <35) at week 4 for all participants and at weeks 16 and 24 for a subset of 200 participants (50 per arm). We will additionally consider CT <30, indicating higher quantity infections, in secondary analyses. We will additionally group bacteria shown to be associated with growth faltering (*Campylobacter* species, Heat Labile Toxin Enterotoxigenic *Escherichia coli*, Enteroaggregative *Escherichia coli*, typical Enteropathogenic *Escherichia coli* and/or *Shigella*)[45] as a single variable in secondary analyses.
11. Acceptability is measured as the proportion of caregivers reporting that administration of the IP was desirable or satisfactory along with perceived trust, safety and comfort in the IP via 5-point Likert scale responses in surveys administered at follow-up visits and via emerging qualitative FGD thematic content from both caregiver and health worker FGDs.
12. Adherence will be defined in two ways: (1) the proportion of caregivers self-reporting that their child consumed some or all of the IP ≥95% of the time over 6-month follow-up using daily pictorial adherence logs and (2) adherence to the recommended dosing based on objectively measured container consumption (± 10% consumption of prescribed IP in all weeks).
13. Incremental cost-effectiveness of the IP will be estimated. Outcomes for the cost-effectiveness analysis include the incremental costs and cost per episode

of diarrhoea averted in each IP arm, compared with the placebo arm. We will conduct primarily bottom-up costing by documenting resources used, amounts used and unit costs/prices for each resource. We will exclude protocol-driven trial costs. Capital and fixed costs will be annualised over the period of implementation. We will report costs in both 2023 US dollars and local currency. Because all children in the study also receive standard-of-care diarrhoeal and malnutrition management, the placebo arm serves as an appropriate standard-of-care proxy.

To determine whether a 16-week course of lactoferrin, lysozyme or a combination of both shortens time to WHO-defined recovery from wasting (MUAC ≥12.5 cm) and reduces the incidence of moderate-to-severe diarrhoea during the subsequent 6 months following presentation to a health facility with diarrhoea among children with moderate/severe childhood wasting

Analysis of primary outcomes will include all randomised children according to the treatment they were randomised to receive but will exclude those who were deemed ineligible, post randomisation (modified intention to treat (ITT)). The rate ratio (RR) of each intervention arm compared with placebo will be determined using a Poisson model with number of new diarrhoea episodes as the outcome and time at risk during the 6-month follow-up period as the model offset and Wald χ^2 tests of the two-way intervention arm comparisons. Time at risk will be censored at the date of last follow-up for children who have died or are lost to follow-up. Also, child time at risk during a moderate-to-severe diarrhoeal episode, and the 48 hours after, will not be included in the denominator. The median time to nutritional recovery will be compared between intervention arms using Kaplan-Meier survival analysis and associated log-rank tests for each two-way comparison of intervention group to placebo. Cox-proportional hazards regression will be used for analysis of time to nutritional recovery with adjustment for inpatient/outpatient status (because randomisation was stratified) and baseline covariates (eg, baseline nutritional status, comorbidities and HIV status) if needed.

A child will be considered lost to follow-up if they did not attend at least one of the follow-up clinic visits (at weeks 4, 10, 16 and 24) and were not available at any of the home visits at week 2 or beyond (at 2, 6, 8, 12, 14, 18, 20 and 22 weeks). We will conduct subgroup analyses to evaluate the effect of interventions in subgroups: (1) severe versus moderate wasting, (2) high/low adherence to the intervention (as defined above) and (3) age <11 months versus 12–24 months. These will be presented as Forest plots and evaluated visually. Children who were completely lost to follow-up, that is those having no follow-up visits, will be excluded from the primary analysis then included in sensitivity analysis assuming they have had the primary outcome (for the diarrhoea analysis) and assuming they did not have the event (for nutritional recovery). In per-protocol analyses (secondary to the ITT), we will compare treatment effects in groups defined by self-reported adherence to the intervention. Per-protocol analyses will also exclude children who were ineligible, those who were lost to follow-up (defined as missing all follow-up visits) and those who withdrew consent. We will assess the pattern of missingness in the data to establish if it appears to be missing at random. If there is substantial missing covariate data (>10%), multiple imputation using the Markov chain Monte Carlo method will be used to impute covariate information where appropriate. Missing outcome data will not be imputed, but participants will be censored at the last follow-up visit therefore contributing some person-time to the analysis. Additionally, in analyses secondary to the primary analysis, we will test for effect modification between IPs using a Wald test. If there is no evidence of effect modification using a conservative alpha of 0.1, we will test the two lactoferrin and two lysozyme arms independently in comparison to the non-lactoferrin and non-lysozyme arms, respectively, to determine the efficacy of each supplement capitalizing on the factorial design.

To explore whether a 16-week course of lactoferrin, lysozyme or combination therapy improves secondary clinical, nutritional, enteric pathogen and enteric function outcomes

Risk of death or first rehospitalisation among children in the placebo arm will be compared between each intervention arm and placebo using Cox proportional hazards regression. Participants will be censored at the date of study completion or loss to follow-up. We will compare the mean change in cumulative duration of diarrhoea, haemoglobin concentration, linear growth, ponderal growth, antitrypsin, myeloperoxidase and calprotectin using multiple generalised linear mixed effects models with random intercepts. The RR of incidence of severe diarrhoea, dysentery, diarrhoea (any severity) and medically attended diarrhoea of each intervention arm compared with placebo will be determined using a Poisson model. Because L:R ratio is only measured after diarrhoea resolution, mean L:R ratio will be compared using a similar approach but omitting the baseline timepoint. Finally, GEE with Poisson link and independent correlation structure will be used to evaluate the prevalence of enteric bacteria, over time in each intervention arm compared with placebo. All analyses will be adjusted for multiple comparisons using the Benjamini and Hochberg method using a false discovery proportion of 0.05.[46]

To evaluate acceptability, adherence and cost-effectiveness of lactoferrin and/or lysozyme administration in Kenya

(1) Acceptability: quantitative acceptability measures will be presented as an average per visit by arm. Time series analysis will be used to understand increases, decreases and correlation between acceptability measures over time, by treatment arm, thus serving as a potential explanatory variable for adherence. FGDs will be transcribed verbatim into the local language if not conducted in English. Five 1 min quality assurance spot checks will be conducted on

each transcript by an independent researcher fluent in the local language to determine quality of the transcription. Transcribed data will be translated into English and reviewed by an independent reader to confirm the quality of the translation. Two independent coders will code the qualitative data in ATLAS.ti using thematic coding and a mix of inductive and deductive coding to identify the constructs influencing acceptability and adherence.[47] A codebook will be prepared prior to data collection and will be added to and updated iteratively. The two independent coders will meet weekly to review coded transcripts, ensure intercoder agreement, to add to or refine the codebook accordingly, and seek agreement on a thematic analysis. (*2) Adherence:* the proportion of caregiver/child dyads who were adherent to the IP will be reported according to each definition above. Multivariate logistic regression will be used to determine if baseline IP acceptability as reported on caregiver questionnaires influences adherence to (1) self-reported adherence of ≥95%, (2) objective container monitoring adherence of ≥95%. (3) *Cost-effectiveness*: we will estimate the incremental cost-effectiveness ratio (ICER) for each IP compared with the placebo. Analyses will be conducted in Treeage Pro 2018 Healthcare Module[48] to estimate moderate-to-severe diarrhoeal events averted among a static population of each trial intervention group compared with the placebo group. We will use a 6-month time horizon to estimate the cost-effectiveness of the intervention on short-term moderate-to-severe diarrhoeal episodes over the duration of the trial. Deterministic and probabilistic sensitivity analyses will be conducted, including to determine if broadening treatment criteria from children with malnutrition to non-wasted paediatric populations affects observed ICERs.

Data and safety monitoring

A Data Safety and Monitoring Committee (DSMC) was established prior to study initiation to monitor SAEs and to evaluate the statistical analysis plan and stopping rules for the trial.[49] The DSMC membership included expertise in clinical trials, statistics, child mortality assessment, ethics and paediatric care in resource-limited settings. SAEs will be monitored in real time and will be summarised and reported to the DSMC safety officer, study investigators and relevant institutional review boards (IRBs) in accordance with IRB-specific regulations. We will also conduct trial audits (not independent of investigator or trial personnel) every 6 months.

A single interim analysis for moderate-to-severe diarrhoea incidence will be performed using an O'Brien-Fleming boundary for harm when 50% of expected person time (150 child years) has been accrued. Interim analyses will be based on two-way comparisons between three intervention arms and placebo. For 37.5 child years accrued in each arm at the interim analysis, we expect to see 38 events of moderate-to-severe diarrhoea in the placebo arm (expected moderate-to-severe diarrhoea incidence of 100 episodes/100 child years,[50] 100/100*37.5=38) and 24 events in each of the three intervention arms (35% reduction in this incidence in any of the interventional arms,[25 31] 0.65*37.5=24). Assuming 62 events will be available at half of the person-time accrual per pairwise comparison, a z-score critical value of 2.797, or p value <0.005, from Wald χ^2 tests of the pairwise intervention arm comparisons with placebo calculated using Poisson regression models will be the cut-off of statistical significance. An interim analysis for harm based on time to nutritional recovery will not be conducted because it is not clear that days difference in time to nutritional recovery is harmful. Time to nutritional recovery and adverse events will be reported descriptively.

The DSMC will consider the totality of evidence from the interim analysis and descriptive data to make a determination about continuing the study. Futility will not be a basis for stopping rules because of the trials' value in understanding mechanisms of postdiarrhoea nutritional recovery and recurring diarrhoeal episodes. Benefit will also not be a basis for interim stopping because the IP is not an immediate life-saving treatment and the placebo arm, or the non-beneficial arm, can still seek care and treatment. Time to nutritional recovery and adverse events will be reported descriptively.

Assuming the DSMC decides to continue the trial after the interim analysis, an alpha of 0.04 will be used as the statistical significance boundary at the final analysis for the primary hypothesis tests.

Statistical power and sample size

To determine whether a 16-week course of lactoferrin, lysozyme or a combination of both shortens time to WHO-defined recovery from wasting (MUAC ≥12.5 cm) and reduces the incidence of moderate-to-severe diarrhoea during the subsequent 6 months following presentation to a health facility with diarrhoea among children with moderate/severe childhood wasting: the total sample size required (600 children, 150 per arm) was calculated based on the comparison of moderate-to-severe diarrhoea incidence between any single intervention arm (lactoferrin, lysozyme or combined therapy) to the control arm (placebo-treated children). We assumed the incidence of moderate-to-severe diarrhoea in the placebo-arm to be approximately 100 episodes/100 child years based on the Global Burden Disease 2019 estimates of under 5 diarrhoea incidence of 167/100 child years[51] and assuming 60% of these episodes would meet the moderate-to-severe definition based on previous data.[50] We assumed a 35% reduction in this incidence in any of the interventional arms based on previous data.[25 31] Using an alpha of 0.05 and assuming 80% power, we will require, at minimum, 134 children per intervention arm. Assuming 2% of children die during follow-up, based on estimates of mortality during convalescence from moderate-to-severe diarrhoea in Kenya,[50] and a 10% loss-to-follow-up, we will enrol an additional 16 children per treatment arm, recruiting 600 children in total (150 per arm). With 134 surviving children in each treatment arm with complete follow-up, we

determined the minimum detectable difference in time to nutritional recovery between each intervention arm and the placebo arm assuming an alpha of 0.05 and 80% power. This conservative estimate is based on not being able to include data prior to censoring so represents the minimal detectable times. Data from CMAM programmes suggest that approximately 70%–85% of children in CMAM programmes recover to a MUAC ≥12.5 cm, with recovery taking between 4 and 8 weeks with SD ranging from 0.2 to 3.2 weeks, based on frequency of anthropometric assessments (studies with less-frequent assessments result in larger SD of time to recovery).[52–56] Based on these assumptions, we will be powered to detect a difference of between 1 day (assuming a 0.2 SD) and 1 week (assuming a 3.2 SD) difference in time to nutritional recovery, respectively.

To explore whether a 16-week course of lactoferrin, lysozyme or combination therapy improves secondary clinical, nutritional, enteric pathogen and enteric function outcomes: assuming 2% of children die during follow-up based on estimates of mortality during convalescence from moderate-to-severe diarrhoea in Kenya and 10% are hospitalised after diarrhoea copresentation with wasting, we will have 80% power to detect an HR of 0.3 or greater (in magnitude) of any two-way comparison of an intervention arm to placebo. Because this analysis will be underpowered, we will focus interpretation on the measure of effect as opposed to p values. Haemoglobin assessment and linear growth power calculations are based on the mean difference likely to be observed over 6 months. Data from the Childhood Acute Illness & Nutrition (CHAIN) cohort suggests that the mean of haemoglobin of children with acute malnutrition and diarrhoea is 10 mg/dL with an SD of 3 mg/dL.[57] Data from previous clinical trials suggest that a prolonged course of lactoferrin may lead to a 1 mg/dL increase and we will have 82% power to detect that difference.[29] The linear growth outcome will be assessed as change in LAZ between baseline and day 180. Over this time frame, we expect children of this age recovering from an acute illness to have a decline in delta LAZ of 0.25 with a SD of 0.1. Assuming a sample size of 134 children per arm with growth data, and an alpha of 0.05, this study would have 90% power to detect change in delta LAZ of 0.05. For the markers of enteric dysfunction, concentration and SD of myeloperoxidase are included in the power calculation as previous evidence suggests that this marker has the greatest variance. Assuming a mean log concentration of 8.9 ng/mL and SD of 1.26 ng/mL, 110 with 0.05 alpha and 50 per treatment group, we will have 80% power to detect a difference of 0.7 mg/L (8%) between two groups at any timepoint, a difference considered clinically meaningful. Anticipating a mean L:R ratio of 0.75 (SD 5.4) in the placebo group as observed in previous work in Kenya, and using data from the recent lactoferrin and lysozyme study in Malawi where there was a 30% improvement in lactulose detection (SD 9% to 14%), we determined that we will be able to detect a difference in L:R ratios of at least 4% and 6%, respectively. We expect that placebo-treated children will have prevalences of EAEC, *Campylobacter* and *Shigella* of 51%, 28% and 15%, respectively. With all children other than those who died or were lost to follow-up having a week 4 sample (134 children per arm), we will be able to detect a 25%, 37% and 50% reduction in EAEC, *Campylobacter* and *Shigella* prevalence, respectively, between intervention arms. In the subset of ~50 children per arm who will have enteric pathogen data at weeks 16 and 24, we will be able to detect prevalence reductions ranging from 35% to 62%.

To evaluate acceptability, adherence and cost-effectiveness of lactoferrin and/or lysozyme in Kenya: the sample size for the qualitative data collection is based on the ability to reach data saturation by the re-emergence of key themes across data sources, time and resources. If during qualitative data collection, we are not able to reach data saturation using the prespecified FGD sample sizes as detailed above, an additional two FGDs will be conducted with caregivers and health workers, respectively, to ensure comprehensiveness and representativeness of the qualitative data.

Study timeline

The trial began on 10 March 2023 and participant recruitment will continue over a 24-month period. The last follow-up visit will occur 6 months after the final enrolment takes place. Data analyses and laboratory assays will be conducted throughout the active trial period and the 6 months after. All data analysis, results dissemination and manuscript preparation will be completed within 2 years after the last participant is recruited (allowing 6 months for last follow-up visit, 6 months for final laboratory assays and 9 months for data analysis and interpretation). This realistic timeline will enable completion and submission of all manuscripts related to the primary aims of the study by quarter four of 2026.

Potential challenges and limitations

If fewer children seek care for diarrhoea than we expected, or if fewer children have wasting than expected, as can occur during years of high harvests, an additional ancillary recruiting site will be opened at nearby health facilities to increase recruitment. Kenya has two rainy seasons where diarrhoea prevalence is highest, in February–March and June,[58] and we will recruit for 2 years' worth of high diarrhoea season. If the interventions fail to demonstrate efficacy at preventing diarrhoea recurrence or shortening duration of nutritional recovery, then the mechanistic objectives described under aim 2 will lose impact. However, the detailed clinical and laboratory data will enable a thorough understanding of why children fail to recover clinically and nutritionally from diarrhoea, and we will be extremely well powered to address these questions with an observational study of 600 children. This trial is focused on children at highest risk of poor outcomes during diarrhoea recovery to optimise our likelihood of finding an effect of the intervention if one

exists. However, children recovering from diarrhoea not yet meeting the definition of wasting, but who are at risk for wasting, may also benefit.

Patient and public involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Ethics and dissemination

The study has received approvals from the IRBs of the KEMRI, the University of Washington, the Kenyan Pharmacy and Poisons Board, and the Kenyan National Commission on Science, Technology and Innovation. The clinical trial is also registered with clinicaltrials.gov (NCT05519254) and Pan African Clinical Trial Registry (PACTR202108480098476). Any modifications to the study protocol or consent materials will be submitted for approval all regulatory authorities before implementation.

The results of this trial will be published in peer-reviewed journals and the key findings will be presented at relevant Kenya-based and international conferences and shared with stakeholders. The clean deidentified dataset from the trial, data dictionary, statistical code and other study documents (CRFs, standard operating procedures, statistical analysis plan, informed consent forms and protocol) will be made public.

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Contributors KDT and PBP conceived this trial, with substantial input from BOS, JW, BAR, IT, JN, ERH, GJ-S and ARM. PBP and BOS are study coprincipal investigators, and KDT is the project director. RT developed the statistical analysis plan with oversight from BAR. ARM developed the acceptability, adherence and cost-effectiveness plan. JN, JL, JAP-M and ERH developed the enteric pathogen testing plan. LK developed procedures related to the investigational product. JO, EY, MO and BOS coordinated and oversaw the implementation of all clinical study procedures. MMD, EO and JN developed the study database, and DR oversees all laboratory procedures. All authors contributed to the development of this manuscript and/or study procedures and to reading and approving the final version for publication. PBP is the guarantor.

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