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Inactivation of Enveloped Viruses and Killing of Cells by Fatty Acids and Monoglycerides

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Lipids in fresh human milk do not inactivate viruses but become antiviral after storage of the milk for a few days at 4 or 23°C. The appearance of antiviral activity depends on active milk lipases and correlates with the release of free fatty acids in the milk. A number of fatty acids which are normal components of milk lipids were tested against enveloped viruses, i.e., vesicular stomatitis virus, herpes simplex virus, and visna virus, and against a nonenveloped virus, poliovirus. Short-chain and long-chain saturated fatty acids had no or a very small antiviral effect at the highest concentrations tested. Medium-chain saturated and long-chain unsaturated fatty acids, on the other hand, were all highly active against the enveloped viruses, although the fatty acid concentration required for maximum viral inactivation varied by as much as 20-fold. Monoglycerides of these fatty acids were also highly antiviral, in some instances at a concentration 10 times lower than that of the free fatty acids. None of the fatty acids inactivated poliovirus. Antiviral fatty acids were found to affect the viral envelope, causing leakage and at higher concentrations, a complete disintegration of the envelope and the viral particles. They also caused disintegration of the plasma membranes of tissue culture cells resulting in cell lysis and death. The same phenomenon occurred in cell cultures incubated with stored antiviral human milk. The antimicrobial effect of human milk lipids in vitro is therefore most likely caused by disintegration of cellular and viral membranes by fatty acids. Studies are needed to establish whether human milk lipids have an antimicrobial effect in the stomach and intestines of infants and to determine what role, if any, they play in protecting infants against gastrointestinal infections.

Human milk contains a number of antiviral factors that are not immunoglobulins (5, 6, 13, 17). Some of these factors are located in the nonlipid fraction of the milk, but most studies found antiviral activity associated with the lipid fraction. Antiviral lipids were best characterized by Welsh et al. (21), who found that free unsaturated fatty acids and monoglycerides in milk inactivated enveloped, but not nonenveloped, viruses. In a recent study (9a), we confirmed and extended the work of Welsh et al. We showed that lipids from fresh breast milk are not antiviral but become active against enveloped viruses upon storage at 4°C and in infant stomachs, probably by the release of fatty acids from milk triglycerides. In the present study, we compared the effect of fatty acids and monoglycerides on enveloped viruses and demonstrated that unsaturated fatty acids disrupt both viral envelopes and cell membranes.

MATERIALS AND METHODS

Cell cultures. Vero cells (African green monkey kidney cell line; Flow Laboratories Inc., McLean, Va.) were grown in Eagle basal medium (BME) (GIBCO Laboratories, Grand Island, N.Y.) with 10% inactivated fetal bovine serum (GIBCO). Sheep fibroblast cultures were obtained from the choroid plexus of a lamb brain and grown in 15% lamb serum (Colorado Serum Co.) in BME. The maintenance medium (MM) for Vero cells was BME with 2% fetal bovine serum; for sheep cells, the MM was 2% lamb serum in BME. Gentamicin (0.1%) was added to all media.

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Viruses. Vesicular stomatitis virus (VSV) strain Indiana and herpes simplex virus type 1 (HSV-1) strain MacIntyre were obtained from the American Type Culture Collection, Rockville, Md., and grown in Vero cells. Visna virus strain K796 (19) was grown in sheep choroid plexus cells. Poliovirus type 1 strain Chat was obtained from R. I. Carp (New York State Institute for Basic Research) and grown in Vero cells.

Virus titration. Viruses were titrated by inoculation of 10-fold dilutions (VSV, poliovirus, and HSV-1 were inoculated into Vero cell cultures, and visna virus was inoculated into sheep choroid plexus cell cultures) in 96-well microtiter tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.). A virus dilution (0.1 ml) in MM was inoculated into each well with four wells per dilution. The plates were kept for 2 to 12 days, depending on the virus, and examined daily for cytopathic effect. Virus titers were calculated by the method of Reed and Muench (14).

Milk samples. Human milk samples 1, 2, and 3 were collected under sterile conditions 1 to 5 months postpartum and kept deep-frozen at −86°C until used in experiments.

Reagents. Fatty acids and monoglycerides were purchased from Sigma Chemical Co., St. Louis, Mo. (purest grade). Immediately before use they were melted and emulsified in liquid form in BME with 10% fetal bovine serum by vortexing at the highest speed for 1 min. The emulsions (100 mg/ml) were diluted to the desired concentrations in MM. Emulsions of short-chain fatty acids were neutralized to pH 7 by addition of 1 M NaOH. Unsaturated fatty acids and monoglycerides were kept under nitrogen, and emulsions were used within a few minutes of preparation. Eserine

TABLE 1. Free fatty acids (FFA) and antiviral activity in milk[a]

Milk sample	Storage temp/time (°C/days)	FFA (mg/ml)	Reduction of VSV titer (log_{10})	Lipoprotein lipase (U/ml)
1	−86/4	0.5	0	336
	23/4	12.0	4.0	
	4/4	7.0	4.0	
3	−86/4	0.5	0	20
	23/4	2.0	0	
	4/4	2.0	0	

[a] The same results were obtained for milk tested fresh or after storage at −86°C.

sulfate (physostigmine; Sigma) and NaCl were dissolved in water and diluted in MM before use in experiments.

Assay of antiviral activity. About 10^5 50% tissue culture infective doses ($TCID_{50}s$) of virus were mixed with a fivefold dilution of milk in MM or with an emulsion of fatty acids and monoglycerides in MM and incubated at 37°C for 30 min. Virus mixed with MM alone was used as a control. After incubation, the infectivity of each mixture was titrated by the serial dilution endpoint method. Dilutions (10-fold) were made in MM. The 10^{-2} to 10^{-5} dilutions were inoculated into monolayers of Vero cells, and the virus titers were determined as described above. The difference between the titer (log_{10}) of the control virus and the titers of milk-virus and lipid-virus mixtures, i.e., the reduction of virus titer, was used as a measure of antiviral activity.

Preparation of virus for electron microscopy. VSV was concentrated and partially purified by differential centrifugation in a Beckman L2-65B ultracentrifuge, and samples (10^{10} $TCID_{50}$/ml) were incubated at 37°C for 30 min in MM with or without emulsified fatty acids. The virus suspensions were applied to carbon-coated grids and negatively stained with 2% phosphotungstic acid, pH 7.0. Specimens were examined by using a Hitachi HS 8-2 electron microscope at 50 kV.

Preparation of cells for electron microscopy. Monolayer cultures of cells were incubated for 30 min at 37°C either in MM alone or with milk or a fatty acid emulsion. The cell layers were then carefully rinsed with Hanks balanced salt solution and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After rinsing in buffer and postfixation with 2% osmium tetroxide, the cells were dehydrated through gradings of ethanol, critical-point dried, and sputter coated with 10.5 nm of gold. They were examined in an ISI-SS40 scanning electron microscope at 20 kV.

Estimation of free fatty acids levels. Lipids from 100 μl of the milk samples were extracted with 0.5 ml of chloroform-methanol (2:1). The upper phase was removed, and an aliquot of the chloroform layer was separated by thin-layer chromatography on Silica Gel G (Merck & Co., Inc., Rahway, N.J.) plates with quantitative standards of oleic acid in a solvent system consisting of hexane-diethyl ether-acetic acid (70:30:1.5). The developed plates were charred after spraying with dichromate-sulfuric acid, and the free fatty acids were quantitated by densitometry.

RESULTS

Relationship between lipolysis and antiviral activity. Previous results (9a) showed that human milk becomes active against enveloped viruses after storage at 4, 23, or −20°C for various lengths of time. The antiviral activity is associated with the cream fraction, but the skim fraction is needed for the lipids to become antiviral. To test whether the appearance of antiviral activity depended on active milk lipases, we stored milk samples 1, 2, and 3 at 4°C for 4 days with or without two lipase inhibitors, 5 mM eserine sulfate and 1 M NaCl (7, 8). The virus titer (VSV) fell from 10^5 to $\leq 10^{1.5}$ $TCID_{50}$ after incubation with milk stored without an inhibitor, thus showing a reduction of $10^{3.5}$ $TCID_{50}$. In contrast, virus incubated in the same way with milk which had been stored with lipase inhibitors showed no loss of infectivity at the concentrations used. The inhibitors had no effect on milk which was already antiviral.

Another indication that the appearance of antiviral activity in stored human milk is associated with lipolysis is shown in Table 1. Deep-frozen human milk sample 1 did not have a detectable level of free fatty acids, but the level increased to 7 and 12 mg/ml upon storage at 4 and 23°C, respectively, for 4 days. Both stored samples were highly antiviral. The free fatty acid level of milk sample 3, on the other hand, increased to only 2 mg/ml upon storage, and the milk did not become antiviral. Compared with milk sample 3, milk sample 1 had much higher levels of lipoprotein lipase, which was previously shown to correlate with the appearance of milk antiviral activity (9, 9a).

Antiviral activity of fatty acids and monoglycerides. A comparison of the antiviral activity of a number of fatty acids found in milk (11) is shown in Table 2. Short-chain (butyric, caproic, and caprylic) and long-chain saturated (palmitic and stearic) fatty acids had no or a very small antiviral effect at the highest concentrations tested. On the other hand, the

TABLE 2. Viral inactivation by incubation with fatty acids at 37°C for 30 min

Fatty acid	Concn[a] in mg/ml (mM)	Reduction of virus titer (log_{10})		
		VSV	HSV-1	VV[b]
Butyric (4:0)[c]	10 (113)	0	ND[d]	ND
Caproic (6:0)	10 (86)	0	ND	ND
Caprylic (8:0)	10 (69)	1.8	ND	≥3.2
Capric (10:0)	4 (22)	≥4.0[e]	≥4.0	≥3.2
Lauric (12:0)	2 (10)	≥4.0	≥4.0	≥3.2
Myristic (14:0)	4 (16)	≥4.0	≥4.0	1.7
Palmitic (16:0)	20 (78)	1.0	1.0	0.7
Palmitoleic (16:1)	2 (15)	≥4.0	≥4.0	≥3.2
Stearic (18:0)	20 (70)	0	ND	ND
Oleic (18:1 *cis*)	2 (7)	≥4.0	≥4.0	≥3.2
Elaidic (18:1 *trans*)	2 (7)	≥4.0	ND	ND
Linoleic (18:2)	1 (3.5)	≥4.0	≥4.0	≥3.2
Linolenic (18:3)	1 (3.6)	≥4.0	≥4.0	≥3.2
Arachidonic (20:4)	0.5 (1.6)	≥4.0	ND	ND

[a] Concentration of fatty acid in virus mixtures incubated at 37°C for 30 min. All fatty acids were tested in a series of twofold concentrations. Shown is either the lowest concentration which reduced the VSV titer by ≥4.0 log_{10} units or the highest concentration tested (butyric, caproic, caprylic, palmitic, and stearic).

[b] VV, Visna virus.

[c] Carbon atoms:double bonds.

[d] ND, Not done.

[e] The titer (log_{10}) of the control virus incubated with mm was 5.5, whereas no virus was detectable in the 10^{-2} to 10^{-5} dilutions of fatty acid-virus mixtures. It was not possible to test these mixtures in lower dilutions (10^{-1} or undiluted) because they were toxic to the cell cultures. Assuming that the 10^{-1} dilution contained infectious virus, the highest possible titer of the fatty acid-virus mixture was $10^{1.5}$ $TCID_{50}$, and the reduction of virus titer (log_{10}) would equal 4.0 (5.5 minus 1.5). If the titers of the mixtures were less than $10^{1.5}$, the reduction of titer would be greater than 4.0.

medium-chain saturated and long-chain unsaturated fatty acids were all antiviral but at different concentrations. Table 2 shows the lowest concentration causing a >10,000-fold reduction in VSV titer. A 2-fold-lower concentration either did not inactivate the virus or caused only a 10-fold reduction in titer. Similar results were obtained for HSV-1 and visna virus, a retrovirus. In contrast, incubation of poliovirus at 37°C for 30 min with capric, lauric, myristic, palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, each at a concentration of 8 mg/ml, did not cause a significant reduction of virus titer compared with the titer of poliovirus incubated without fatty acids ($10^{4.7}$ $TCID_{50}$). The sodium salts of oleic and linoleic acids had antiviral effects similar to those of the free acids.

Other products of lipolysis, e.g., 1-monoglycerides of fatty acids, were also tested for antiviral activity (Table 3). All the monoglycerides tested except monomyristin and monoolein were antiviral in concentrations 5 to 10 times lower (millimolar) than those of the corresponding fatty acids.

Effect of fatty acids on viral particles. To study the effect of fatty acids on virus particles, VSV was concentrated, partly purified, and then incubated at 37°C for 30 min in MM with or without linoleic acid. Negative staining of virus incubated without fatty acids showed an abundance of characteristic bullet-shaped particles covered with spikes and containing coiled nucleocapsids (Fig. 1a). Incubation with 0.5 mg of linoleic acid per ml caused leakage of viral envelopes, allowing the stain to enter many particles (Fig. 1b). The effect was far more pronounced with 1 mg of linoleic acid per ml (Fig. 1c), causing particle disintegration. Titration of the samples used for electron microscopy showed a <10-fold reduction in virus titer with 0.5 mg of linoleic acid per ml, whereas 1 mg/ml caused a ≥1,000-fold reduction. Similar results were obtained by negative staining of VSV incubated with low concentrations of arachidonic acid.

Disintegration of cell membranes by fatty acids. Negative staining of VSV treated with fatty acids suggested that virus inactivation results from disruption of the viral envelope, which is derived from the host cell plasma membrane. To study the effect on cell membranes, monolayers of Vero cells or sheep fibroblasts were incubated at 37°C for 30 min in MM with or without 1 mg of linoleic acid per ml and examined by scanning electron microscopy. Control cells incubated in MM without fatty acids showed intact cell membranes (Fig. 2c), whereas in cell layers treated with 1 mg of linoleic acid per ml, the cell membranes were partly or completely disintegrated (Fig. 2d), causing cell lysis. The same effect was seen by incubation of cells with human milk which had been stored at 4°C for 4 days (Fig. 2b). This milk sample (no. 1) (Table 1) contained 7 mg of fatty acids per ml and was highly antiviral. On the other hand, milk sample 1 stored at

TABLE 3. Viral inactivation by incubation with monoglycerides at 37°C for 30 min

Monoglyceride	Concn[a] in mg/ml (mM)	Reduction of virus titer (log_{10})	
		VSV	HSV-1
Monocaprylin (8:0)[b]	2.0 (9)	≥4.0	ND[c]
Monocaprin (10:0)	0.5 (2)	≥4.0	≥3.7
Monolaurin (12:0)	0.25 (0.9)	≥4.0	≥3.7
Monomyristin (14:0)	2.0 (13)	3.0	ND
Monoolein (18:1)	1.0 (2.8[d])	2.3	ND
Monolinolein (18:2)	0.25 (0.7)	≥4.0	ND

[a] Lowest concentration causing ≥3.0 log_{10} reduction in virus titer.
[b] Carbon atoms:double bonds.
[c] ND, Not done.
[d] Highest antiviral activity of the concentrations tested (0.5 to 4 mg/ml). The same results were obtained when the monoglyceride was dissolved in ethanol and diluted 1:100 in mm before being added to the virus.

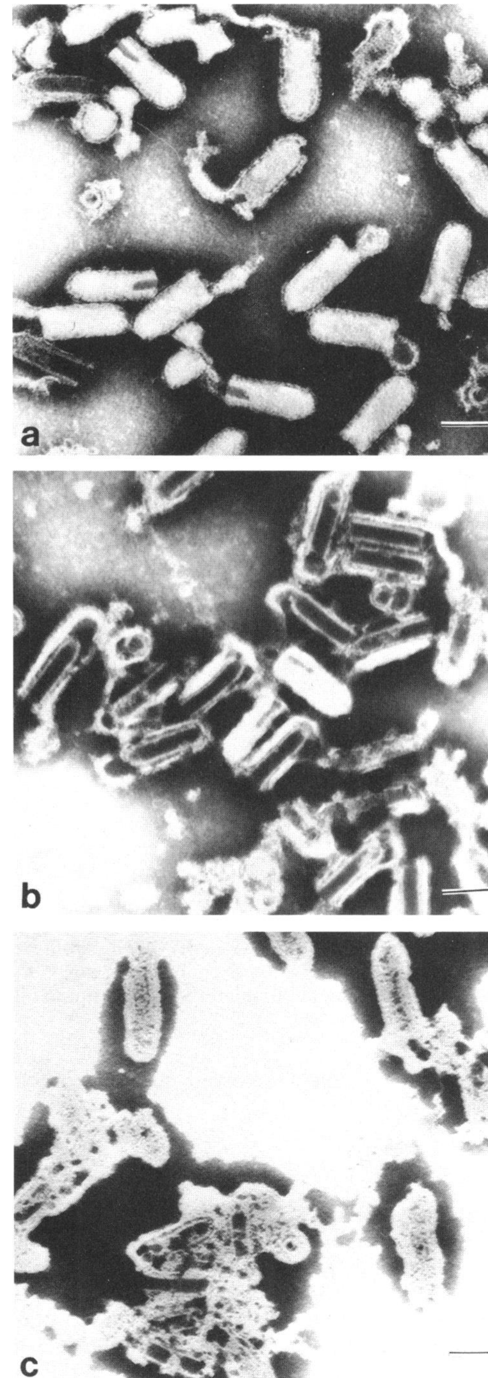

FIG. 1. Negative staining of VSV particles showing the effect of linoleic acid. VSV was incubated at 37°C for 30 min in (a) MM, (b) linoleic acid (0.5 mg/ml of MM), and (c) linoleic acid (1 mg/ml of MM). (a) Normal intact particles covered with spikes. (b) Viral envelope no longer intact, allowing penetration of stain into most particles. (c) Virus particles in various stages of disintegration. Bar = 0.1 μm.

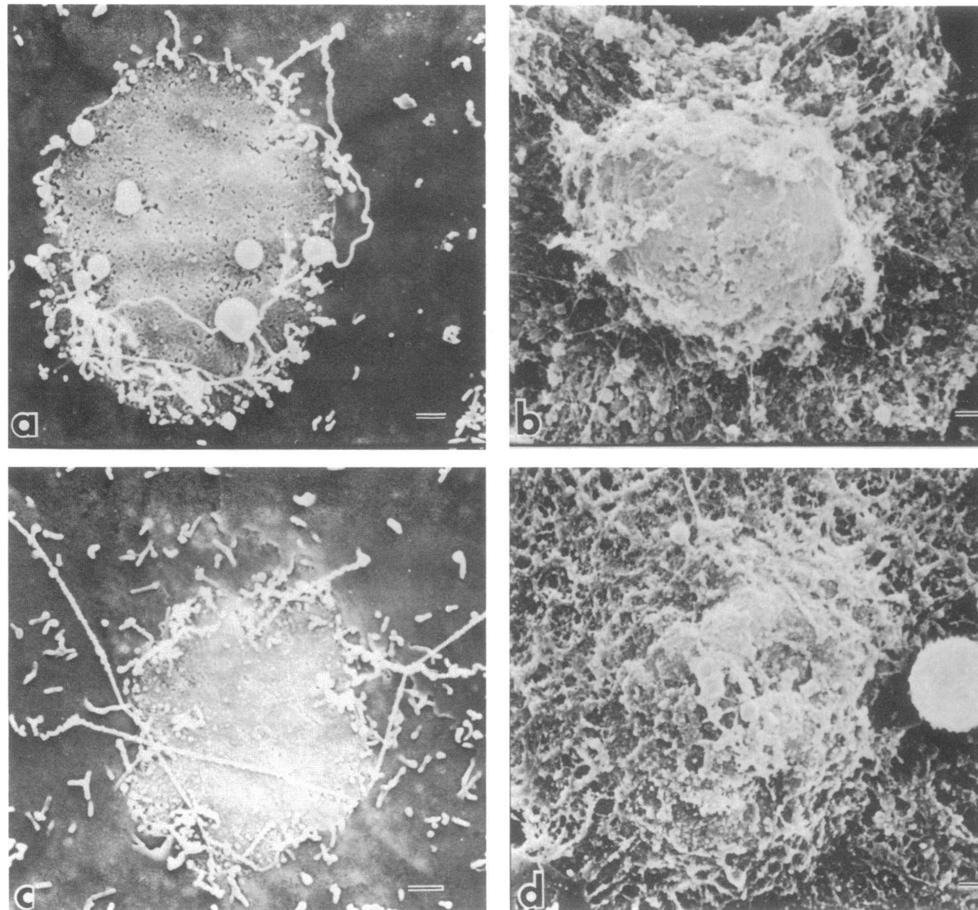

FIG. 2. Scanning electron micrographs of cell cultures showing the effect of human milk and linoleic acid. Vero cells were incubated at 37°C for 30 min in (a) human milk, (b) milk stored at 4°C for 4 days, (c) MM, or (d) linoleic acid (1 mg/ml of MM). Milk samples were diluted 1:5 in MM. (a and c) Intact cell membranes with microvilli. (b and d) Cell membranes partly or completely disintegrated. Bar = 1.0 μm.

−86°C for 4 days (Table 1) showed no effect on cell membranes (Fig. 2a).

DISCUSSION

We have shown (9a) that human milk becomes antiviral not only upon storage but also in the stomach of infants within 1 h of feeding. The appearance of antiviral activity in stored milk is related to the level of lipoprotein lipase in the milk, indicating that it is caused by the release of fatty acids or other products of lipid hydrolysis. Similar results were previously reported by Welsh et al. (20, 21). In this study, we present more data which indicate that the antiviral effect of stored human milk is caused by lipolysis and show that of the nine fatty acids most commonly found in human milk (11), seven are highly active in killing enveloped viruses. The polyunsaturated long-chain fatty acids were the most active, but medium-chain saturated fatty acids, particularly lauric and myristic acids, also showed activity. Monocaprin and monolaurin were active in concentrations 10 times lower than those of the corresponding free acids, but monomyristin was consistently less active. Long-chain saturated fatty acids, which make up about 30% of the fatty acids in human milk, and short-chain fatty acids, which are more common in cow milk (11), were not, or were very slightly, antiviral. The concentrations of fatty acids found to reduce viral titers by ≥10,000-fold in vitro (Table 2) are in the same range of fatty acid concentrations found in human milk (11). Our results indicate that as lipolysis of milk triglycerides proceeds, either during storage or in the gastrointestinal tract, two types of antiviral lipids, monoglycerides and free fatty acids, are produced. It is possible that these two classes of lipid differ in efficacy against intestinal pathogens. This may also be true for the members of each lipid class.

Our results are similar to those of earlier studies with different viruses (6, 10, 16, 20) and further establish the marked antiviral effect of most fatty acids found in milk. The electron microscope study suggests that the antiviral effect is caused primarily by disintegration of viral envelopes by fatty acids. Similar findings were reported by Sarkar et al. (17), who treated mouse mammary tumor virus with the cream fraction of human milk and noted degradation of the viral envelope. Our study also shows disintegration of the plasma membrane of cultured cells with concentrations of fatty acids and stored human milk that inactivate enveloped viruses. The fatty acids and monoglycerides which we found to be strongly antiviral were shown to induce fusion of cell membranes (1). Although the exact mechanism is not clear, it has been suggested that the fatty acids and their monoesters are incorporated into the lipid membrane, causing destabilization of the bilayer (3). A similar mechanism might lead to the complete disintegration of cell membranes and viral

envelopes we observed. We did not compare the sensitivity of cells and enveloped viruses at various fatty acid concentrations. However, a study using hydrophobic alcohols showed that viruses are killed at concentrations that apparently had no effect on cultured cells (18).

Several studies have indicated a lower incidence of infections, particularly gastrointestinal, in breast-fed versus bottle-fed infants (4, 12). However, the role of milk fatty acids and their derivatives in protecting babies against illness is not established, despite their well-known antimicrobial effect in vitro. Although most known gastrointestinal viruses are nonenveloped, necrotizing enterocolitis in infants is caused by an enveloped virus, i.e., a human enteric coronavirus (15). *Giardia lamblia*, an intestinal protozoan parasite infecting children, is killed by milk fatty acids in vitro (9), suggesting the possibility of a giardiacidal effect of fatty acids in the intestines. Since fatty acids lyse cells by disrupting their plasma membranes, it is likely that they kill not only giardia but also other parasitic protozoa. Although a few studies have demonstrated antimicrobial activity of human and animal stomach contents after milk feeding (2, 9a), much more work is needed to characterize the active factors and to establish their role in prevention of, and recovery from, gastrointestinal infections.

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