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(71) Applicant *(for all designated States except US)*: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FAHL, William, E. [US/US]; 2411 Tawhee Drive #112, Madison, WI 53711 (US). LETCHWORTH, Geoffrey, J. [US/US]; 1022 Waban Hill Drive, Madison, WI 53711 (US). MUELLER, Gerald, C. [US/US]; 3926 Plymouth Circle, Madison, WI 53705 (US). SAVAGE, Adam, K. [US/US]; 311 North Blair Street, Madison, WI 53703 (US). LOO, Deborah [US/US]; 1400 University Avenue, Madison, WI 53706 (US).

(74) Agents: REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).

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(57) Abstract

Neonates during their first thirty days of life are particularly susceptible to pathogens because their immune system is not yet fully functional. Adults may also be unusually susceptible to pathogens when their immune system has been compromised by disease or when they have been acutely exposed to a bolus of GI pathogen. The invention provides a method of immunizing neonates and adults to pathogens by orally administering recombinant probiotic bacteria that express antibodies to the pathogens. These recombinant bacteria may be optionally administered with antibodies immunologically specific to the pathogens. The invention further provides a composition of recombinant probiotic bacteria that express antibodies to pathogen, and a method to use this composition to immunize neonates and adults.

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GASTROINTESTINAL BACTERIAL ANTIBODY FACTORIES

This application claims priority to U.S. Provisional Application No. 60/094,697, filed 30 July 1998; the entirety of which is incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the United States Department of Agriculture Grant No. 94-37204-0857; Hatch Nos. 3948 and 3822; and the National Institutes of Health Grant Nos. CA07175 and CA22484.

FIELD OF THE INVENTION

The present invention relates to the field of genetically engineered bacteria for therapeutic and prophylactic use. In particular, the invention provides recombinant probiotic bacteria expressing antibodies to common gastrointestinal pathogens, and methods for their use in disease treatment and prevention.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application in parentheses in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications is incorporated by reference herein.

For the first thirty days or so of life, humans and animals have no functional immunity to pathogens. As a result, infants can be at significant risk of serious disease or death, due to infection by pathogenic organisms, which, later in life, may be overcome by the action of a functional immune system.

For instance, in humans, cattle and swine, infection with rotavirus is one of the most significant causes of life-threatening diarrheal infections during the first few months of life. Roughly twenty percent of newborn calves and piglets in the United States die from such infection. In chickens, recurrent infection of entire broods with *Salmonella* is a common and recurring problem, causing death in a significant proportion of the chicken population as well as illness within humans when the *Salmonella* is carried over into the human food chain.

Immunosuppression in adults, as caused, for example, by certain diseases such as acquired immune deficiency syndrome (AIDS) or by treatment with immunosuppressive drugs, also increases vulnerability to pathogenic organisms to which the individual normally would be able to mount an immune defense. Such organisms include pathogenic *Escherichia coli*, *Cryptosporidium*, *Salmonella*, *Vibrio cholera*, *Listeria monocytogenes* and *Helicobacter pylori* (associated with ulcer development), among others. In addition, healthy adults exposed to an unusual pathogen or a large dose of pathogen (e.g., *Cryptosporidium* in contaminated drinking water or *Salmonella*-contaminated food and milk) would benefit by a means to reduce or prevent GI infection by the pathogen. Such large-dose infections have been known to occur in isolated incidences in the United States (e.g. patients in nursing homes and passengers on cruise ships), and certainly occur with greater regularity in more extreme conditions, such as refugee gatherings due to war, famine and the like. In this regard, persons such as military personnel who may be exposed deliberately to extreme doses of infective biological agents (i.e., "biological warfare") would also benefit by a means for reducing or preventing such infection.

Accordingly, a need exists to provide immune-mediated protection to neonates and/or immunosuppressed adults, to treat or avoid the serious and life-threatening diseases associated with infection by certain gastrointestinal (GI) pathogens.

Three basic approaches have been explored in an attempt to protect neonate animals against GI pathogens. One approach is to immunize (vaccinate) pregnant mothers using antigens associated with the pathogenic organism (e.g., pathogenic strains of *Escherichia coli*). The objective is to stimulate production of antibodies against the pathogen in the mother's colostrum, the milk that is fed to neonates in the first day or two postpartum. The antibody consumed by a neonate of some species may be retained for a period of time (i.e., absorbed into the blood and circulated), providing some immune coverage against subsequent exposure to the pathogen. In human neonates, none of the colostrum antibodies are retained and so this approach is not effective.

A second approach has been to immunize the neonate against pathogens using antigens associated with the pathogenic organisms. The oral administration of live heterologous and homologous rotavirus protected neonate and adult mice against a virulent murine rotavirus challenge (Feng et al., 1994, J. Virology 68:7766-7773; Burns et al., 1995, Virology 207:143-153). However, where the neonatal immune system is essentially non-functional this approach has met with very limited success. In human neonates in particular the immune system is only slightly functional, and so humans are unlikely to be benefitted by neonate immunization.

A third approach has been to administer a bolus, usually by nasal injection, of antibody or semi-purified antisera, directed against the pathogen of

interest, to the neonate animal. If the concentration of antibody is high enough on the GI mucosal surface, and the injections are repeated frequently enough to keep the antibody concentration high, then this procedure would afford the animal a means of protection against GI pathogens. However, this approach is neither cost-feasible nor time/personnel- feasible in an animal production facility of any significant size, inasmuch as it requires repeated handling of individual neonates, as well as multiple administrations of purified antibodies or serum, which can be expensive.

From the foregoing discussion, it can be seen that none of the approaches attempted thus far has provided a good solution to the problem of providing immune protection to infants or other immunosuppressed individuals. It is an object of the present invention to provide such a solution; i.e., an effective and feasible means for imparting immunity to neonates and other immunosuppressed individuals against GI and other pathogens.

SUMMARY OF THE INVENTION

This invention provides compositions and methods for supplementing or replacing an immune response against selected pathogens in individuals requiring such treatment. Generally, the invention is directed toward newborn infants, either animals or humans, or to immunosuppressed or immunodeficient adults, or to healthy individuals acutely exposed to a bolus of GI pathogen.

The compositions of the invention comprise probiotic microorganisms which have been genetically modified to express recombinant antibodies (rAB) directed against selected pathogenic organisms. In preferred embodiments of the invention, the probiotic microorganisms are GI tract-colonizing or -associated

bacteria, such as *Lactococcus*, *Bifidobacteria*, *Eubacteria*, non-pathogenic strains of *Escherichia coli*, *E. coli* F18 and *E. coli* strain Nissle 1917, or various strains of *Lactobacillus*, such as *L. casei*, *L. plantarum*, *L. paracasei*, *L. acidophilus*, *L. fermentum*, *L. zeae* and *L. gasseri*. In preferred embodiments, the pathogenic organisms are GI pathogens, such as rotavirus, caliciviruses, reoviruses, coronaviruses, enteroviruses, adenoviruses, Norwalk-type viruses, enterotoxigenic *Escherichia coli*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Cryptosporidium* spp., *Giardia lamblia*, *Entamoeba histolytica*, *Heliobacter pylori*, *Isospora belli*, *Clostridium spp.* and *Vibrio cholera* (the latter two causing GI distress *via* secretion of toxins); and non-GI pathogens, such as coxsackieviruses, poliovirus, hepatitis A virus, Salmonella and Shigella spp., *Listeria monocytogenes* and strongyloides. In preferred embodiments, the antibodies expressed by the probiotic bacteria are adapted for expression in bacteria. Though any one bacterial cell produces only a single species of recombinant antibody, multiple clones of producing bacteria can be mixed to provide probiotic populations that produce recombinant antibodies against multiple pathogens at the same time. This is an additional advantage conferred by this invention because vaccination of animals generally allows for only a single, productive immune response to a single antigen to occur at the same time. The probiotic bacteria may express a mixed population of antibodies immunologically specific to one pathogen, or to more than one pathogen. In a particularly preferred embodiment, the probiotic organism is expressing a rotavirus-neutralizing antibody. In another preferred embodiment, the composition of the

invention further comprises lactose, maltodextrin and/or a desiccant.

The invention is practiced by feeding the antibody-expressing probiotic organisms to an animal or human in need of such treatment. The organisms colonize or otherwise become associated with the GI tract of the individual, whereupon they grow and multiply, continuing to produce and secrete anti-pathogen antibodies. Thus, a steady-state immune condition with respect to the selected pathogens is created in the GI tract of the individual, who is thereby protected from infection by the pathogen(s), even though the individual is unable to generate its own immune response. In preferred embodiments, the individual requiring this treatment is a neonate, immunosupressed or immunodeficient adult, or a healthy individual acutely exposed to a bolus of one or more pathogens. In a preferred embodiment, a supplemental bolus of antibodies immunologically specific to at least one pathogen is administered with the antibody-expressing probiotic organism. In another preferred embodiment, the antibody-expressing probiotic organism is administered orally to a neonate in a bolus comprising up to 40 X 10^9 colony forming units (cfus), two times a day, for up to eight weeks of probiotic bacteria, and may additionally comprise up to 25 mg/kg body weight/day of at least one antibody immunologically specific to at least one pathogen. In another preferred embodiment, the antibody-expressing probiotic organism is administered orally to an adult in a bolus comprising up to 80 X 10^9 colony forming units, three times a day, for ten days or until the external threat of the pathogen is removed. The bolus may additionally comprise up to 25 mg/kg body weight/day of at least one antibody immunologically specific to at least one pathogen.

Other features and advantages of the present invention will be understood from the description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. DNA sequence analysis of the ~ 750 base pair Sfi1-Not1 insert in the pCANTAB 5E cloning/expression vector (Clone 11)(SEQ ID NO:1). The open reading frame within the insert contains the mouse variable heavy chain (V_H) and variable light chain (V_L) domains which are found in the monoclonal antibody secreted by the M159 hybridoma. The open reading frame also contains the 13 amino acid E tag domain that enables immunodetection and immunoaffinity purification of the rAb molecule. The *Sfi*1 and *Not*1 sites the define the rAb insert region, the V_H domain showing 97-99% sequence identity to other mouse heavy chain genes, the linker region, the V_L domain showing 95-99% sequence identity to other mouse light chain genes and the 13 amino acids E tag domain are all indicated.

Figure 2. DNA sequence analysis of the ~750 base pair Sfi1-Not1 insert in the pCANTAB 5E cloning/expression vector (Clone 22)(SEQ ID NO:2). The sequence is identical to that found in Clone 11 except for two codons within the linker region, and two codons within the V_H region that could provide minor differences in recognition and binding affinity for the encoded rAb. The *Sfi*1 and *Not*1 sites the define the rAb insert region, the V_H domain showing 97-99% sequence identity to other mouse heavy chain genes, the linker region, the V_L domain showing 95-99% sequence identity to other mouse light chain genes and the 13 amino acids E tag domain are all indicated.

Figure 3. Western blot analysis of recombinant antibody levels in bacterial media taken from induced cultures of clone 11 and clone 22. Also shown is purified recombinant antibody following immunoaffinity chromatography on a column to which anti-E tag antibody was conjugated. Recombinant antibody is visualized in this blot by probing the blot with an anti-E tag antibody to which horseradish peroxidase is conjugated; color development is done using ABTS reagents. Lane 1 contains 1 µg recombinant antibody standard Lane 2 contains TCA-precipitated 1 ml of clone 11 media. Lane 3 contains TCA-precipitated 1 ml of clone 22 media. Lane 4 contains an aliquot of purified clone 11 rAb eluted from anti-E tag immunoaffinity column.

Figure 4. Purification of the recombinant antibody from media of clone 11 using an anti-E tag immunoaffinity column. An aliquot of Fraction 6 was analyzed by western blot; it is shown in lane 4 in the Figure 4 immunoblot.

Figure 5. The percent of neonate mice with diarrhea after a gastric bolus containing 0 µg, 25 µg, 8 µg and 1 µg of M159 rotavirus monoclonal antibody followed by an oral rotavirus challenge dose (10^7 pfu).

Figure 6. The percent of neonate mice with diarrhea at three days after a gastric bolus containing 25 µg of M159 rotavirus antibody followed by an oral rotavirus challenge dose 0 h, 8 h, 16 h and 25 h later. The control treatment consisted of a gastric bolus without M159 rotavirus antibody with an oral rotavirus challenge dose 25 h later.

DETAILED DESCRIPTION OF THE INVENTION

The following basic steps should be followed in order to practice the present invention: (1) identify a pathogen or combination of pathogens against which immune protection in a neonate or other at-risk individual is desired; (2) in a conventional system (e.g., mouse, rabbit, sheep), generate neutralizing antibodies against the pathogen(s); (3) clone the antibody genes in a form suitable for expression in selected probiotic bacteria; (4) transform the bacteria with the cloned antibody genes, such that the bacteria express and secrete the antibodies; and (5) feed the recombinant antibody-producing bacteria to animal or human subjects for which the immune protection is desired. The sections below describe each of these steps in greater detail.

This invention is applicable to the prevention or control of disease caused by any pathogen against which a gastrointestinal immune response is effective. It is particularly applicable to GI pathogens, but can also provide a measure of protection (i.e., a first line of defense) against ingested pathogens whose natural target is not the GI tract.

GI pathogens contemplated as targets for practice of the present invention include, but are not limited to: rotavirus, caliciviruses, reoviruses, coronaviruses, enteroviruses, adenoviruses, Norwalk-type viruses, enterotoxigenic *Escherichia coli*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Clostridium* spp., *Vibrio cholera*, *Cryptosporidium* spp., *Giardia lamblia*, *Entamoeba histolytica*, *Heliobacter pylori*, and *Isospora belli*. Non-GI pathogens that enter the body through the GI tract contemplated as targets for practice of the present invention include, but are not limited to coxsackieviruses, poliovirus, hepatitis A virus,

Salmonella and Shigella spp., *Listeria monocytogenes* and strongyloides.

Neutralizing antibodies against one or more selected pathogens can be produced using any standard methodology. They can be generated against any antigenic determinant of the pathogen, including the intact pathogen, specific surface antigens of the pathogen, epitopes of those surface antigens, or any other portion of the organism capable of eliciting an immune response. In a preferred embodiment, recombinant antibodies from a particular species are used to immunize the same species.

In a preferred embodiment of the invention, monoclonal antibodies immunologically specific for a particular antigen of the pathogen are produced according to standard methodologies. Some such antibodies are already known and available. For instance, Example 1 describes a neutralizing monoclonal antibody against rotavirus serotype G3. In addition, a hybridoma is available from the American Type Culture collection (ATCC Accession No. HB 8178), which secretes a monoclonal antibody against the K99 pilus protein found on enterotoxigenic strains of *E. coli*. The advantage of using a monoclonal antibody is that it is directed to a single antigen or epitope that can be pre-screened as "neutralizing", i.e. binding to an antigen on the pathogen important for infection, replication, etc. Other monoclonal antibodies and fragments thereof of interest include, but are not limited to, those specific to the hepatitis B surface antigen (Passafiume et al., 1998, FEBS Let. 441:407-412), the Puumala virus (Liang et al., 1997, Virology 235:252-260), HIV-1 (Burton et al., 1994, Science 266:1024-1027) and the human rhinovirus (Condra et al., 1990, J. Biol. Chem. 265:2292-2295).

A mixed population of monoclonal antibodies, or a population of polyclonal antibodies may also be used in the invention. For instance, instead of using a single

hybridoma cell line, spleens from immunized mice may be harvested and used to clone a mixed population of antibodies against the antigen used for immunization. The advantage of this approach is that the mixed antibody population targets a diverse array of epitopes on the pathogen, which will be able to neutralize the pathogen at different sites within the physiologically changing conditions of the mammalian or avian GI tract.

Finally, recombinant antibodies suitable for use with the invention may be synthetic mutants. One or more recombinant antibodies specific to a site on a selected pathogen may be isolated by starting with a library of recombinant antibodies. In this antibody library, the three short domains within the variable region of the heavy chain (residues 31-33, 50-54, 95-98) and the three short domains within the variable region of the light chain (residues 32, 50, 91-96), which determine high affinity binding to antigen, have been deliberately mutagenized using partially degenerate primers to create a synthetic library with enormous diversity (Pini et al., 1998, J. Biol. Chem. 273:21769-21776).

Methods for cloning antibodies for expression in bacteria are well known in the art. For instance, as described in greater detail in the Examples, a kit for cloning antibodies into phagemids for expression in *E. coli* is commercially available. In a preferred embodiment of the invention, that kit and the protocols set forth therein are used to clone selected antibodies and test them *in vitro* for their ability to neutralize the target pathogen(s). The cloned antibodies are then adapted for transformation and expression in the selected probiotic bacterium. In another preferred embodiment, genes encoding recombinant antibodies isolated in the first rounds of selection are deliberately subjected to random mutagenesis using degenerate primers to both the

heavy chain and light chain antigen binding domains to isolate mutant variants with enhanced binding affinities for neutralizing sites on the pathogen of interest (Pini et al., 1998, J. Biol. Chem. 273:21769-21776).

After isolation, the sequence of the antibody protein may be deliberately altered to increase the affinity of binding to the neutralizing sites on the pathogen and/or stability in the GI tract. In a preferred embodiment, a residue within the linker sequence between the V_H and V_L domains is mutagenized to substitute a cysteine residue in order to enable the formation of disulfide bonds between rAb monomers to yield dimeric rAbs with increased binding affinity. In another preferred embodiment, codons within protease digestion sites are changed to structurally neutral amino acids to eliminate the digestion site. In this embodiment, if the site occurs within one of the known antigen recognition domains on the recombinant antibody, then mutagenesis is combined with re-selection to identify variants that have lost the protease site and have retained high affinity antigen binding.

Antibodies immunologically specific for selected pathogens can also be administered in combination with the recombinant probiotic bacteria expressing the antibodies. These antibodies may be isolated, or may be administered while still inside the antibody-expressing cell. The isolated antibodies may be secreted by the recombinant probiotic organism or may be purified natural or recombinant antibodies. The administration of antibodies with the recombinant probiotic bacteria expressing antibodies has the advantage of establishing immune protection immediately, while the probiotic bacteria establish themselves in the gastrointestinal tract and begin to secrete recombinant antibodies.

Probiotic bacteria suitable for the present invention include any non pathogenic, preferably physiologically beneficial, bacteria that colonize or are otherwise associated with or resident in the gastrointestinal tract of the selected subject animal or human. Preferably, these bacteria are species of the genus *Lactobacillus*. However, other genera may be utilized, including, but not limited to: *Lactococcus*, *Bifidobacteria*, *Eubacteria* and non-pathogenic strains of *Escherichia coli* (see Gibson and Roberfroid, J. Nutrition 125: 1401-1412, 1995). Non-pathogenic *Escherichia coli* strains of particular interest include, but are not limited to, *E. coli* F18 (Cohen et al., 1983, Infect. Immun. 40:62-69; Myhal et al., 1982, Eur. J. Clin. Microbiol. 1:186-192), *E. coli* strain Nissle 1917 (*E. coli* DSM 6601, Mutafluor®)(Lodinová- ádniková and Sonnenborn, 1997, Biol. Neonate 1:224-232, incorporated by reference herein).

The choice of probiotic strain(s) to be used with the present invention may be determined by a combination of factors, including the normal site of infection for a given pathogen and the normal site for colonization of the antibody-producing bacteria. For example, rotavirus typically infects GI epithelium in the last 20-30 % of the GI tract. One strategy for neutralizing rotavirus infection would include feeding a mixture of *E. coli* F18 or K12 cells secreting anti-rotavirus recombinant antibody that will colonize and secrete antibody in the lower GI tract, overlapping the site of rotavirus infection, mixed with *Lactobacillus braevis* cells secreting anti-rotavirus recombinant antibody that will colonize and secrete antibody in the very upper portion of the GI tract. While not limiting the function of this embodiment to any one mode, with

this approach the ingested virus will be engaged and bound by antibody early in the GI tract, and antibody concentration will be further boosted by F18 *E.coli* cells colonized at the normal site of infection.

Probiotic bacterial strains suitable for delivering presynthesized antibody are strains that do not secrete the recombinant antibodies, but rather sequester them inside the cell membrane or wall. Such strains include, but are not limited to, laboratory strains of *E. coli* that have been derived from the original probiotic strain *E. coli* K12. These laboratory strains of *E. coli*, such as HB2151, are advantageously selected for their weakened cell walls. While not limiting the function of this embodiment to one mode, it is contemplated that these strains of *E. coli* expressing at least one recombinant antibody will lyse in the upper GI tract when exposed to the normal bile salts. In lysing, these cells will spill their contents of recombinant antibody into the GI lumen where it will mix with other lumenal contents. This embodiment provides an inexpensive way to deliver pure recombinant antibody to the upper GI tract.

The Lactobacilli are a large and diverse group of Gram-positive bacilli that are common components of the normal indigenous flora of humans and other animals. Lactobacilli are considered "health promoting," but in any event are rarely pathogenic, making them good candidates for use in the present invention. Indeed, Lactobacilli have been exploited recently as vaccine delivery vehicles, i.e. expressing antigens in the GI tract of immune-functional individuals for purposes of eliciting an immune response (see, e.g., Rush et al., Chapter 6 in Gram-Positive Bacteria as Vaccine Vehicles for Mucosal Immunization, (G. Pozzi & J.M. Wells, eds.), Landes Bioscience, 1997).

Different strains and species of *Lactobacillus* are differentially capable of colonizing or becoming otherwise associated with the GI tract of a particular animal. Two approaches can be used in selecting appropriate strains for delivery of antibodies to a particular animal species (including humans): (1) selection of strains known to be capable of colonizing mucosal surfaces of the animal (e.g., *Lactobacillus GG* is known to colonize the human GI tract); and/or (2) selection of strains naturally found in foods, which may have limited interactions with the host GI tract, but nevertheless may inhabit the GI tract for significant periods of time.

The fate of viable *Lactobacillus* cells within the GI system of mammals depends on a number of variables. At one extreme, the bacterial cells simply mix with the undigested fiber of ingested food and move along the GI system secreting antibody into the mucosal matrix that covers the inner surface of the GI epithelium. In this situation there is little colonization by the *Lactobacillus* cells. At the opposite extreme, cells will find host sites within the mucosal matrix, stay there and replicate (i.e., "colonize"), and continue to secrete antibody into the mucosal matrix around them. The factors that determine the ability of cells to colonize or merely populate sites within the GI system are not fully understood. Some parameters that affect the ability of probiotic bacteria to colonize include: the pH of the particular GI site (acidophilus means "acid lover" meaning these strains can accommodate the low pH of the stomach and duodenum); the oxygen content of the site; the strain of bacteria; and the ability to compete for nutrients with other types of bacteria that are encountered on the mucosal surface of GI epithelium. Inasmuch as transient or colonizing

strains will be able to perform the intended function of secreting antibodies, selection of appropriate strains can be made on the basis of how long such secretion is desired in the individual undergoing the treatment. Alternatively, combinations of different strains, some transient and others colonizing, can be used to provide short and long-term immune protection.

Many *Lactobacillus* species have been demonstrated amenable to transformation and expression/secretion of recombinant proteins. Lactobacilli can be genetically modified by way of two mechanisms: (1) *via* introduction of an ectopic plasmid carrying a foreign gene; or (2) *via* stable integration of a foreign gene into the genome. One advantage to this latter approach is that it avoids potential loss of the foreign gene, which can result from plasmid shedding during expansion of the bacterial cultures. Thus, the potential lower expression associated with stable integration of the foreign gene could be offset by the more stable retention of that gene and resulting ability of the bacteria to express and secrete recombinant antibodies for an extended period of time.

There are numerous examples in the art of cloning and expression vectors designed for use in *Lactobacillus* species. Moreover, the art describes the successful introduction and expression of foreign genetic material in a variety of *Lactobacillus* species, including *L. casei, L. braevis,L. plantarum, L. paracasei, L. acidophilus, L. fermentum* and *L. zeae*, among others (Rush et al., 1997, *supra;* Rush et al., Microbiol. Biotechnol. 47: 537-542, 1997; Hols et al., Microbiology 143: 2733-2741, 1997). Example 3 below describes vector design and a protocol for transformation of an *L. gasseri* strain that colonizes the murine upper GI tract. This protocol follows standard protocols for transformation of

Lactobacillus by electroporation (see, e.g., Aukrust et al., Chapter 20 in Methods in Molecular Biology, Vol. 47, (J.A. Nickoloff, ed.), Humana Press Inc., Totowa, N.J.).

The stable integration of foreign genes into the *Lactobacillus* genome via the temperate phage mv4 has also been described (Dupont et al., J. Bact. 177: 586-595, 1995; Avuray et al., J. Bact. 179: 1837-1845, 1997). This bacteriophage, which naturally occurs in *L. delbruckii* subsp. *bugaricus* has been modified for use in integrating foreign genes into the genome of *L. plantarum*, *L. casei* and *L. lactis*.

The vectors presently available in the art for transforming *Lactobacillus* were not designed for consumption by humans or animals. In particular, most of the vectors utilize antibiotic resistance as a selection means. Since it is undesirable to introduce antibiotic resistance into an animal or human patient, the currently available vectors should be modified to comprise a different selection means, such as a color indicator. Selection means that do not rely on antibiotic resistance are known in the art and are available.

Once the recombinant antibody-expressing probiotic bacteria are produced, they are used to protect against invasion by the selected pathogen in neonates or other individuals requiring such treatment. The mode of administration is by oral or nasal injection, or by feeding (alone or incorporated into the subject's feed or food). For use in neonates, the bacteria preferably are introduced into the GI tract at birth, and thereafter periodically until the infant's immune system becomes functional. In this regard, it should be noted that the *Lactobacillus acidophilus* that is presently widely available commercially is sold as gel capsules containing a lyophilized mixture of bacterial cells and a solid support such as mannitol. When the gel capsule is

ingested with liquid, the lyophilized cells are re-hydrated and become viable, clonogenic bacteria. Thus, in one preferred embodiment, *Lactobacillus* cells are added to the neonate's milk substitute by sprinkling in the powdered, lyophilized preparation. The re-hydrated, viable *Lactobacillus* cells will then populate and/or colonize at sites throughout the upper and lower gastrointestinal system, and by constitutively secreting recombinant antibody, provide a blanket of protection against the pertinent pathogens.

For other individuals, the bacteria should be administered as soon as, or prior to (if possible), immunosuppression or exposure to extreme doses of a GI pathogen occurs. For instance, for protection of military troops in transport to a war zone, probiotic strains of the invention that populate the GI tract can be continuously consumed as a gel capsule for as long as a potential threat remains. After consumption of such capsules is terminated, the probiotic cells are slowly diluted away from the GI tract. For infrequent but recurrent metropolitan water contamination with, e.g., *Cryptosporidium* or *Salmonella*, the probiotic bacteria of the invention are used to abruptly stop the widespread morbidity associated with such public health infections rather than to allow an extended, slow disappearance of infections as the pathogen is brought under control by various means, if any. In the case of *Cryptosporidium*, the method of treatment of the invention is particularly important as no chemotherapy is currently available. In this setting, distribution and consumption of probiotic bacteria secreting the appropriate antibodies can provide immediate immune coverage for the population, thereby quickly reducing the number of infectable hosts and greatly accelerating the disappearance of the metropolitan epidemic.

The composition of the invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the composition appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art (Lodinová- adniková and Sonnenborn, 1997, Biology of the Neonate 71:224-232).

The composition comprising a probiotic microorganism expressing the antibody or antibodies of choice preferably is administered orally, with or without supplementary antibodies. The bacteria and optional antibodies may be hydrated or lyophilized. Several options exist for formulating these boluses, including tablets, capsules, solutions, syrups, elixirs, suspensions, magmas, gels and powders. The composition may also include diluents, disintegrants, desiccants, coatings and colorants. Enteric coatings may be used to delay dissolution of the tablet until it has passed into the intestine.

The composition of the invention may further comprise food products. A bolus of lyophilized, engineered, probiotic bacteria which is generally tasteless, could also be dispersed into foods to expedite consumption by adult livestock or humans. Lyophilized bacteria have shelf lives of approximately one year when stored in a refrigerator and about 3-4 months when stored without refrigeration. As is currently the case for non-engineered probiotic bacteria, the recombinant antibody-expressing bacteria could also be used to produce foods such as yogurt or kiefer, or they could be added to the

finished food products to provide the desired protective role once consumed.

For neonates, both livestock and humans, the most common method of administering the composition of the invention will comprise oral dosing of a composition comprising lyophilized bacteria, lactose or maltodextrin as a carrier/filler and stir the tasteless powder into milk substitute (livestock) or formula (humans) that is being fed to the neonates multiple times each day. For adult humans, gel capsules or compressed tablets containing the same mixture of lyophilized bacteria and carrier/filler used in neonates is contemplated as the most efficient way to consume the probiotic bacteria.

Examples in which lyophilized probiotic bacteria are administered as described above are well known in the art and are also commonly used by health food consumers in today's marketplace. Current recommendations from manufacturers suggest that adult humans should consume between 9 - 90 X 10^9 colony forming units of probiotic bacteria per day in order to re-colonize a human gastrointestinal tract following a 10 day course of antibiotics. In commercial preps of lyophilized probiotic bacteria, there are about 10^{10} colony forming units per gram of dried, fibrous powder. Therefore, the daily dose can be easily consumed either in three gel capsules, as a powder stirred into food or infant formula, or as a standard constituent of a food product such as yogurt.

In a preferred embodiment, the probiotic microorganism composition is administered to a neonate over the period of time during which the animals are immunologically at-risk. Generally, a bolus comprising 3 X 10^9 - 40 X 10^9 colony forming units is be administered two times a day, for up to eight weeks. Optionally, antibodies may be added to the neonate bolus at a

concentration between 0.1 mg - 25.0 mg/kg body weight/day, depending upon the relative binding affinity of the respective antibodies.

In another preferred embodiment, the probiotic bacterial composition is administered to an adult in a bolus comprising 12 X 10^9 - 80 X 10^9 colony forming units, three times a day for at least ten days. The actual duration of administration will be largely determined by the duration of the external pathogen threat. The composition of the probiotic material administered to adults will be the same as previously described for neonates, and the method by which it is administered will include gel capsules, compressed capsules and dispersal in foods. The lyophilized bacteria preparations may be prepared at up to 10^{10} colony forming units per gram and then diluted out with filler/carriers like maltodextrin to simplify handling.

In humans, initial doses of probiotic bacteria and antibody is based upon the body weight doses optimized in prior work with laboratory animals and with clinical studies in livestock animals. Based upon the data from the mouse experiments set forth in Example 4, it is extrapolated that a dose of monoclonal antibody in the range of 0.1-2.5 mgs per kg body weight per day is expected to protect livestock or humans against GI infection with rotavirus or other pathogens of interest. This extrapolated dose would be higher, e.g., 1.0 - 25 mgs per kg body weight per day for recombinant antibodies with lower binding affinities.

The appropriate dosage will be one that diminishes the symptoms of infection and/or protects against infection. The levels of the antibody can are monitored in stool samples over defined periods of time to determine the effectiveness of the dosage.

The following examples are provided to describe the invention in greater detail. They are not intended to limit the invention in any way. Unless otherwise specified, general cloning, biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) ("Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) ("Ausubel et al.") are used.

EXAMPLE 1
Preparation of Anti-Rotavirus Antibodies for Expression and Secretion in *E. coli*

In this example, we describe the cloning a mouse anti-rotavirus monoclonal antibody (Mab) for expression in *E. coli*. This protocol utilizes a commercially available kit, the "Recombinant Phage Antibody System" (Pharmacia Biotech, Piscataway, New Jersey), and essentially follows the manufacturer's instructions.

A murine hybridoma cell line (#159) expressing a rotavirus serotype G3-neutralizing monoclonal antibody was obtained as a gift from H.B. Greenberg, Stanford University. A culture of the #159 cell line containing ~5 x 10^6 viable cells was prepared. Messenger RNA was isolated from the cultured cells using the QuickPrep® mRNA purification kit (Pharmacia, Piscataway N.J.) according to the manufacturer's instructions.

The mRNA was ethanol-precipitated, resuspended in 20 µl RNAse free water and heated to remove any secondary structure. First strand cDNA synthesis was then performed according to the manufacturer's instructions. The first strand antibody cDNA was used as a template for PCR amplification to generate suitable quantities of antibody heavy and light chain DNA for

cloning. This was accomplished by standard PCR protocols (as set forth in the manufacturer's instructions), using primers specifically hybridizing to appropriate heavy chain and light chain-encoding DNA. The amplified heavy and light chain PCR fragments were separated from other components of the mixture by preparative agarose gel electrophoresis, followed by excision of the separated bands and elution of the fragments from the agarose. The purified products were then gel quantified.

The antibody heavy and light chain DNA were joined into a single chain with a linker DNA segment provided by the manufacturer. The resultant combined DNA segment (referred to in the manufacturer's instructions as the ScFv DNA) was amplified by PCR, using primers containing either *NotI* or *SfiI* restriction sites. The final product was separated from other components of the PCR mixture by centrifugation in a MicroSpin column, after which the ScFv DNA segment was quantitated.

The ScFv DNA was subjected to digestion with *NotI* and *SfiI* for ligation into a similarly digested phagemid vector, pCANTAB 5E, provided with the kit. The phagemid displays the recombinant antibody domain because it is fused to the external, filamentous gene 3 protein.

Competent *E. coli* TG1 cells were then transformed with the pCANTAB 5E vector containing the antibody ScFv insert. Two clones, clone 11 and clone 22, were selected for further study. The ~750 bp *Sfi*1 - *Not*1 rAb inserts of clone 11 (SEQ ID NO:1; Fig. 1) and clone 22 (SEQ ID NO:2; Fig. 2) were sequenced. TG1 cells contain a stop-codon suppressor that causes a portion of the recombinant antibody to remain attached to the phage. Recombinant phage were rescued from the transformed *E. coli* according to the manufacturer's instructions.

Immunoblotting showed the level of recombinant antibody in the media from *E. coli* clones 11 and 22, and

a purified fraction of rAb from an anti-E immunoaffinity column (Fig. 3). Cultures of HB2131 cells were induced with 1 mM IPTG for 4 hours. Cells were pelleted by centrifugation, and 1 ml media aliquots were precipitated with 10% TCA on ice. Proteins were neutralized with Tris base, dissolved in SDS sample buffer and electrophoresed on a 4-12% polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with an anti-E tag antibody conjugated with horseradish peroxidase. Color development of bands was done using ABTS reagents. In Fig. 3, one ml of bacterial media is shown to contain about 0.3 ug of recombinant antibody. In this culture, there were ~ 2 X 10^9 bacteria/ml which extrapolates to 0.2 pg recombinant antibody produced per cell per hour.

Recombinant phage were tested for expression of the anti-rotavirus antibody by immunological interaction with intact rotavirus or with the epitope for which the murine Mab is specific, if it is known. Following this confirmation, *E. coli* may be reinfected with the phage clones. The *E. coli* strain HB2151 (which does not contain the stop codon suppressor) was used for the purpose of producing soluble recombinant anti-rotavirus antibodies, in accordance with the manufacturer's instructions. Soluble rAb was purified on an anti-E tag immunoaffinity column as per the manufacturers instructions (Fig. 4). Fifty ml of bacterial media from IPTG-induced clone 11 cells was applied to column; column was washed and then eluted with 0.1 M glycine (pH 3.0).

In connection with the panning and confirmation steps described above, we have obtained MA-104 cells which are host cells that allow replication of RRV2-1 rotavirus in tissue culture. The MA-104 cells are infected with the RRV2-1 rotavirus, letting the lytic viral expansion occur in the tissue culture dishes, and then harvesting the virus particle-laden media

supernates. The virus-containing media is subjected to both fluorocarbon extraction and sucrose density centrifugation to yield highly concentrated viral stocks which are then titred to determine the number of infectious viral particles per ml using MA-104 monolayer cultures in a standard plaque-forming assay. After a week in culture, these plaques are scored by staining the monolayer with crystal violet and scoring the number of clear plaques formed. The number of clear plaques equals the number of infectious virus particles applied to the plate. Viral stocks typically exceed 1 x 10^9 plaque-forming units (pfu) per ml.

The titered tissue culture supernates are used for at least four purposes. First, in the initial isolation of clones of *E. coli* cells that are expressing recombinant antibody against rotavirus, there will presumably be minor differences between clones as to the exact specificity and binding characteristics of the recombinant antibody being made. To identify an *E. coli* clone that is producing the recombinant antibody with the most desirable binding characteristics, the virus particles are adsorbed on to the bottom of a multiwell plate, and then media is added that contains individual clones of *E. coli* cells that are known to be making recombinant antibody. At this stage, the recombinant antibody is still fused to the end of a phage protein that is extended from the surface of the *E. coli* cell. Thus, by binding the recombinant antibody to the adsorbed rotavirus, i.e., panning, one can also recover the *E. coli* cell and the recombinant gene that encodes the recombinant antibody.

Second, at the stage of selecting soluble antibodies, a more refined manner is employed for selecting soluble, recombinant antibodies that have desirable characteristics for the binding of rotavirus,

now in suspension rather than adsorbed to a plastic surface. Here, rotavirus particles that have been detectably labeled (e.g., biotinylated) are added to individual suspensions of recombinant antibody. The conditions of the binding assay can be varied to select for recombinant antibodies that have particular binding characteristics that might be desirable when they are secreted from *Lactobacillus* cells in the lumen of an animal's intestine. This might include, for instance, the ability to bind rotavirus or the K99 antigen of ETEC *E. coli* at a reduced pH representative of food leaving the stomach. Panning can actually be done in the presence of dilutions of previously sterilized GI lumenal contents to accurately reflect the conditions under which antigen-recombinant antibody binding occurs. Binding of soluble, recombinant antibody to biotinylated rotavirus particles can be demonstrated by adding avidin coated beads to bind the biotinylated rotavirus, and pelleting. The resuspended beads are incubated with, e.g., a fluorescein-labeled anti-mouse antibody. If primary, recombinant antibody is bound to the rotavirus particles, then the fluorescein-labeled secondary antibody will also bind and this is monitored using a standard 96 well fluorescence monitor.

Third, the neutralizing ability of recombinant antibodies can be screened by adding known numbers of plaque-forming units from RRV stocks to MA-104 monolayers to achieve a known number of clear plaques per tissue culture well. Dilutions of individual recombinant antibodies are preincubated with the titered virus supernates prior to adding the virus to MA-104 supernates. A reduction in the number of clear plaques is indicative of the ability of the recombinant antibody to neutralize binding and infection of natural host cells. In the first stage of such screening, test cells

such as MA-104 cells are examined. These results are used to infer the neutralizing effect of the antibodies in normal GI epithelial cells of host organisms. For the RRV2-1 strain of virus, normal host organisms, i.e., those animals that develop severe GI distress when infected, include at least mice, humans and monkeys.

Fourth, in animal model systems, bacteria (first laboratory and probiotic strains of *E. coli* (HB2151, F-18, Nissle 1917), and subsequently strains of *Lactobacillus,* e.g., *Lactobacillus gasseri* which are known to colonize in mice) secreting the recombinant antibodies are fed to mice, and then titered aliquots of virus-laden supernate (typically 10^7 pfus) are given to the neonate mice to assess whether, and to what extent, the antibody-secreting bacteria suppress or eliminate diarrheal infection in the neonate mice. Mice are scored for the presence of a fulminant RRV infection by gently pressing the abdomen and examining the anal region for the presence of yellow watery stool or for the presence of yellow stool in the last 5 cm of the colon.

EXAMPLE 2
Modification of DNA Encoding a Recombinant Anti-Rotavirus Antibody for Expression and Secretion in *Lactobacillus*

The anti-rotavirus-encoding SvFc DNA described in Example 1 is used to produce a plasmid for transformation and expression in *Lactobacillus*. The *Lactobacillus* expression vector, pCMR102 (Rush et al., Microbial. Biotechnol. 47: 537-542, 1996) is used as the base plasmid. This plasmid is a derivative of the plasmid pZN17, which contains a broad host-range *Lactococcus lactis* plasmid pSH71 replicon and is a low copy number plasmid both in *E. coli* and *Lactococcus lactis*. The pCMR102 plasmid further comprises a

synthetic derivative, EZZ, of the protein A gene from *Staplococcus aureus*. The pCMR102 plasmid has been shown to direct production and export of a heterologous protein in several *Lactobacillus* species (Rush et al., 1996, *supra*).

The MOMP VD4-encoding segment of pCMR102 is excised from the plasmid by restriction digestion with *EcoRI* and *PstI*, and replaced with the rotavirus antibody-encoding ScFv DNA, which has been end-modified to contain the appropriate restriction sites. The new plasmid comprises a DNA segment encoding an in-frame fusion protein of the anti-rotavirus antibody and the EZZ protein.

Once efficient expression and secretion of the recombinant antibody are achieved using the plasmid-encoded gene, the purified plasmid DNA is digested with appropriate restriction enzymes to remove an intact DNA cassette that contains 5' and 3' regulatory sequences and the coding sequence for both the recombinant antibody, as well as a gene product that will enable colorimetric or immune screening for bacterial colonies that are synthesizing the encoded gene products. The expression cassette is electroporated under the previously optimized conditions into the appropriate strain of log-phase growing probiotic host cells. The cells are then plated under non-selective conditions, and a large number of probiotic bacterial colonies are screened to detect colonies that are (1) synthesizing and secreting the recombinant antibody and/or (2) expressing the originally attached gene that would enable colorimetric screening.

The above-mentioned latter step of transferring in a DNA fragment to the bacterial cells and then growing the cells to enable integration of the gene expression cassette into a neutral or favored site within the genome of the selected probiotic bacterium is done for two

reasons: (1) so that the bacteria will have no drug resistance genes in them when they are released and consumed as a product by otherwise healthy individuals, and (2) so that the likelihood of shedding the recombinant antibody gene from the bacterial genome is greatly reduced or eliminated, as compared to placement of the gene on an ectopic plasmid. Methods are available in the literature for introducing recombinant genes on DNA fragments into favored sites within the *Lactobacillus* genome that enable extensive cell growth without loss of the recombinant gene (e.g., Raya et al., J. Bact. 174: 5584-5592, 1992; DuPont et al., J. Bact. 177: 586-595, 1995). Such methods are expected to be equally useful for other probiotic bacteria.

EXAMPLE 3
Transformation of *Lactobacillus gasseri*

Lactobacillus species are amenable to transformation by electroporation. In this example we describe a protocol devised for the transformation of *Lactobacillus gasseri*.

Media:

1) MRS broth: 10.0 g peptone, 8.0 g meat extract, 4.0 g yeast extract, 20.0 g glucose, 1 mL monooleate (Tween 80), 2.0 g K_2HPO_4, 5.0 g sodium acetate·3H20, 2.0 g $(NH_3)_3$-citrate, 0.2 g $MgSO_4 \cdot 7H_20$, 0.05 g $MnSO_4 \cdot 4H_20$, distilled water to 1 L.

2) MRS plates: MRS broth solidified with 1.5% agar.

3) MRSSM: MRS with 0.5 M Sucrose, 0.1 M $MgCl_2$.

Electroporation solutions:

1) SM: 326 g sucrose (952 mM), 0.71 g $MgCl_2 \cdot 6H_2O$ (3.5 mM), distilled water to 1 L.

2) DNA: Dissolve plasmid pGK12 in TE (10mM Tris-HCl, 1 mM EDTA, pH 7.5) to 0.10-1.0 µg/µL. Ligation mixtures

should be ethanol precipitated and washed, then dissolved in TE before electroporation.

Methods:

A 25- mL preculture of *L. gasseri* in exponential growth phase was used to inoculate 100 mL of MRS or MRS supplemented with glycine. Inoculation was to A_{600} = 0.25; cultures were incubated at 30 C until the A_{600} = 0.6. Cells were harvested by centrifugation at minimum speed (1500 g for 5 min) and the supernatant decanted.

Cells were resuspended in 100 mL of SM (or 100 mL of 1 mM $MgCl_2$ according to an alternate procedure), then washed and re-pelleted as above. Pellets were again resuspended in 100 mL of SM or, in an alternate procedure, 30% PEG, then washed and re-pelleted as above.

Cells were resuspended in 1 mL of SM (or 30% PEG according to an alternate procedure). Aliquots containing 10^9-10^{10} cells/mL were transferred to micro tubes for electroporation.

The DNA in a volume 1/20 of the cell suspension volume was added immediately before electroporation. The mixture was transferred to an ice cold electroporation cuvette with a 2-mm electrode gap. Different electric pulses were delivered as shown in Table 1 below.

Immediately following the discharge, 1 mL of MRSSM was added to the cuvette and the resulting diluted cell suspension transferred to a microfuge tube and incubated at 30 C for 2 hrs. Undiluted and serial dilutions of the cell suspension were spread on MRS plates containing appropriate selection ingredients (e.g., antibiotics, color indicators). Plates were incubated at 30 C for up to 3 days, to allow for growth of colonies of transformed cells.

Results of the electroporation of *L. gasseri* are shown in Table 1.

Table 1: Electroporation of pGK12 plasmid into *L. gasseri* strain 100-5

Resistance Settings	# of Chloramphenicol^R colonies
200	15
400	13
600	35
800	>100

a. 5.0 µg/mL chloramphenicol was used for selection
b. 2-mm electrode gap cuvettes were used in electroporations
c. The resistance setting was the only variable parameter; other settings were: voltage, 1.5 kV and capacitance, 25 µF
d. 3 µg of pGK12 plasmid and 100 µL of 100-5 cells were used in all electroporations
e. No colonies were seen on negative control plates, which consisted of 100-5 cells that were not mixed with pGK12 but were subjected to the same electroporation conditions as stated above.

EXAMPLE 4
Use of Rotavirus Antibody to Confer Immunity in Mouse Neonates

Oral administration of the rotavirus monoclonal antibody M159 prevents the development of the symptoms of diarrhea in mouse. A feeding tube was used to administer a gastric bolus to 7 day old mouse pups, followed by an oral rotavirus challenge dose. A gastric bolus containing 50 µg to less than 1 µg of M159 rotavirus antibody gave complete protection against the oral dose of rotavirus (7.5 X 10^6 pfus) that immediately followed (Fig. 5). The unprotected mouse pups exhibited 100% infection at three days. A gastric bolus of 25 µg M159 antibody gave complete protection against an oral rotavirus challenge (7.5 X 10^6 pfus) administered up to 24 hours later, while control mouse pups exhibited 100% infection at three days (Fig. 6).

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

What is claimed is:

1. A composition for supplementing or replacing an immune response against one or more selected pathogens in individuals requiring such treatment, which comprises a probiotic microorganism genetically modified to express recombinant antibodies immunologically specific for at least one selected pathogen.

2. The composition of claim 1, which further comprises a supplemental bolus of antibodies which are immunologically specific for one or more selected pathogens.

3. The composition of claim 2, in which the antibodies of the supplemental bolus are immunologically specific for the same pathogen as the antibodies produced by the probiotic microorganism.

4. The composition of claim 2, in which the antibodies of the supplemental bolus are immunologically specific for a different pathogen from the antibodies produced by the probiotic organism.

5. The composition of claim 1, wherein the pathogen is a gastrointestinal pathogen.

6. The composition of claim 5, wherein the pathogen is selected from the group consisting of rotavirus, caliciviruses, reoviruses, coronaviruses, enteroviruses, adenoviruses, Norwalk-type viruses, enterotoxigenic *Escherichia coli*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Clostridium* spp., *Vibrio cholera*, *Cryptosporidium* spp., *Giardia lamblia*, *Entamoeba histolytica*, *Heliobacter pylori*, and *Isospora belli*.

7. The composition of claim 1, wherein the pathogen is not a gastrointestinal pathogen.

8. The composition of claim 7, wherein the pathogen is selected from the group consisting of coxsackieviruses, poliovirus, hepatitis A virus, Salmonella and Shigella spp., *Listeria monocytogenes* and strongyloides.

9. The composition of claim 1, wherein the probiotic organism is selected from the group consisting of *Lactococcus*, *Bifidobacteria*, *Eubacteria*, non-pathogenic strains of *Escherichia coli*, *E. coli* F18 and *E. coli* strain Nissle 1917.

10. The composition of claim 1, wherein the probiotic organism is from the genus *Lactobacillus*.

11. The composition of claim 10, wherein the species of *Lactobacillus* is selected from the group consisting of *L. casei*, *L. plantarum*, *L. paracasei*, *L. acidophilus*, *L. fermentum*, *L. zeae* and *L. gasseri*.

12. The composition of claim 1, wherein the recombinant antibodies have been adapted for expression in bacteria.

13. The composition of claim 1, wherein the recombinant antibodies are polyclonal.

14. The composition of claim 1 wherein the recombinant antibodies are monoclonal.

15. The composition of claim 1, wherein the recombinant antibodies comprise a mixed population of antibodies immunologically-specific for one pathogen.

16. The composition of claim 1, wherein the recombinant antibodies comprise a mixed population of antibodies immunologically-specific for more than one pathogen.

17. The composition of claim 1, comprising a plurality of probiotic organisms wherein each organism produces at least one recombinant antibody immunologically specific for at least one pathogen.

18. The composition of claim 1, wherein the probiotic organism expresses a rotavirus-neutralizing monoclonal antibody.

19. The composition of claim 1, further comprising at least one compound selected from the group consisting of lactose, maltodextrin and a desiccant.

20. A method for supplementing or replacing an immune response against one or more selected pathogens in individuals requiring such treatment, which comprises administering to the individuals a probiotic bacterium genetically modified to express one or more recombinant antibodies immunologically specific for the selected pathogens, in a manner enabling the microorganism to secrete the recombinant antibodies in the GI tract of the individual, for a time and in an amount effective to impart an immune response against the selected pathogens.

21. The method of claim 20, wherein a supplemental bolus of antibodies which are

immunologically specific for one or more selected pathogens is additionally administered.

22. The method of claim 20, wherein the individual requiring the treatment is selected from the group consisting of newborn infant animals or humans, immunosuppressed or immunodeficient adults, and healthy individuals acutely exposed to a bolus of one or more pathogens.

23. The method of claim 20, wherein the individuals are neonates and the probiotic bacteria are administered orally, in a bolus comprising 3 X 10^9 - 40 X 10^9 colony forming units, two times a day.

24. The method of claims 23, wherein the bolus additionally comprises 0.1-25.0 mg/kg body weight/day of at least one antibody immunologically specific to at least one pathogen.

25. The method of claim 20, wherein the individuals are adults and the probiotic bacteria are administered orally in a bolus comprising 12 X 10^9 - 80 X 10^9 colony forming units, three times a day.

26. The method of claim 25, wherein the bolus additionally comprises 0.1-25.0 mg/kg body weight/day of least one antibody immunologically specific to at least one pathogen.

27. A method for administering the composition of claim 1, wherein the composition is administered orally.

1/1 Sfi1 site
GCG GCC CAG CCG GCC ATG GCC CAG GTG CAA CTG CAG CAG TCT GGG GGA GGC TTA GTG CAG
A A Q P A M A Q V Q L Q Q S G G G L V Q

61/21
CCT GGA GGG TCC CGG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TTT GGA
P G G S R K L S C A A S G F T F S S F G

121/41 V_H Heavy Chain Variable Region
ATG CAC TGG GTT CGT CAG GCT CCA GAG AAG GGG CTG GAG TGG GTC GCA TAC ATT AGT AGT
M H W V R Q A P E K G L E W V A Y I S S

181/61
GGC AGT AGT ACC CTC CAC TAT GCA GAC ACA GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC
G S S T L H Y A D T V K G R F T I S R D

241/81
AAT CCC AAG AAC ACC CTG TTC CTG CAA ATG ACC AGT CTA AGG TCT CAG GAC ACG GCC ATG
N P K N T L F L Q M T S L R S Q D T A M

301/101
TAT TAC TGT GCA AGA TGG GGT AAC TAC CCT CAC TAT GCT ATG GAC TAC TGG GGC CAA GGG
Y Y C A R W G N Y P H Y A M D Y W G Q G

361/121 Linker Region
ACC ACG GTC ACC GTC TCC TCA AGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC
T T V T V S S S G G G S G G G G S G G G

421/141
GGA TCG GAC ATC GTG CTC ACT CAG TCT CCA GCC TCC CTA TCT GCA TCT GTG GGA GAA ACT
G S D I V L T Q S P A S L S A S V G E T

481/161
GTC ACC ATC ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT TAT TTA GCA TGG TAT CAA CAG
V T I T C R A S E N I Y S Y L A W Y Q Q

541/181
AAA CAG GGA AAA TCA CCT CAG TTC CTG GTC TAT AGT GCA AAA ACC TTA GCA GAA GGT GTG
K Q G K S P Q F L V Y S A K T L A E G V

601/201 V_L Light Chain Variable Region
CCA TCA AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG
P S R F S G S G S G T Q F S L K I N S L

661/221
CAG CCT GAA GAT TTT GGG AAT TAT TAC TGT CAA CAT TAT TAT GGT ACT CCG CGC ACG TTC
Q P E D F G N Y Y C Q H Y Y G T P R T F

721/241 Not1 Site E Tag Sequence
GGT GCT GGG ACC AAG CTG GAG CTG AAA CGG GCG GCC GCA GGT GCG CCG GTG CCG TAT CCG
G A G T K L E L K R A A A G A P V P Y P

781/261
GAT CCG CTG GAA CCG CGT GCC GCA TAG
D P L E P R A A *

Figure 1

1/1 Sfi1 site
GCG GCC CAG CCG GCC ***ATG GCC CAG GTG CAA CTG CAG CAG TCT GGG GGA GGC TTA GTG CAG***
A A Q P A M A Q V Q L Q Q S G G G L V Q

61/21
CCT GGA GGG TCC CGG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TTT GGA
P G G S R K L S C A A S G F T F S S F G

121/41 ***V_H Heavy Chain Variable Region***
ATG CAC TGG GTT CGT CAG GCT CCA GAG AAG GGG CTG GAG TGG GTC GCA TAC ATT AGT AGT
M H W V R Q A P E K G L E W V A Y I S S

181/61
GGC AGT AGT ACC CTC CAC TAT GCA GAC ACA GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC
G S S T L H Y A D T V K G R F T I S R D

241/81
AAT CCC AAG AAC ACC CTG TTC CTG CAA ATG ACC AGT CTA AGG TCT CAG GAC ACG GCC ATG
N P K N T L F L Q M T S L R S Q D T A M

301/101
TAT TAC TGT GCA AGA TGG GGT AAC TAC CCT CAC TAT GCT ATG GAC TAC TGG GGC CAA GGC
Y Y C A R W G N Y P H Y A M D Y W G Q G

361/121 Linker Region
ACC AGG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC
T R V T V S S G G G G S G G G G S G G G

421/141
GGA TCG GAC ATC GAG CTC ACT CAG TCT CCA ACC ACC ***CTA TCT GCA TCT GTG GGA GAA ACT***
G S D I E L T Q S P T T L S A S V G E T

481/161
GTC ACC ATC ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT TAT TTA GCA TGG TAT CAA CAG
V T I T C R A S E N I Y S Y L A W Y Q Q

541/181
AAA CAG GGA AAA TCT CCT CAG TTC CTG GTC TAT AGT GCA AAA ACC TTA GCA GAA GGT GTG
K Q G K S P Q F L V Y S A K T L A E G V

601/201 ***V_L Light Chain Variable Region***
CCA TCA AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG
P S R F S G S G S G T Q F S L K I N S L

661/221
CAG CCT GAA GAT TTT GGG AAT TAT TAC TGT CAA CAT TAT TAT GGT AGT CCG CGC ACG TTC
Q P E D F G N Y Y C Q H Y Y G S P R T F

721/241 Not1 site ***E Tag Sequence***
GGT GCT GGG ACA AAG TTG GAA ATA AAA CGG GCG GCC GCA ***GGT GCG CCG GTG CCG TAT CCG***
G A G T K L E I K R A A A G A P V P Y P

781/261
GAT CCG CTG GAA CCG CGT GCC GCA TAG
D P L E P R A A *

Figure 2

1 2 3 4

Figure 3

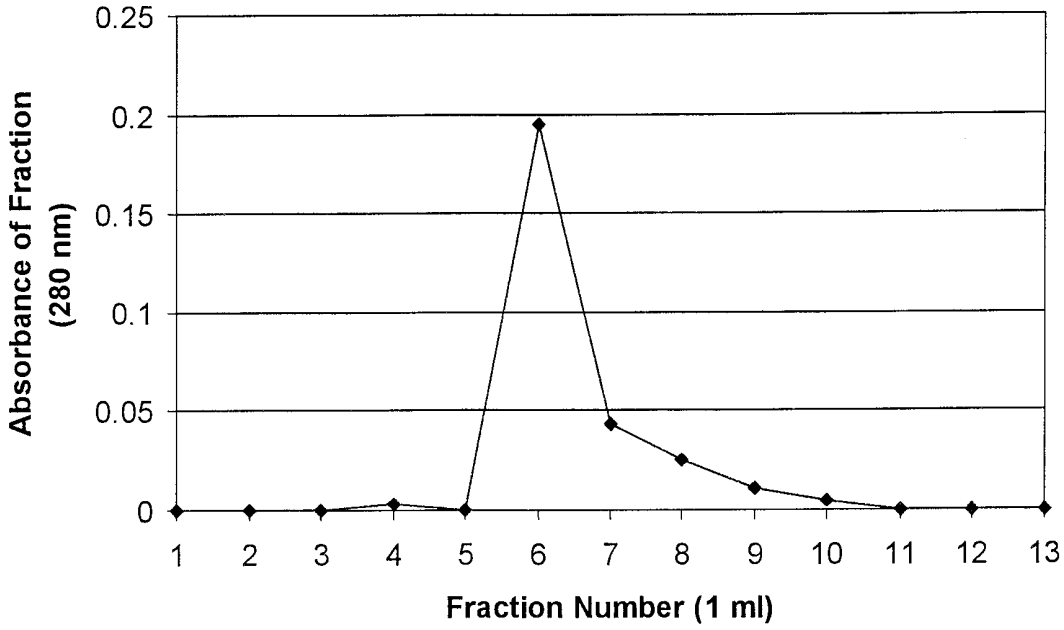
0.25
0.2
0.15
0.1
0.05
0
Absorbance of Fraction (280 nm)
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction Number (1 ml)

Figure 4

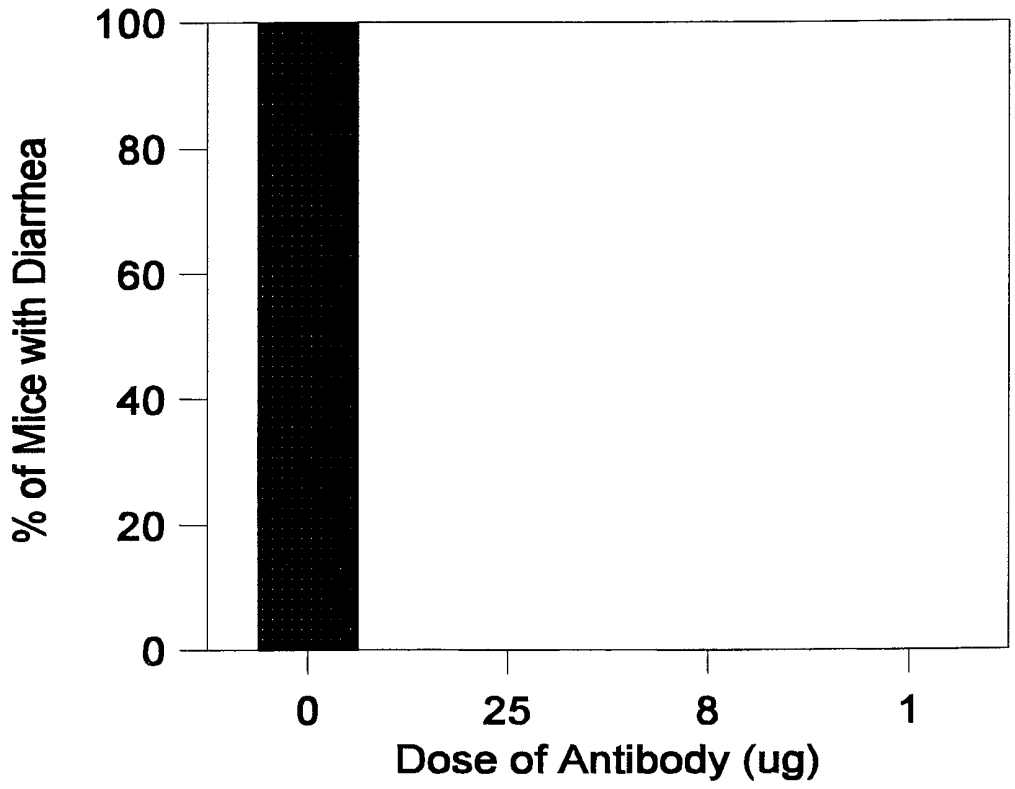
100
80
60
40
20
0
% of Mice with Diarrhea
0
25
8
1
Dose of Antibody (ug)

Figure 5

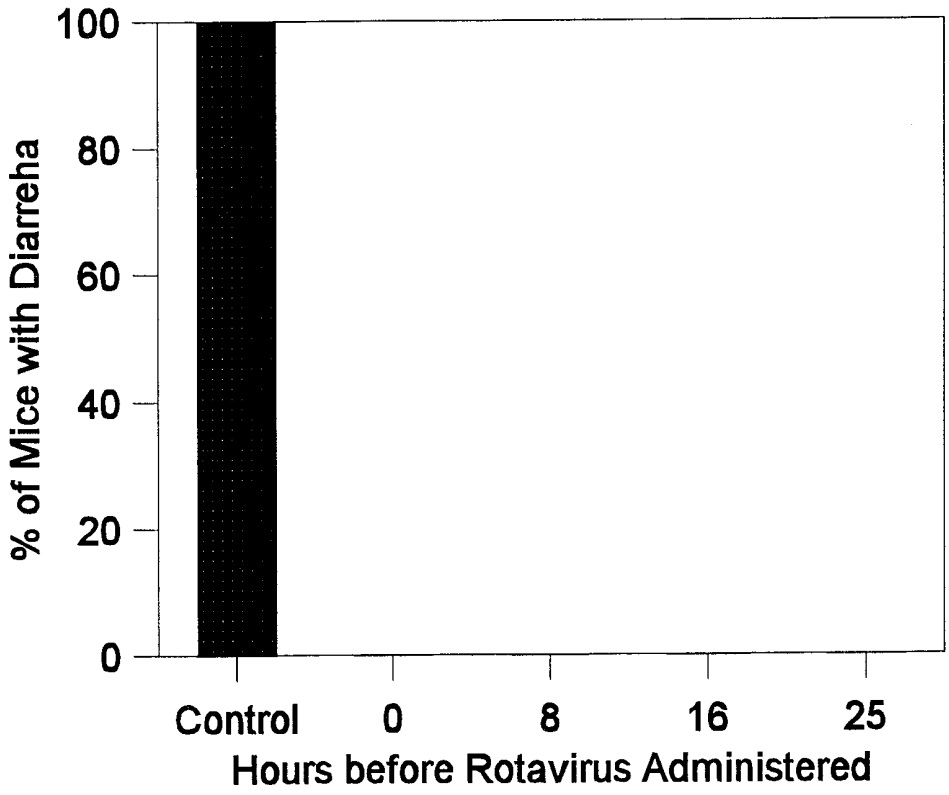
100
80
60
40
20
0
% of Mice with Diarreha
Control
0
8
16
25
Hours before Rotavirus Administered

Figure 6

SEQUENCE LISTING

<110> Wisconsin Alumni Research Foundation
Fahl, William E.
Letchworth, Geoffrey J.
Mueller, Gerald C.
Savage, Adam K.
Loo, Deborah

<120> Gastrointestinal Bacterial Antibody Factories

<130> WARFP98073WO; F086PC

<150> 60/094,697
<151> 1998-07-30

<160> 2

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 807
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed nucleotide encoding mouse anitbody heavy chain variable region -- linker region-- mouse antibody light chain variable region -- E Tag. Antibody from mouse hybidoma line #159.

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<222> (15)...(348)
<223> Heavy chain variable region

<221> misc_feature
<222> (349)...(453)
<223> Linker, Pharmacia Biotech "Recombinant Phage Antibody System"

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<222> (454)...(750)
<223> Light chain variable region

<221> misc_feature
<222> (760)...(78)
<223> E tag, Pharmacia Biotech "Recombinant Phage Antibody System"

<400> 1

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ggatcggaca tcgtgctcac tcagtctcca gcctccctat ctgcatctgt gggagaaact 480
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aaacagggaa aatcacctca gttcctggtc tatagtgcaa aaaccttagc agaaggtgtg 600

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ccatcaaggt tcagtggcag tggatcaggc acacagtttt ctctgaagat caacagcctg     660
cagcctgaag attttgggaa ttattactgt caacattatt atggtactcc gcgcacgttc     720
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gatccgctgg aaccgcgtgc cgcatag                                        807
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<210> 2
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<212> DNA
<213> Artificial Sequence

<220>
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<221> misc_feature
<222> (15)...(348)
<223> heavy chain variable region

<221> misc_feature
<222> (349)...(456)
<223> linker, Pharmacia Biotech "Recombinant Phage Antibody System"

<221> misc_feature
<222> (457)...(750)
<223> light chain variable region

<221> misc_feature
<222> (760)...(799)
<223> E tag, Pharmacia Biotech "Recombinant Phage Antibody System"

<400> 2

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cctggagggt cccggaaact ctcctgtgca gcctctggat tcactttcag tagctttgga     120
atgcactggg ttcgtcaggc tccagagaag gggctggagt gggtcgcata cattagtagt     180
ggcagtagta ccctccacta tgcagacaca gtgaagggcc gattcaccat ctccagagac     240
aatcccaaga acaccctgtt cctgcaaatg accagtctaa ggtctcagga cacggccatg     300
tattactgtg caagatgggg taactaccct cactatgcta tggactactg gggccaaggc     360
accagggtca ccgtctcctc aggtggaggc ggttcaggcg gaggtggctc tggcggtggc     420
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ccatcaaggt tcagtggcag tggatcaggc acacagtttt ctctgaagat caacagcctg     660
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gatccgctgg aaccgcgtgc cgcatag                                        807
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17296

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 21/06; C12N 15/00
US CL : 435/69.1, 172.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 5,733,540 A (LEE) 31 March 1998, see columns 1-2, 6, col. 7, lines 23-30, col. 9-10, col. 11, lines 11-36, col. 16, lines 8-14, claims 1-28, see entire document.	1,5-14,17, 18,20, 22, 27 ------- 19,23,25
Y	US 5,531,988 A (PAUL) 02 July 1996, see col. 2, col. 3, lines 42-47, col. 5, lines 1-19, col. 7, lines 13-17, see entire document.	1-2,15-16, 19, 21-26
A	US 5,637,677 A (GREEN et al) 10 June 1997, see abstract, claims and entire document.	1-27
A	US 4,956,452 A (SNYDER et al) 11 September 1990, see abstract, claims and entire document.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search
02 NOVEMBER 1999

Date of mailing of the international search report
06 DEC 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
GINNY PORTNER
Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17296

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,950,595 A (MASUHO et al) 21 August 1990, see abstract, claims and entire document.	1-27
A	US 4,571,385 A (GREENBERG et al) 18 February 1986, see abstract, claims and entire document.	1-27
A	US 5,895,758 A (MAJNARICH et al), 20 April 1999, see abstract, claims and entire document.	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17296

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST; DIALOG; STN
search terms: chimeric, recombinant, monoclonal, polyclonal, probiotic, pro-biotic, biotherapeutic, immunoglob?, antibod?, host cell, lactobacillus, rotavirus