

Supplementation of Infant Formula With the Probiotic *Lactobacillus reuteri* and Zinc: Impact on Enteric Infection and Nutrition in Infant Rhesus Monkeys

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ABSTRACT

Gut colonization by *Lactobacillus reuteri* may have beneficial effects on infant health or capacity to resist infectious disease. Zinc supplementation has also been proposed to increase infants' resistance to disease; however, many studies have yielded conflicting results.

Objectives: To study effects of probiotic supplementation of infant formula (with or without supplemental zinc) on nutritional status, gut colonization and the ability to resist gastrointestinal infection in an infant rhesus monkey model.

Methods: Infant monkeys were fed control infant formula (5 mg Zn/L), control formula with *L. reuteri* or control formula with *L. reuteri* and supplemental zinc (15 mg Zn/L) from birth to 4 months. Growth, nutritional status, mineral absorption, intestinal colonization and frequency and severity of enteropathogenic *Escherichia coli*-induced gastroenteritis were monitored.

Results: Gastrointestinal *L. reuteri* colonization was achieved and was associated with increased ileal villous surface area and improved hematocrit, with no adverse effects on growth or nutritional indices. Fortification to 15 mg Zn/L reduced plasma copper, erythrocyte Cu/Zn-superoxide dismutase, hemoglobin, and iron absorption. Infants fed *L. reuteri*-supplemented formula had reduced diarrhea severity throughout the study period and recovered more rapidly from acute diarrhea than the other groups.

Conclusion: *L. reuteri*-supplementation of infant formula is safe, improves iron status and decreases diarrhea severity in infant rhesus monkeys and thus may help protect formula-fed human infants from infection and nutritional deficiencies. *JPGN* 35:162–168, 2002. **Key Words:** Infants—Probiotics—Lactobacilli—Zinc—Iron—Diarrhea—EPEC. © 2002 Lippincott Williams & Wilkins, Inc.

INTRODUCTION

Breast-fed infants suffer from fewer infections and infections of shorter duration than formula-fed infants (1). This beneficial effect is attributed to immunostimulatory and antimicrobial properties of breast milk (2), and to the characteristic gastrointestinal microflora (3) of breastfed infants in which lactic acid producing bacteria (*Lactobacillus* spp. and *Bifidobacterium* spp.) predominate (4). Gut colonization by these microorganisms aids in resistance to gastrointestinal infections in animal models and humans (5,6), possibly due to the maintenance of normal intestinal flora (7,8), creation of an environment with lower pH or the production of antibacterial substances, such as organic acids, nisin (9), and reuterin (10). Recent investigations have demonstrated that probiotic colonization of the gastrointestinal

tract is likely to have other beneficial effects on an individual's overall nutritional status including increased micronutrient absorption from enhanced mucosal integrity (11,12), reduced circulating cholesterol (13), enhanced mucosal immune system (14), and prevention of cancer and cancer recurrence (15,16).

Oral zinc supplementation in infants has also been reported to improve growth and nutrition and to reduce the prevalence and incidence of acute and chronic diarrheal disease (17,18). However, many zinc-supplementation studies have yielded conflicting results (18). Although the mechanism behind this protection is not established, it is likely that zinc both enhances immune function and improves the mucosal barrier function. We have developed an infant rhesus monkey model to study enteropathogenic *Escherichia coli* (EPEC)-induced gastroenteritis. We have used this model to investigate the hypothesis of the present study, that *L. reuteri*-supplementation of infant formula would have a positive effect on growth, nutritional status, and mineral absorption and that the increased level of zinc supplementation would have no additional beneficial effect. We

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monitored food intake, growth, iron status (hemoglobin, hematocrit), zinc status (plasma zinc), copper status (plasma copper, erythrocyte Cu/Zn superoxide dismutase), lactate dehydrogenase and circulating lactate, blood urea nitrogen, ^{59}Fe , ^{47}Ca and ^{65}Zn absorption, gut morphology, and response to induced gastroenteritis diarrhea from birth to 4 months.

MATERIALS AND METHODS

All procedures were approved by the Animal Care and Use Committee and the Radiation Use Authorization Committee at the University of California, Davis.

Experimental Design

Diets

The experimental formulas were based upon a dry-blended, whey-predominant hydrolysate formula (Profylac, Alk Abelló, Denmark, commercially available) containing 5.3 g fat, 1.9 g protein, and 11 g carbohydrate/100 kcal and 5 mg zinc/L according to proximate analysis. The control formula was modified as follows: 1) formula supplemented with zinc sulfate (to a final level of 15 mg zinc/L) and *L. reuteri* (9×10^6 colony forming units (cfu)/g) (*L. reuteri* + zinc) and 2) formula (5 mg zinc/L, normal level) supplemented with *L. reuteri* (9×10^6 cfu/g) (*L. reuteri*). The *L. reuteri* strain M164 used was host-homologous isolated from rhesus monkeys (*Macaca mulatta*).

Infants

Twenty-one infant rhesus monkeys were obtained at birth from the breeding colony maintained at the California Regional Primate Research Center at the University of California, Davis. They were given three oral doses of clindamycin and clarithromycin (10.6 mg of each per dose) on the first day of life to eliminate maternally transferred *Lactobacilli*. They were maintained indoors under the constant care of nursery and veterinary staff. The infants were bottle-fed one of the experimental diets ad libitum, from birth until 4 months ($n = 7$ /group). From birth to 1 month, the infants were individually housed in polycarbonate isolettes with a surrogate mother (a terry cloth dummy); from 1 to 4 months of age they were paired and housed in stainless steel cages. No solid food was given during the study. To avoid cross-inoculation of the probiotic, infants fed *L. reuteri*-supplemented formulas were housed in a separate facility from those fed control formula.

Food intake and stool consistency was recorded daily and growth (weight and length) was measured weekly. Rectal swabs were collected biweekly into Cary-Blair Culture Swab Transport Medium system and stored at -80°C until analysis. Blood was drawn at birth and monthly for analysis.

Calcium absorption (at ~ 1.5 mo) and iron and zinc absorption (at 2 months and 3.5 mo) were assessed following radioisotope administration. Monkeys were fasted for 4 hours before oral-gastric intubation of the radiolabeled formula ($\sim 1 \mu\text{Ci}$ $^{47}\text{CaCl}_2$ (RISØ National Laboratory, Roskilde, DK), $^{59}\text{FeCl}_2$ or $^{65}\text{ZnCl}_2$ (Amersham Pharmacia Biotech)/3 ml diet). Immedi-

ately after dosing, each animal was placed inside a whole body counter (Institute of Toxicology and Environmental Health, University of California, Davis) equipped with two 10×20 -cm sodium iodide crystals and a multichannel analyzer (ND-66: Nuclear Data, Schaumburg, IL) to determine the amount of radioactivity administered. No food was given for 2 hours after dosing. Radioactivity in infants was recounted after 4 days (calcium) or 7 days (iron and zinc) to determine the amount of isotope retained. Whole body calcium or iron and zinc absorption were calculated as the amount of radioactivity retained after 4 or 7 days, respectively, taking into account individual isotopic decay.

At 3.5 months, the infants were given an infectious dose of 10^8 colony forming units (cfu's) enteropathogenic *Escherichia coli* O127 (nalidixic acid resistant strain 2348/69, EPEC) in 3 ml of infant formula by oral-gastric gavage to simulate gastric infection. Incidence of infection was monitored on day 0 (prior to inoculation), and daily to 7 days post-inoculation by the veterinary staff by assessing rectal temperature, stool consistency, and hydration. Rectal swabs were collected on day 0 (prior to inoculation) and day 1, 2, 3, and 6 days post-inoculation. The presence of EPEC was determined in swab samples on Mac Conkey agar containing 100 $\mu\text{g/ml}$ nalidixic acid. Biopsies of the gastric, ileal, and rectal mucosa were taken at ~ 2.5 months (before EPEC challenge) and ~ 4 months (after EPEC challenge).

Experimental Methods

Hematology

Hemoglobin and hematocrit were quantified with an automated electronic cell counter (Baker 9010 Analyzer; Serono-Baker, Allentown, PA).

Mineral analysis

Plasma samples were digested with 0.1% (v/v) ultra-pure nitric acid as described previously (19). Plasma copper and zinc were analyzed by flame atomic absorption spectrophotometry (Thermo Jarrell Ash, Franklin, MD). Bovine liver preparations were used as reference materials (standard reference material 1577: US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) to validate the mineral analyses.

Lactate Dehydrogenase (LDH)/L-Lactic Acid

Lactate dehydrogenase activity was determined in blood by the reduction of pyruvate to lactate using a commercially available spectrophotometric kinetic assay (Sigma, St. Louis, MO). L-lactic acid was determined by the peroxidase-catalyzed condensation of a chromogen as a function of H_2O_2 produced by conversion of lactate to pyruvate using a commercially available spectrophotometric assay (Sigma).

Blood Urea Nitrogen (BUN)/Cholesterol

Blood urea nitrogen was determined using a commercially available spectrophotometric assay (Sigma) based on the reaction of urea with diacetylmonoxime. Total cholesterol was de-

terminated by spectrophotometric assay using a commercially available kit (Sigma).

Red Blood Cell (RBC) Superoxide Dismutase Activity (SOD)

RBC Cu,Zn-superoxide dismutase activity was determined by inhibition of the auto-oxidation of pyrogallol (23).

Histology

Infants were fasted for 24 hours before surgery. Golytely, a mild purgative (30 ml/kg), was given via oral-gastric gavage to completely void intestinal contents (Braintree Pharmaceuticals, Braintree, MA). Infants were provided an additional 30 ml/kg Golytely supplemented with a citrus-flavored drink for ad libitum consumption to maintain hydration. Intestinal biopsies were performed under general anesthesia (inhalant isoflurathane) by the veterinary staff at the California Regional Primate Research Center, at University of California, Davis. Ileal biopsies (2 samples of 2-mm tissue) were obtained by surgical removal. Gastric and colonic biopsies (2 samples/site of 2-mm tissue) were obtained by endoscopy. Samples were fixed in 10% formalin, pH 7.2, sectioned, and stained for morphometry and pathology evaluation. Concurrent samples were fixed in 0.1% cacodylic acid buffer and evaluated visually by scanning electron microscopy.

Microbiological Enumeration

Diet. Viability of *Lactobacillus reuteri* in infant formula throughout storage was assessed by enumeration on Man, Rogosa, and Sharpe with sodium acetate (MRS-3) agar. Plates were incubated for 48 to 72 hours anaerobically and the number of cfu/g was determined.

Stool. Lactic acid bacteria (LAB) enumeration of stool was determined by mixing frozen rectal swab into 1 ml of MRS broth at room temperature for 15 minutes and serial dilution. *L. reuteri* was enumerated by plating on MRS-3 agar with vancomycin (50 µg/ml). Total lactobacilli was enumerated on LBS (Lactobacilli selection) agar. Plates were incubated for 48 to 72 hours anaerobically and the number of cfu/swab was determined. Total *Enterobacteria* were enumerated by plating dilutions of stool samples on Mac Conkey Agar. Enteropathogenic *E. coli* (EPEC) was monitored by plating on Mac Conkey Agar with nalidixic acid (100 µg/ml).

Diarrhea

Stool consistency was monitored throughout the experimental period and characterized as normal (1), semi-solid (4), liquid (7), or bloody (8) by the veterinary staff at the California Regional Primate Research Center.

Statistical Analysis

Statistical analysis was performed by using one-way ANOVA. Analyses were performed with Graphpad Instat version 2.01 (San Diego, CA) and significance was determined at $P < 0.05$.

RESULTS

Anthropometry

Infants fed *L. reuteri* + zinc had significantly smaller crown-rump length than infants fed Control formula at 1 and 4 months ($P < 0.05$) (Fig. 1) and weighed less than infants fed other formulas from 1 month, although significance was not reached. These differences were not related to food intake, which was similar between groups.

Biochemical Tests

Infants fed *L. reuteri* + zinc had significantly higher plasma zinc from 1 month ($P < 0.05$) to 4 months ($P < 0.001$) than infants fed the other diets (Fig. 2). At 1 month, infants fed *L. reuteri* + zinc had significantly higher plasma copper than infants fed *L. reuteri* ($P < 0.05$) or Control ($P < 0.001$). Concurrently, infants fed *L. reuteri* + zinc had significantly lower Cu/Zn-SOD activity ($P < 0.05$) at 4 months (Fig. 3). At 4 months, infants fed *L. reuteri* had higher hemoglobin than infants fed *L. reuteri* + zinc ($P < 0.05$) and hematocrit than infants fed *L. reuteri* + zinc or Control ($P < 0.01$) (Table 1). There were no differences observed in total cholesterol or blood urea nitrogen. At 3 months, infants fed *L. reuteri* + zinc had significantly lower circulating L-lactate concentrations than infants fed *L. reuteri* ($P < 0.05$) or Control ($P < 0.01$) (Table 2). At 4 months, infants fed *L. reuteri* + zinc had lower L-lactate than infants fed Control ($P < 0.01$), but the difference between infants fed *L. reuteri* and *L. reuteri* + zinc disappeared. At 3 months, infants fed *L. reuteri* + zinc had significantly higher LDH than Control ($P < 0.05$). At 4 months, infants fed *L. reuteri* + zinc had higher LDH than infants fed *L. reuteri* and Control ($P < 0.05$) (Table 2).

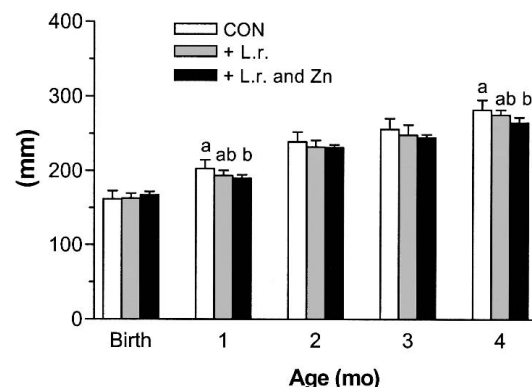


FIG. 1. Longitudinal growth of infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc) from birth through 4 months of age. Numbers represent mean \pm SD, $n = 7$ /diet. Means without a common letter differ, $P < 0.05$.

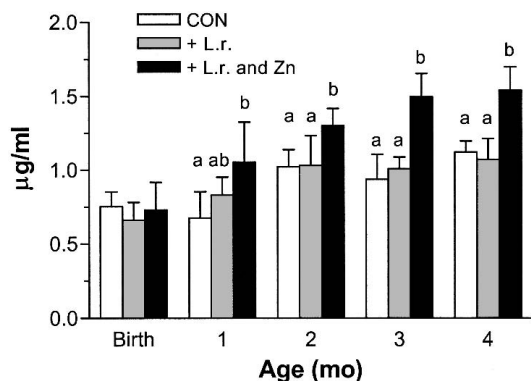


FIG. 2. Plasma zinc concentrations of infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc) from birth through 4 months of age. Numbers represent mean ± SD, n = 7/diet. Means without a common letter differ, P < 0.05.

Mineral Absorption

There were no significant differences in calcium absorption between treatment groups. Infants fed *L. reuteri* + zinc had lower zinc (P < 0.01) and iron (P < 0.05) absorption than infants fed either *L. reuteri* or Control at 3.5 months, but not at 2 months of age (Fig. 4).

Gut Colonization

Total Lactobacilli

There was no significant difference in number of total lactobacilli (cfu/swab) through 16 weeks of age. At 18 weeks of age, infants fed Control (P < 0.01) and *L. reuteri* + zinc (P < 0.05) had significantly higher total lactobacilli than infants fed *L. reuteri*.

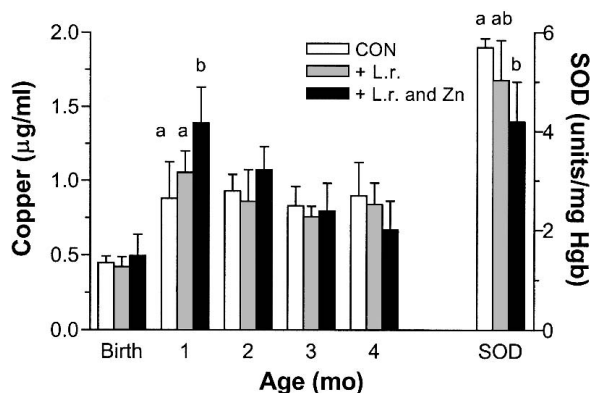


FIG. 3. Plasma copper from birth through 4 months of age and red blood cell superoxide dismutase activity (SOD) at 4 months of infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc). Numbers represent mean ± SD, n = 7/diet. Means without a common letter differ, P < 0.05.

TABLE 1. Hemoglobin, hematocrit, blood urea nitrogen (BUN), and plasma cholesterol in infant rhesus monkeys

Diet		¹ Hemoglobin (g/dL)	% Hematocrit	BUN (mg/dL)	Cholesterol (mg/dL)
Birth	Control	19.0 ± 1.7 ^a	54.1 ± 4.7 ^a	17.5 ± 3.9 ^a	41.7 ± 15.5 ^a
	+ <i>L.r.</i>	18.1 ± 2.9 ^a	50.9 ± 8.5 ^a	18.2 ± 3.9 ^a	42.7 ± 13.3 ^a
	+ <i>L.r.</i> and Zn	18.9 ± 2.1 ^a	53.1 ± 6.2 ^a	17.1 ± 5.8 ^a	37.6 ± 13.5 ^a
1 mo	Control	14.3 ± 0.9 ^a	41.4 ± 2.6 ^a	19.3 ± 6.0 ^a	104.6 ± 26.2 ^a
	+ <i>L.r.</i>	13.4 ± 0.8 ^a	39.2 ± 2.4 ^a	16.9 ± 3.0 ^a	101.0 ± 14.2 ^a
	+ <i>L.r.</i> and Zn	14.4 ± 0.6 ^a	41.1 ± 1.5 ^a	14.6 ± 2.4 ^a	97.3 ± 18.8 ^a
2 mo	Control	13.5 ± 0.6 ^a	39.9 ± 1.5 ^a	12.5 ± 1.2 ^a	100.3 ± 9.7 ^a
	+ <i>L.r.</i>	14.1 ± 0.5 ^a	41.9 ± 1.3 ^a	14.0 ± 7.1 ^a	107.2 ± 13.2 ^a
	+ <i>L.r.</i> and Zn	13.8 ± 1.5 ^a	41.1 ± 4.4 ^a	18.7 ± 3.1 ^a	100.4 ± 6.9 ^a
3 mo	Control	12.5 ± 1.3 ^a	36.7 ± 3.8 ^a	9.2 ± 4.0 ^a	109.8 ± 19.6 ^a
	+ <i>L.r.</i>	12.9 ± 0.7 ^a	38.6 ± 1.2 ^a	12.8 ± 3.5 ^a	113.0 ± 9.6 ^a
	+ <i>L.r.</i> and Zn	13.9 ± 1.1 ^a	40.9 ± 2.9 ^a	9.1 ± 2.4 ^a	102.6 ± 13.0 ^a
4 mo	Control	13.3 ± 0.8 ^{ab}	39.4 ± 2.2 ^a	10.5 ± 2.9 ^a	105.5 ± 14.8 ^a
	+ <i>L.r.</i>	14.3 ± 0.7 ^b	43.6 ± 1.7 ^b	8.6 ± 0.9 ^a	105.4 ± 15.6 ^a
	+ <i>L.r.</i> and Zn	12.8 ± 1.0 ^a	37.4 ± 3.2 ^a	9.2 ± 3.7 ^a	91.8 ± 19.0 ^a

* Fed control infant formula or formula supplemented with *Lactobacillus reuteri* (+*L.r.*) or *Lactobacillus reuteri* and zinc (+*L.r.* and Zn) from birth to 4 months of age.

¹Numbers represent mean ± SD, n = 7 infants/group. Means without a common letter for each variable at each age differ, P < 0.05.

Lactobacillus Reuteri

After 2 weeks of age, *L. reuteri* was not detected in feces of any monkeys fed Control. There was no difference in the number of *L. reuteri* cfu/swab between infants fed *L. reuteri* + zinc or *L. reuteri* from 2 to 18 weeks of age.

Total Enterobacteria and Enteropathogenic E. coli

After EPEC challenge, monkeys fed *L. reuteri* + zinc had significantly lower total *Enterobacteria* (P < 0.05) than monkeys fed the other diets through 7 days post-EPEC challenge. Infants fed *L. reuteri* + zinc had lower number of EPEC/swab (P < 0.01) than infants fed Control or *L. reuteri*, while infants fed *L. reuteri* had lower number of EPEC/swab than infants fed Control (P < 0.01) (Fig. 5).

Diarrhea

Stool consistency was monitored daily throughout the study to assess severity of diarrhea. Infants receiving

TABLE 2. Lactate dehydrogenase activity and L-lactate concentration in plasma from infant rhesus monkeys*

	¹ L-lactate (mg/dL)		¹ Lactate dehydrogenase (units/L)	
	3 mo	4 mo	3 mo	4 mo
Control	61.6 ± 22.4 ^b	61.7 ± 26.4 ^b	364.3 ± 73.5 ^a	815.1 ± 108.4 ^a
+ <i>L.r.</i>	57.7 ± 11.7 ^b	39.5 ± 19.3 ^a	431.7 ± 49.1 ^{ab}	798.2 ± 71.8 ^a
+ <i>L.r.</i> and Zn	30.5 ± 11.0 ^a	32.4 ± 16.7 ^a	552.4 ± 33.1 ^b	2514.0 ± 1019.0 ^b

* Fed control infant formula or formula supplemented with *Lactobacillus reuteri* (+*L.r.*) or *Lactobacillus reuteri* and zinc (+*L.r.* and Zn) at 3 and 4 months of age.

¹Numbers represent mean ± SD, n = 7 infants/group. Means without a common letter for each variable at each age differ, P < 0.05.

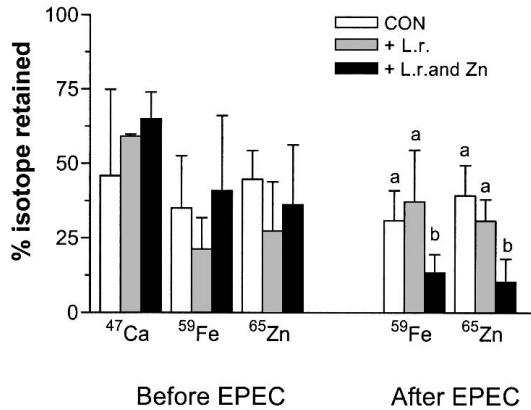


FIG. 4. Calcium, iron, and zinc absorption before EPEC infection and iron and zinc absorption after EPEC infection in infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc) formula. Numbers represent mean \pm SD, $n = 7$ /diet. For each isotope, means without a common letter differ, $P < 0.05$.

L. reuteri-supplemented formula had severe diarrhea during 24% of the trial days which was a significantly shorter period ($P < 0.01$) as compared with groups receiving *L. reuteri* + zinc (33%) or Control (34%) formula. There were no effects of EPEC administration on hydration or temperature. However, differences were observed in diarrhea severity. Infants fed Control formula had a delay in severe diarrhea as compared to infants fed *L. reuteri* + zinc or *L. reuteri*. Infants fed *L. reuteri* + zinc and *L. reuteri* had immediate and acute diarrhea after 2 days post-EPEC challenge; however, infants fed *L. reuteri* began to resolve diarrhea before infants fed *L. reuteri* + zinc or Control (Fig. 6).

Histology

By light microscopy we detected no pathologic changes in any infants. Unfortunately, due to the limited size of the intestinal biopsies, ileal villi were often not observed and it was not possible to get quantitative information. However, we did observe through scanning electron microscopy that ileal villi from infants fed *L. reuteri* had a larger surface area than infants fed *L. reuteri* + zinc or Control before challenge with EPEC. After EPEC-challenge, ileal villi in infants fed *L. reuteri* + zinc remained healthy and presented a more expanded surface (Fig. 7). Unfortunately, it was not possible to get such information for the infants fed *L. reuteri* or Control after EPEC-challenge.

DISCUSSION

Probiotic supplementation with *Lactobacillus* spp. and *Bifidobacteria* spp. has been suggested to be an effective method for establishing a 'host-friendly' intestinal mi-

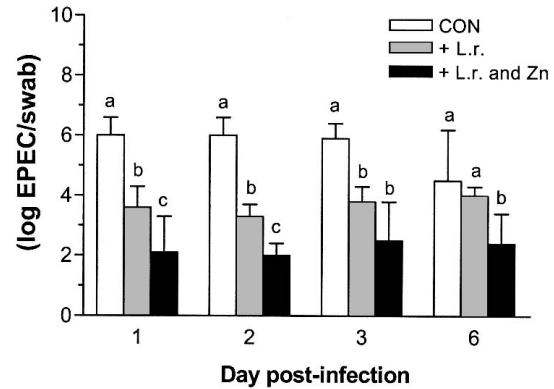


FIG. 5. Effect of dietary treatment on infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc) formula on EPEC collected by rectal swab and enumerated by standard microbiologic plating techniques on selective medium through 6 days after EPEC infection. Numbers represent mean \pm SD, $n = 7$ /diet. Means without a common letter differ, $P < 0.05$.

croflora, potentially aiding in the prevention of disease and enhancement of overall health (14,20). Although we found no differences in total lactobacilli using standard plate enumeration techniques, successful colonization of *L. reuteri* was achieved in groups fed *L. reuteri*-supplemented formula. Colonization with *L. reuteri* has been associated with changes in intestinal morphology (11). In this study we observed that infants fed *L. reuteri*-supplemented formula had a larger ileal villous surface area than infants fed *L. reuteri* + zinc-supplemented formula or control formula. Similar results were observed by Allori et al. (12) after supplementation of malnourished mice with *L. casei*. Due to the limited biopsy size, it was not possible to get information about the morphology of the ileal villi after EPEC-challenge in infants fed *L. reuteri*-supplemented formula or control formula.

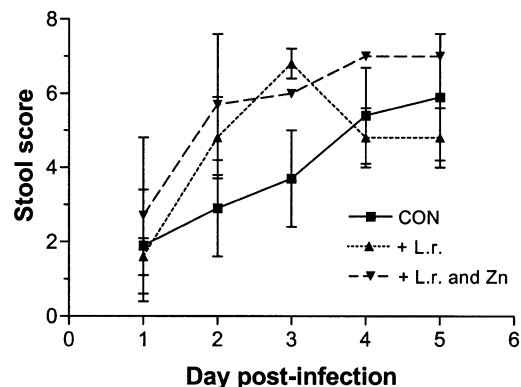


FIG. 6. Effect of dietary treatment on infants fed Control (CON), *Lactobacillus reuteri*-supplemented (+ *L.r.*) and *Lactobacillus reuteri* and zinc-supplemented (+ *L.r.* and zinc) formula on stool consistency as qualitatively assessed and rated per 8 point stool score after EPEC infection. Numbers represent mean \pm SD, $n = 7$ /diet.

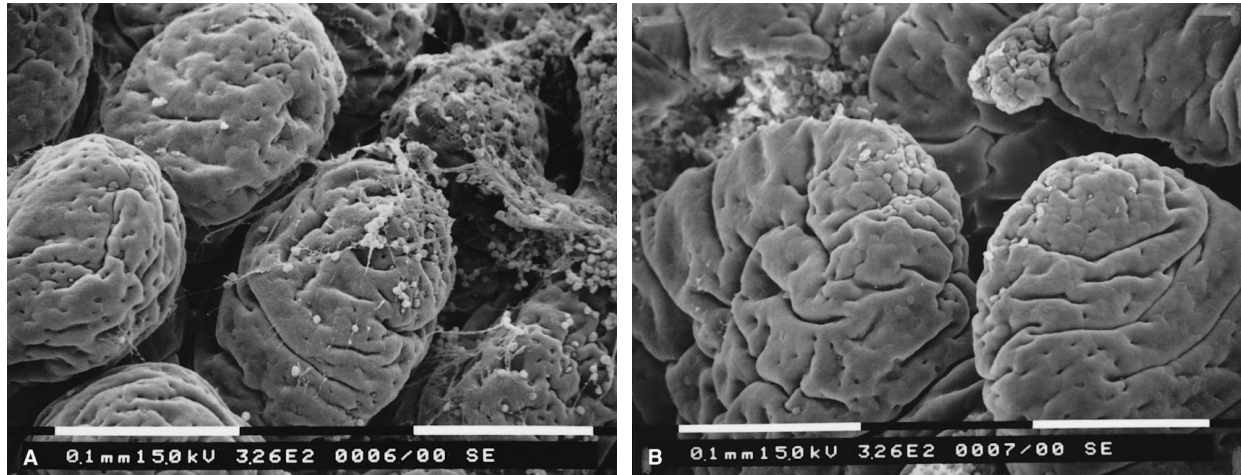


FIG. 7. Electron micrograph (326-fold magnification, bar represents 100 μ m) of ileal villi from infant monkey fed *L. reuteri* + zinc. Panel A) before challenge with enteropathogenic *E. coli* (EPEC). Panel B) after challenge with EPEC.

However, the fact that ileal villi remained healthy and had a more expanded surface morphology post-EPEC challenge in infants fed *L. reuteri* + zinc-supplemented formula suggests that *L. reuteri* could play a role in the protection of the ileal villous integrity during gastrointestinal infection. Additionally, *L. reuteri*-colonization positively affected the ability of these infants to recover from experimentally induced gastroenteritis, possibly resulting in earlier resolution of diarrhea.

There is concern that supplementation of foods with lactobacilli might increase luminal lactic acid concentration and produce lactic acidemia in treated individuals. We observed no differences in L-lactate levels between infants fed *L. reuteri*-supplemented formula and control formula at 3 months of age. However, at 4 months of age, the L-lactate level was actually lower in infants fed *L. reuteri*-supplemented formula indicating that supplementation of infant formula with *L. reuteri* did not increase blood L-lactate in this model. In accordance with Stangl and Kirchgessner (21), we found an increase in lactate dehydrogenase (LDH) activity and a decrease in circulating L-lactic acid concurrently with reduced iron status in infants fed *L. reuteri* + zinc-supplemented formula. Contrary to our results, studies in adult humans have shown decreased cholesterol in individuals fed lactobacilli-supplemented foods (19). This difference could be related to age differences between the infants in our study and studies in older populations as well as the duration of the treatment.

Zinc supplementation has been proposed to help protect infants from infectious disease. However, as Bhutta et al. (17) observed, zinc supplementation may interfere with copper absorption, which carries its own negative impact on health. Furthermore, Doherty et al. (18) found that 6 mg zinc/kg body weight given orally for 30 days to malnourished children was associated with an increased mortality when compared to mortality in children fed the same or

lower dose for only 15 days. In the present study, infant rhesus monkeys fed infant formula with a higher than normal zinc concentration (15 mg Zn/L) had decreased crown-rump length through 4 months of age, compared to infants fed the other experimental diets. Although negative effects of zinc deficiency on growth have been well-documented (22), negative effects on growth of zinc supplementation in non-zinc-deficient infants have not been documented and may be a consequence of negative interactions between zinc and other micronutrients. The effects of elevated zinc intake on copper and iron status have been well-documented (23) and have been postulated to be a result of competition for mucosal transport (24). Infants fed *L. reuteri* + zinc-supplemented formula had lower iron absorption than the other groups at 3 months of age. The lower iron status of these infants was most likely a consequence of the lower iron absorption from the zinc-supplemented formula. Furthermore, plasma copper and Cu/Zn superoxide dismutase activity were decreased in infants fed *L. reuteri* + zinc-supplemented formula suggesting a secondary copper deficiency. Copper deficiency may be associated with growth retardation primarily due to alterations in connective tissue formation and bone growth (25). Additionally, infants fed *L. reuteri* + zinc-supplemented formula had lower zinc absorption at 3 months, possibly a function of down-regulated zinc transport at the mucosal surface as a consequence of elevated dietary zinc. The mechanisms underlying zinc absorption are just beginning to be elucidated, and there is evidence that this regulation could be mediated by changes in expression or processing of zinc transport proteins (26) or divalent metal transporter 1 (DMT1) (27) at the mucosal surface. Therefore, zinc supplementation of infants with adequate zinc status should be approached with caution. Interestingly, infants fed *L. reuteri*-supplemented formula had increased hematocrit levels indicating a positive effect of *L. reuteri*-supplementation on iron status.

In conclusion, we found that supplementation of infant formula with *L. reuteri* results in probiotic colonization of the gastrointestinal tract with no apparent adverse effects on growth or nutritional indices. In fact, positive effects on iron status were noted in infants fed *L. reuteri*-supplemented formula. Increased levels of L-lactic acid were not observed in infant rhesus monkeys fed *L. reuteri*-supplemented infant formula. Increasing fortification of infant formula from 5 mg to 15 mg Zn/L had negative effects on iron and copper status by reducing plasma copper, RBC Cu/Zn SOD, hemoglobin, hematocrit and iron absorption. Lower zinc absorption was also noted possibly indicating activation of homeostatic mechanisms for protection against zinc overload. Administration of *L. reuteri*-supplemented formula reduced the severity of diarrhea during the study period. After challenge with enteropathogenic *E. coli*, acute diarrhea was observed; however, the pattern of clinical presentation was different for dietary treatments. Infants fed control formula had slower progression of diarrhea severity, while infants fed both *L. reuteri*-supplemented formula had immediate, acute diarrhea. Resolution of diarrhea began earlier in the infant monkeys fed *L. reuteri*-formula without supplemental zinc (5 mg Zn/L) than in the other two groups. We speculate that supplementation of infant formula with *L. reuteri* may provide protection to formula-fed human infants from diarrheogenic microorganisms and improve iron status without negatively affecting overall nutritional status.

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