

Efficacy of Rice-based Oral Rehydration Solution Containing Recombinant Human Lactoferrin and Lysozyme in Peruvian Children With Acute Diarrhea

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ABSTRACT

Objective: To compare glucose and rice-based oral rehydration solution with rice-based oral rehydration solution containing recombinant human lactoferrin and recombinant human lysozyme in diarrhea outcomes.

Patients and Methods: We conducted a randomized, double-blind controlled trial in children with acute diarrhea and dehydration. One hundred and forty children 5 to 33 months old were block randomized to receive low osmolality WHO-ORS (G-ORS), rice-based ORS (R-ORS), or rice-based ORS plus lactoferrin and lysozyme (Lf/Lz-R-ORS). Intake and output were monitored for 48 h in the ORU, with continued monitoring through home and clinic follow-up for 14 d.

Results: The G-ORS and R-ORS groups did not show any differences in diarrhea outcomes and were therefore combined as the control group. Intent-to-treat analysis showed a

significant decrease in duration of diarrhea (3.67 d vs 5.21 d, $P=0.05$) in the Lf/Lz-R-ORS group as compared with the control group and a significant increase in the number of children who achieved 48 h with solid stool, 85% vs 69% ($P<0.05$). There were also decreases in volume of diarrhea and the percentage of children who had a new diarrhea episode after achieving the endpoint.

Conclusions: Addition of recombinant human lactoferrin and lysozyme to a rice-based oral rehydration solution had beneficial effects on children with acute diarrhea. *JPGN* 44:258–264, 2007. **Key Words:** Acute diarrhea—Oral rehydration solution—Human lactoferrin—Human lysozyme. © 2007 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

INTRODUCTION

Diarrhea remains 1 of the 2 most important causes of morbidity and mortality in children under 5 years of age. The World Health Organization (WHO) estimates that as many as 1.8 million children die from diarrhea each

year (1). Peru has a high rate of diarrheal disease and it is estimated that 20% to 25% of the 36,000 pediatric deaths each year in Peru are caused by diarrhea (2). In Peru and other less privileged countries, diarrhea is generally of infectious origin and can be associated with *Escherichia coli*, *Giardia*, rotavirus, *Shigella*, *Salmonella*, and *Campylobacter*, among others (3). Repeated attacks of diarrhea in children can lead to undernutrition, growth failure, and compromised immune function (2,4). The cornerstone of treatment for children with diarrhea and associated dehydration is oral rehydration therapy. Since the development of a glucose-based oral rehydration solution (ORS) (4), millions of childhood deaths have been avoided (5–7). One limitation of the original WHO-ORS formulation was failure to reduce the severity of diarrhea. Substitution of a rice-based ORS (R-ORS) was successful in reducing the volume of diarrhea in patients with cholera (8). One suggested mechanism of action was the reduction of osmolality in the R-ORS. Recently, WHO recommended a new formulation with low osmolality, 245 mOsm/L as

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compared with 311 mOsm/L in the original formulation, which has proven to be more efficacious (9). In Mexico R-ORS was able to reduce the rate of intravenous fluid intervention; however, for other diarrhea outcomes, low osmolarity G-ORS and R-ORS were similar (10).

Breast-fed infants have a lower incidence of diarrhea, as well as other infections, when compared with non-breast-fed infants (4,11). In vitro data suggest that the milk proteins lactoferrin and lysozyme play an important part in the protective role of breast milk (12). Lactoferrin and lysozyme individually and in combination have demonstrated activity against a wide spectrum of bacteria, viruses, parasites, and fungi (12). The addition of human lactoferrin and lysozyme to an ORS may result in a reduced duration of diarrheal disease and enhanced rate of recovery. The availability of an ORS that provides bioactive proteins found in breast milk, along with rehydration, would provide a potential major advance in the management of infectious diarrhea.

To produce human lactoferrin and lysozyme in large quantities, recombinant technology has been used with rice as the host organism (13,14). This was achieved by inserting the genes for human milk lactoferrin and lysozyme, respectively, into rice. The recombinant proteins produced were tested extensively in the laboratory and found to be substantially equivalent to the native proteins in all biochemical and functional tests (13,14). Expression of human lactoferrin and lysozyme in rice is an attractive approach because rice is among the first foods recommended for introduction to infants. It has good nutritional value and low allergenicity. Thus, any residual materials from rice introduce no risk and may be viewed as nutritionally sound. Because human lactoferrin and lysozyme are a major part of the diet of breast-fed infants, we hypothesized that the addition of recombinant human lactoferrin and lysozyme to ORS at concentrations found in breast milk may provide additional benefits in the treatment of diarrhea.

PATIENTS AND METHODS

Design, Setting, Participants

The study was designed as a prospective, double-blind, randomized protocol conducted at the Oral Rehydration Units (ORU) of the Instituto Especializado de Salud del Niño (IESN), Lima, Peru, and the Hospital Belén, Trujillo, Peru. The study enrolled male children ages 5 to 33 months with acute diarrhea (≥ 3 watery stools per day) of < 72 h duration without antibiotic treatment or evidence of blood in the stool. Children exclusively breast-fed, children with severe malnutrition (weight for height z-score < 2 SD below the National Center for Health Statistics reference population (15), and children with chronic or severe illnesses were excluded from the study. Subjects were enrolled after written informed consent by the parent or guardian. Children were randomly assigned to 1 of 3 treatment groups

in blocks of 6 (<http://www.randomization.com>). Treatment material was randomized and consecutively labeled by the formulator.

Ethical Considerations

The protocol was approved by the Instituto Nacional de Salud (INS) from the Ministry of Health of Peru, the Ethics Committees of the IESN, Instituto de Investigación Nutricional (IIN), and the University of California, Davis.

Interventions

The study included 3 treatment groups: glucose-ORS based on the WHO recommended low-osmolarity formulation (G-ORS), an R-ORS CeraLyte 70 (Cera Products, Columbia, MD), and the R-ORS with added recombinant human lactoferrin and human lysozyme (Lf/Lz-R-ORS), expressed in rice as described earlier. The transgenic rice was grown and harvested; recombinant lactoferrin was purified from rice powder using salt extraction and ion exchange chromatography (16). Following purification, the iron was removed from the lactoferrin creating apo-Lf. The final lactoferrin product for inclusion in the ORS was 96% protein, 92% lactoferrin, and 0.013 mg/g solid total iron. Recombinant lysozyme was also purified by salt extraction and ion exchange chromatography. The final lysozyme product for inclusion in the ORS was 79% protein and 83.6% lysozyme. The final composition of the 3 ORS solutions in 1 L of boiled water is shown in Table 1.

Study Procedure

Children with acute diarrhea and mild to moderate dehydration were assessed by a pediatrician at the ORU. The ORS solutions were packaged in multipacket bags, randomized by the sponsor, and then numbered consecutively. An eligible child was given the next consecutive number and the ORS packets were labeled with that number. During the first 4 h of the rehydration phase, children were given ORS according to WHO guidelines, 50 to 80 mL/kg body weight. At the end of

TABLE 1. Final composition of ORS solutions in 1 L of boiled water

Ingredients	G-ORS*	R-ORS†	Lf/Lz-R-ORS‡
Sodium, mmol/L	75	70	70
Potassium, mmol/L	20	20	20
Chloride, mmol/L	80	65	65
Citrate, mmol/L	10	10	10
Glucose, g/L	20.0	—	—
Rice carbohydrate, g/L	—	41.0	41.0
rh lactoferrin, g/L	—	—	1.0
rh lysozyme, g/L	—	—	0.2
Osmolarity, mOsm/L§	224 $\pm$ 25.5	234 $\pm$ 10.4	232 $\pm$ 12.4

* G-ORS based on WHO-recommended low-osmolarity formulation.

† Premixed R-ORS solution with low osmolarity.

‡ Premixed R-ORS solution with recombinant human lactoferrin and lysozyme added at levels found in human milk.

§ Actual measurements on final formulation.

the 4-h period, the children were clinically reassessed, 24 children (17.1%) needed 1 to 2 more hours for rehydration, with no difference by treatment group. The maintenance phase was initiated and continued until the children had completed 48 h in the ORU. During the maintenance phase ORS was given to replace measured loss of stool; at this time children were offered water and age-appropriate food ad libitum.

Monitoring of Participants

All intake and output was measured and recorded until the cessation of diarrhea or a maximum of 14 d or upon withdrawal from the study. Stool output was measured throughout the study using preweighed disposable diapers. Urine was collected in urine bags during the first 48 h while the children were in the rehydration unit. Stool consistency was recorded using a scale of 1 to 5: solid, soft, semiliquid, brown liquid, and watery. A score of 1 or 2 for 48 h was considered end of diarrhea. The presence of blood in the stool was detected by visual inspection. ORS was prepared in 1-L gradation marked bottles. Parents were instructed to boil and cool water before mixing the ORS and to discard unused material after 24 h.

Children were monitored in the hospital ORU for the first 48 h. At the end of the 48 h, children were examined by the pediatrician before discharge and returned home. Families were visited daily from days 3 to 6 by a health worker from the IIN. They collected diapers, recorded ORS intake, and provided more sachets of ORS if necessary. On day 7, children returned to the clinic for evaluation. If the child was still experiencing diarrhea, then a health worker continued to visit on days 9, 11, and 13. All of the children returned to the clinic for a final visit on day 14. On day 14, a capillary blood sample was taken and hemoglobin was measured immediately using a portable HemoCue B-hemoglobin photometer (HemoCue AB, Ängelholm, Sweden).

Stool Culture

Stool samples, collected at study entry, were placed in stool culture transport vials (Cary-Blair) and sent from the ORU at the IESN to the IIN laboratory; in Trujillo stool culture was done at the reference laboratory of the National Institute of Health, Ministry of Health, Region La Libertad. For isolation of *Salmonella*, *Shigella*, *E coli*, and *V. cholerae*, samples were cultured directly onto commonly used selective enteric media (SS agar, XLD agar, MacConkey agar, and TCBS agar plates) enriched in selenite broth and alkaline peptone water. For isolating *Campylobacter*, samples were cultured directly onto Butzler agar. *E coli* isolates were tested using a Multiplex Real Time SYBR Green-based polymerase chain reaction for detection of enterotoxigenic, enteropathogenic, Shiga toxin-producing, enteroinvasive, enteroaggregative, and diffusely adherent bacteria (17). *E coli* polymerase chain reaction analyses were performed on samples from 130 children in the Lima group.

Rotavirus Detection

Presence of rotavirus was assessed with an enzyme immunoassay for detection of rotavirus (Group A) in human fecal specimens from IDEIA Rotavirus (DakoCytomation, Carpinteria, CA).

Parasitology

For parasite analysis, merthiolate-iodine-formaldehyde was used for preserving, staining, and fixing the stool specimens (<http://www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm>). We used an enzyme immunoassay for detection of *Giardia*-specific antigen (GSA 65) in aqueous extracts of fecal specimens (ProSpecT *Giardia* Microplate assay, Alexon-Trend, Lenexa, KS).

Statistical Analysis

Data were analyzed using SAS version 9.0 (SAS, Cary, NC). χ^2 tests were used to compare categorical variables and ANOVA tests were used to compare continuous variables among the 3 groups. For nonparametric variables the Mann-Whitney *U* test was used. *z* Scores were calculated using Epi-Info version 3.2.2 (Centers for Disease Control and Prevention, Atlanta, GA). A Cox proportional hazards model was used to analyze and display the proportion of unresolved diarrhea. There were no significant differences between the 2 conventional ORS treatment groups (G-ORS and R-ORS) and data were therefore pooled (CC-ORS) for comparisons with the experimental group (Lf/Lz-R-ORS).

RESULTS

The 3 ORS were similar in content of electrolytes; the R-ORS contained 41 g rice carbohydrate/L, equivalent to 20 g glucose/L in the G-ORS. The experimental Lf/Lz-R-ORS contained recombinant human lactoferrin at 1 g/L and recombinant human lysozyme at 0.2 g/L, concentrations similar to those found in human milk. Osmolarity was similar in the 3 ORS studied (Table 1).

One hundred and forty boys ages 5 to 33 months were randomized into the study; 130 children were enrolled at the IESN Hospital, Lima, and 10 at Belén Hospital, Trujillo. Of these, 135 completed the 14-d study. A flowchart of the study is shown in Fig. 1. Two subjects were lost to follow-up, 2 were excluded from analysis because inclusion criteria were not met, and 1 subject withdrew because of an adverse event unrelated to the test product. No child was severely malnourished (W/H) and 76 of 135 children (56%) had anemia at study completion (hemoglobin <110 g/L) with no differences between groups. The 3 groups were comparable in age, entry weight, hemoglobin, degree of dehydration, and duration of diarrhea at enrollment (Table 2). More children in the Lf/Lz-R-ORS group had an identified pathogen in the stool and the pathogen was more frequently bacterial and *E coli* (Table 3). Clinical outcomes in the Lf/Lz-R-ORS group compared with the combined controls (CC-ORS) are shown in Table 4.

Stool Volume and Duration of Diarrhea

Duration of diarrhea was shorter ($P = 0.05$) in the Lf/Lz-R-ORS group than in the CC-ORS group, 3.67 versus 5.21 d. Stool volume did not significantly differ between

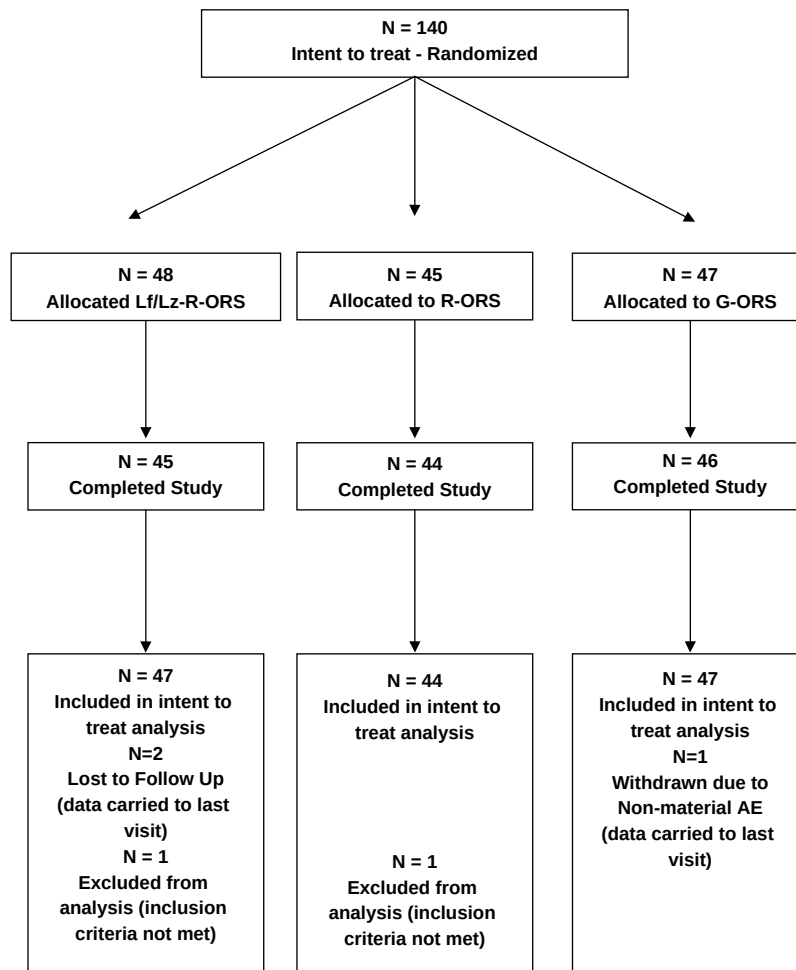


FIG. 1. Flowchart of enrollment

groups, but percentage of children with solid stool for 48 h was significantly higher ($P = 0.04$) in the Lf/Lz-R-ORS group (85.1%) than in the control (CC-ORS) group (69.2%). The percentage of children with relapse after 48 h was lower in the Lf/Lz-R-ORS group than in controls (8.5 vs 18.7%); however, because of large interindividual variations, this difference was not significant. There were no significant differences in total intake of ORS.

The proportion of unresolved diarrhea by duration of treatment (Kaplan-Meier plot) for the 3 groups is shown in Fig. 2. The Lf/Lz-R-ORS group was lower than the other groups at days 1 and 2 and after day 6 of treatment.

Adverse Events

Twelve children experienced 16 adverse events; 11 were mild respiratory infections, 2 had vomiting and from this group 1 required intravenous therapy, 1 swallowed a coin, 1 had periorbital cellulitis, and 1 had solid skin lesions consistent with insect bites. No adverse events were related

to the treatment group. None of the children had persistent diarrhea.

DISCUSSION

This randomized, double-blind study showed that in 5- to 33-month old boys with acute diarrhea and mild to moderate dehydration, the addition of 2 recombinant human milk proteins, lactoferrin and lysozyme, to a rice-based ORS significantly decreased the duration of diarrhea and increased the percentage of children with solid stool for 48 h. For other endpoints, the Lf/Lz-R-ORS subjects showed improvement compared with the controls; however, the differences did not reach statistical significance. There were no adverse events associated with Lf/Lz-R-ORS.

In this study, we evaluated both the standard G-ORS treatment (regular clinical practice) and a commercial R-ORS. Use of R-ORS has been shown to have a significant beneficial effect on the outcome of cholera

TABLE 2. Baseline characteristics of children in the 3 treatment groups

	Lf/Lz-R-ORS (N = 47)	R-ORS (N = 44)	G-ORS (N = 47)
Age, mo			
Mean $\pm$ SD	13.4 $\pm$ 5.5	13.9 $\pm$ 6.0	13.6 $\pm$ 7.0
Range	6–28	5–33	5–32
3–12 mo (no., %)	22 (46.8%)	20 (45.5%)	27 (57.5%)
13–24 mo (no., %)	24 (51.1%)	21 (47.7%)	14 (29.8%)
25–36 mo (no., %)	1 (2.1%)	3 (6.8%)	6 (12.8%)
Entry weight, kg			
Mean $\pm$ SD	9.8 $\pm$ 1.7	9.7 $\pm$ 1.9	9.8 $\pm$ 1.8
Range	6.3–14.9	6.1–13.9	7.0–14.7
z Score (weight for age)	–0.60	–0.73	–0.61
Entry height, cm			
Mean $\pm$ SD	76.9 $\pm$ 6.7	78.0 $\pm$ 8.1	77.3 $\pm$ 8.0
Range	65–92	66–106	65–98
z Score (height for age)	–0.34	–0.27	–0.10
Hemoglobin (g/L $\pm$ SD)	106.9 $\pm$ 12	108.6 $\pm$ 12.9	107.0 $\pm$ 11.7
Dehydration			
Mild (no., %)	35 (75%)	33 (75%)	33 (70%)
Moderate (no., %)	12 (25%)	11 (25%)	13 (28%)
Duration of diarrhea pre-enrollment, h			
Mean $\pm$ SD	34.1 $\pm$ 16.8	34.3 $\pm$ 18.2	33.4 $\pm$ 18.5
Range	6.6–70.0	3.0–72.0	5.9–79.9

diarrhea (18,19), but has not been found to result in significantly improved outcomes in noncholera diarrhea in children (18–20). Our results are similar; we did not find any significant difference between these 2 control treatments in any outcome studied. The R-ORS was included as a control because the recombinant lactoferrin and lysozyme were produced in rice. If this treatment is proven effective, then extensive purification of the proteins from rice may not be needed for inclusion in ORS, which would reduce production costs and thus the cost of the product.

TABLE 3. Pathogens identified in stool samples

	LF/LZ (N = 47)	Rice (N = 44)	WHO (N = 47)	P value
Pathogens*				
None	6 (12.8%)	13 (29.5%)	19 (40.4%)	0.01 [†]
1	26 (55.3%)	21 (47.7%)	17 (36.2%)	0.17
2	12 (25.5%)	9 (20.5%)	7 (14.9%)	0.44
3	3 (6.4%)	1 (2.3%)	4 (8.5%)	0.44
4	0 (0%)	0 (0%)	1 (2.1%)	
Rotavirus	9 (19.1%)	8 (18.2%)	7 (14.9%)	0.85
Bacteria	37 (78.7%)	24 (54.5%)	25 (53.2%)	0.02 [†]
<i>Campylobacter</i>	9 (19.1%)	6 (13.6%)	10 (21.3%)	0.62
<i>E coli</i>	24 (51.1%)	16 (36.4%)	15 (31.9%)	0.14
<i>Blastocyst</i>	10 (21.3%)	5 (11.4%)	9 (19.1%)	0.43
<i>Klebsiella</i>	1 (2.1%)	1 (2.3%)	0 (0%)	0.61
<i>Shigella</i>	0 (0%)	1 (2.3%)	1 (2.1%)	0.46
Other	2 (4.3%)	0 (0%)	1 (2.1%)	0.52
Parasites	3 (6.4%)	3 (6.8%)	2 (4.3%)	0.92
<i>Giardia</i>	2 (4.3%)	3 (6.8%)	2 (4.3%)	0.73
<i>Ascaris</i>	1 (2.1%)	0 (0%)	0 (0%)	0.48

* Patients may have >1 pathogen; hence, the total may exceed the number of patients.

[†] Significant P value.

It has been shown that breast-feeding is associated with a lower incidence of illness in infants, even in affluent countries (21). Furthermore, the duration of illness was found to be significantly shorter in breast-fed than in non-breast-fed infants. Although it has not been proven that lactoferrin and lysozyme are the factors in breast milk responsible for the reduced duration of illness, it is interesting to note that addition of these proteins significantly decreased the duration of diarrhea. Lactoferrin has been shown to have both bacteriostatic and bactericidal activity against a broad spectrum of pathogens, regardless of its iron saturation (22). Lactoferrin has also been shown to have antiviral activity (23). Lysozyme is known to be able to kill gram-positive bacteria in vitro by hydrolyzing the bacterial outer membrane (24). In addition, Ellison and Giehl have shown in vitro that these 2 proteins can kill pathogens in a synergistic fashion, with lactoferrin releasing and binding lipopolysaccharide from the outer membrane of gram-negative bacteria, literally opening holes in the membrane, allowing lysozyme access to the inner

TABLE 4. Clinical outcome measures

Outcome measure	Lf/Lz-R-ORS (N = 47)	CC-ORS (N = 91)	P value
Total ORS intake, mL	1910	2162	0.18
Duration after randomization, d	3.67	5.21	0.05
Total stool volume, g/kg body weight	97.2	102.1	0.24
Percentage with solid stool for 48 h	85.1	69.2	0.04
Percentage with relapse	8.5	18.7	0.11

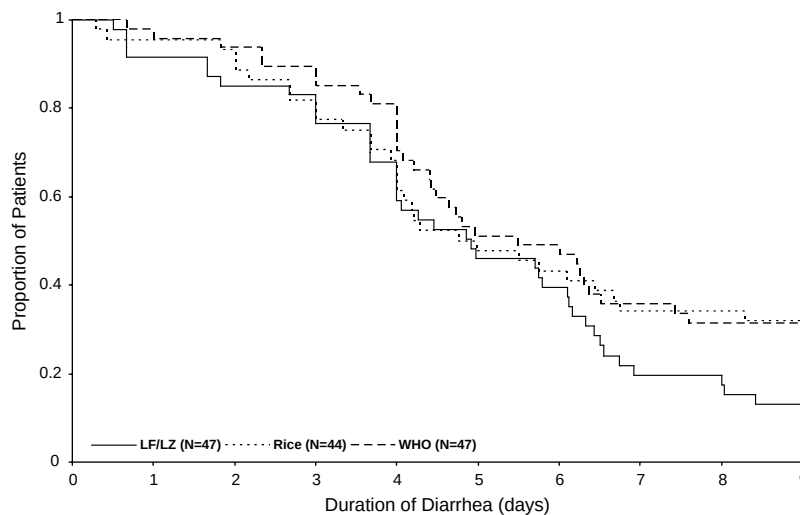


FIG. 2. Kaplan-Meier survival curves showing proportion of subjects with unresolved diarrhea

proteoglycan matrix (25). Similarly, lactoferrin and lysozyme have been shown to have antistaphylococcal activity (26). Although we do not know the mechanism(s) behind the reduced duration of diarrhea we observed in the Lf/Lz-R-ORS group in this study, it is plausible that these bioactive proteins affected the pathogens and their viability. The higher frequency of bacterial pathogen isolation in the stool of children receiving the Lf/Lz-R-ORS would support this mechanism. Activity against *E coli* was demonstrated by both proteins during the characterization studies (13,14). Because the study was done during the warmer months, there were not enough children with a viral pathogen to allow a separate statistical analysis. In addition, 15 of the 24 children with rotavirus had bacterial or parasitic pathogen(s) isolated. It is also possible that these proteins facilitated the development of a beneficial gut microflora. Lactoferrin has been shown to stimulate the growth of *Bifidobacteria*, which have health benefits and are frequently used in probiotic applications (27).

We did not observe any adverse effects of adding these proteins to R-ORS. The recombinant form of lysozyme is identical to native lysozyme because this protein is not posttranslationally modified (ie, no glycosylation or phosphorylation). The lactoferrin protein is also identical to native human lactoferrin (14); however, because lactoferrin is a glycosylated protein, the glycans attached to the protein backbone are of rice origin (16). This means that some terminal carbohydrate residues consist of xylose, which is not the case in human proteins, and sialic acid is lacking, which is a common terminal residue in native human lactoferrin. It has previously been shown that recombinant human lactoferrin produced in rice is functionally equivalent to native human lactoferrin with regard to iron-binding, anti-microbial activity and receptor binding (14). Although not all of the functions of

lactoferrin may be related to its intestinal receptor (28), it is evident that absence of the glycans, differences in glycosylation pattern, or composition do not affect receptor binding (29). Thus, it is highly likely that recombinant human lactoferrin, even with a somewhat different glycosylation pattern than native human lactoferrin, will have the same biological function in vivo.

It is not certain that the effects observed were caused by the combination of lactoferrin and lysozyme, even though in vitro data support this notion. Lysozyme alone could have exerted an antibacterial effect because of its enzyme activity; however, lysozyme is not known to have any antiretroviral activity and is therefore more narrow in its anti-infectious role than lactoferrin, which has been shown to have both antiviral and antibacterial activities (22,23,28). It should also be noted that lactoferrin is known to affect the immune function of the host, either locally (gut mucosal immunity) or systemically (30). Furthermore, proteolytic digestion of human lactoferrin has been shown to result in the formation of a well-characterized antibacterial peptide, lactoferricin (31), and possibly several such peptides (32). When examining the proportion of unresolved diarrhea by duration of treatment (Fig. 2), it is possible that there is an immediate antimicrobial effect (days 1–2) and a later effect (days 7–9) that appears more pronounced and because of the time delay may be mediated by an effect on immune function. This speculation needs to be investigated further.

The design of our study does not allow any estimate of the relative potency of the lactoferrin and lysozyme because only 1 concentration of each was used. It is possible that lower concentrations may achieve a similar effect because breast milk concentrations vary considerably among lactating women. It is also possible that higher concentrations may achieve a more

pronounced effect. Further studies are needed to resolve these questions.

In conclusion, the addition of recombinant forms of the human milk proteins lactoferrin and lysozyme to ORS had a beneficial effect on acute diarrhea in children. In this study we used concentrations of these proteins similar to those found in human breast milk. It is not yet known whether lower concentrations would be as effective; future studies should evaluate this question.

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