Protective effect of bifidus milk on the experimental infection with *Salmonella enteritidis* subsp. *typhimurium* in conventional and gnotobiotic mice


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A.M. SILVA, E.A. BAMBIRRA, A.L. OLIVEIRA, P.P. SOUZA, D.A. GOMES, E.C. VIEIRA AND J.R. NICOLI. 1999. The ability of *Bifidobacterium bifidum* from a commercial bifidus milk to antagonize *Salmonella enteritidis* subsp. *typhimurium in vivo*, and to reduce the pathological consequences for the host, was determined using conventional and gnotobiotic mice. Conventional animals received daily, by gavage, 0.1 ml bifidus milk containing about 10^9 cfu *B. bifidum* and germ-free animals received a single 0.1 ml dose. The conventional and gnotobiotic groups were challenged orally with 10^2 cfu of the pathogenic bacteria 5 and/or 10 d after the beginning of treatment. Control groups were treated with milk. Bifidus milk protected both animal models against the challenge with the pathogenic bacteria, as demonstrated by survival and histopathological data. However, to obtain the protective effect in gnotobiotic animals, the treatment had to be initiated 10 d before the challenge. In experimental and control gnotobiotic mice, *Salm. enteritidis* subsp. *typhimurium* became similarly established at levels ranging from 10^8 to 10^9 viable cells g^-1 of faeces and remained at these high levels until the animals died or were sacrificed. It was concluded that the protection against *Salm. enteritidis* subsp. *typhimurium* observed in conventional and gnotobiotic mice treated with bifidus milk was not due to the reduction of the intestinal populations of the pathogenic bacteria.

INTRODUCTION

Gastrointestinal disease is often the consequence of a myriad factors which disturb the complex ecosystem of the gastrointestinal tract. Antibiotics are the agents most commonly responsible for acute diarrhoea due to the loss of the protective role of the normal intestinal microbiota against pathogenic organisms (Van der Waaij et al. 1982). Other aetiologies of diarrhoea are due to infection not associated with antibiotic use (e.g. toxigenic *Escherichia coli*, *Salmonella enteritidis*, *Entamoeba histolytica*, *Giardia duodenalis*, or viruses). In an effort to prevent or treat these cases, innovative approaches have been tried using live, biotherapeutic agents such as yeast (*Saccharomyces*) and bacterial species (*Lactobacillus, Bifidobacterium*) or faecal enemas (Fuller 1992).

Lactobacilli have the longest history as biotherapeutic agents (probiotics) and are still the most common ingredients among those intended for consumption by farm animals. This choice of probiotic bacteria seems appropriate because the normal gastrointestinal microbiota of these animals is particularly rich in lactobacilli (Tannock 1997). *Bifidobacterium* spp. are now almost as common as lactobacilli in yoghurts and bifidus milk, presumably as a result of the realization that the human intestinal tract harbours larger and more stable...
populations of bifidobacteria than lactobacilli (Kimura et al. 1998). Bifidobacteria are also particularly attractive as potential probiotic agents for humans since they constitute the predominant colonic microbiota of breast-fed infants (Benno et al. 1984) and are thought to be one of the mechanisms by which breast feeding is protective against diarrhoea (Saavedra 1995).

The potential mechanisms by which probiotic agents might exert their protective or therapeutic effect against infectious diarrhoea include competition for nutrients or adhesion receptors (Bernet et al. 1994), production of inhibitory metabolites or antimicrobial agents against pathogens (Klaenhammer 1988), modulation of toxin production or action (Corthier et al. 1985; Brandão et al. 1998), and immunomodulation (Hatcher and Lambrecht 1993). The latter three mechanisms were suggested for bifidobacteria but are based only on in vitro experiments. The likely contribution of each of these mechanisms is difficult to determine in the wider presence of a complex gastrointestinal ecosystem. The use of a gnotobiotic animal model with a simplified intestinal microbial status allows the in vivo observation of interactions between micro-organisms such as a probiotic and its possible pathogenic target bacteria.

As B. bifidum has been shown to be capable of inhibiting multiplication of enteropathogenic bacteria under certain conditions in vitro, it was decided to determine whether this lactic bacterium was capable of antagonizing Salmonella enteritidis subsp. typhimurium in vivo, and of reducing the pathological consequences for the host, using conventional and gnotobiotic mice.

MATERIALS AND METHODS

Mice

Germ-free NIH (Taconic, Germantown, NY, USA) 21-day-old mice were used in this study. The animals were housed in flexible plastic isolators (Standard Safety Company, Pallatine, IL, USA) and handled according to established procedures (Pleasants 1974). The animals were fed an autoclavable commercial diet for rodents (Nuvital, Curitiba, Brazil) ad libitum.

Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvastaal B.V., Zevenaar, The Netherlands). The conventional animals were originally derived from the germ-free colony and kept in a conventional animal room for many generations before use.

Micro-organisms

Bifidobacterium bifidum was used in a lyophilized commercial form (DVS – Christian Hansen Laboratory, Horsholm, Denmark) to prepare the bifidus milk for the treatment of conventional mice. An isolate of B. bifidum from the lyophilized commercial preparation was used to inoculate the bifidus milk for the treatment of gnotobiotic animals. The Salmonella strain of human origin was obtained in pure culture form from Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil) and the identification was confirmed by Institut Pasteur (Paris, France) as Salmonella enteritidis subsp. typhi-murium. The isolated bacteria were maintained at −70 °C in medium containing 20% glycerol.

Experimental infections

Salmonella enteritidis subsp. typhimurium was grown in liquid brain heart infusion (BHI) medium (Difco) at 37 °C. Mice were inoculated by the oro-gastric route with 0.1 ml of the bacterial suspension containing about 10^2 viable cells.

Treatments

Bifidus milk was prepared from 9.5% reconstituted skim milk as recommended by the manufacturer. A single dose of 0.1 ml bifidus milk containing about 10^6 colony forming units (cfu) of B. bifidum was administered to gnotobiotic mice by gavage, 10 and 5 d before the challenge with the pathogenic bacteria, or simultaneously with the challenge. The same dose was administered daily to the conventional animals, 5 d before the challenge or simultaneously with the challenge, and then throughout the remaining experimental period. The control conventional and gnotobiotic groups were treated with 9.5% reconstituted skim milk according to the same schedule as the corresponding experimental groups.

Microbial counts in gnotobiotic groups

Freshly collected faeces were introduced into an anaerobic chamber containing an atmosphere of 85% N2, 10% H2 and 5% CO2 (Forma Scientific Company, Marietta, OH, USA), diluted 100-fold in regenerated sterile buffered saline and homogenized by hand. Serial 10-fold dilutions were obtained and 0.1 ml amounts were plated onto MacConkey agar and de Mann, Rogosa and Sharp (MRS) agar (Merck) for Salmonella and Bifidobacterium counts, respectively. The Petri dishes were cultured at 37 °C outside (24 h) and inside (72 h) the anaerobic chamber, respectively, after which colonies were counted.

Experimental design

Each experimental or control group consisted of 10 conventional or six germ-free mice. The population levels of the pathogenic and lactic bacteria in the faeces, and cumulative mortality, were recorded during the experiments. At the end of the experiments, all remaining mice were sacrificed by ether inhalation.

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Histopathological examination

Tissues samples from mice killed by the infection or sacrificed at the end of the experiments were fixed in 4% formaldehyde and processed for paraffin embedding. The histopathological sections (3–5 μm) were stained with haematoxylin-eosin. The slides were coded and examined by a single pathologist, who was unaware of the experimental conditions for each group.

Statistical analysis

Data for faecal population levels of both bacteria and for cumulative mortality of mice were evaluated by analysis of variance and Fisher’s exact test, respectively. Statistical analysis was performed with the EPISTAT software (TL Gustafson, Round Rock, TX, USA) with the level of significance set at $P < 0.05$.

RESULTS

Figure 1 shows that survival on day 28 after the oral challenge with *Salmonella enteritidis* subsp. *typhimurium* was significantly higher ($P < 0.05$) in both experimental groups (55 and 80% survival in conventional mice with treatment beginning 5 d before, or simultaneously with, the challenge, respectively) when compared with the control group (10% survival). There was no significant difference between the two experimental groups ($P > 0.05$).

Histopathological examination of organs from conventional experimental and control groups confirmed the survival data. The experimental animals had milder intestinal, liver and spleen lesions than the controls. The intestinal lesions were markedly expressed as dilatation of the intestinal loop with a loss of villus relief. The intestinal villus pattern was relatively well preserved in the experimental animals when compared with control mice (Fig. 2a,b). In gnotobiotic mice (Fig. 2c,d), milder lesions in the experimental animals were also observed but only for mice treated 10 d before the challenge compared with the other three groups (treated 5 d before, simultaneously with the challenge, or not treated at all). However, the lesions were generally less severe in the conventional mice compared with the corresponding gnotobiotic animals.

Figure 3 shows that *B. bifidum* became established in the digestive tract of both experimental gnotobiotic groups (germ-free mice treated 5 d before, or simultaneously with, the challenge) and the number of cfu was about $10^{10}$ g$^{-1}$ faeces. After the challenge with the pathogen, the *B. bifidum* faecal population decreased to levels ranging from $10^8$ to $10^9$ cfu g$^{-1}$ faeces.

The kinetics leading to the establishment of *Salmonella enteritidis* subsp. *typhimurium* in the experimental and control groups are shown in Fig. 4. In both experimental gnotobiotic groups harbouring *B. bifidum* (administered 5 d before, or simultaneously with, the challenge), the pathogenic bacteria became established at levels fluctuating between $10^9$ and $10^{10}$ cfu g$^{-1}$ and remained at these high levels until the animals died or were sacrificed. These levels were equivalent to those observed in gnotobiotic mice harbouring the pathogenic bacteria alone. There was no statistical difference in faecal population levels of *Salmonella enteritidis* subsp. *typhimurium* between control and experimental groups ($P > 0.05$). Similar results were obtained with mice treated 10 d before the challenge.

DISCUSSION

In a double-blind, placebo-controlled trial, Saavedra *et al.* (1994) demonstrated that supplementation of infant formula with *B. bifidum* and *Streptococcus thermophilus* reduced the incidence of acute diarrhoea and rotavirus shedding in infants admitted to hospital. In adults, bifidobacteria reduced the side-effects of antibiotics (Colombel *et al.* 1987). The therapeutic use of an antibiotic-resistant *B. longum* by men exposed to high-dose γ-irradiation also apparently protected against opportunistic enteric pathogens (Korschunov *et al.* 1996). Antagonism through toxic metabolites (lactic acid, H$_2$O$_2$) or substances (bacteriocin, antibiotic-like) is one of the most common hypotheses used to explain the protective effect of probiotic against microbial infection. Growth inhibition of several enteropathogenic bacteria by lactic acid bacteria has been shown but generally only in *in vitro* experiments (Klaenhammer 1988). The *Bifidobacterium* strain used in the present study also produced a diffusible antagonistic substance against *Salmonella enteritidis* subsp. *typhimurium in vitro* (data not shown). Like other probiotics, *B. bifidum* was drastically eliminated from the digestive tract of mammals harbouring a
complex intestinal microbiota and for this reason, daily ingestion of these bacteria is necessary to maintain high artificial levels in the gastrointestinal tract of conventional mice. On the other hand, its implantation was possible in germ-free animals using a single dose. For these reasons, the gnotobiotic mouse provides an *in vivo* simplified system which allows the observation of ecological interactions such as antagonism in the gastrointestinal tract between few microbial strains inoculated in this ecosystem.

*Salmonella* spp. are usually described as facultative intracellular parasites that grow primarily inside the macrophages of liver and spleen. Carter and Collins (1974) demonstrated experimentally in mice that the terminal ileum is the primary site of *Salmonella* invasion. Recent work has shown that within 30 min of infection, invasive *Salm. typhimurium* exclusively entered M cells found within the follicle-associated epithelium (FAE) of Peyer’s patches. This indicates that *Salmonella*, like many other microbes, may exploit the host...
M cell antigen uptake system as a route of entry. At 60 min, internalized bacteria were cytotoxic for the M cells and the dead cell formed a gap in the FAE which allowed organisms to invade adjacent enterocytes (Jones et al. 1994).

As described previously by Nardi et al. (1990), the Salm. enteritidis subsp. typhimurium strain used in these experiments induced enteroinvasive disease that was more severe in gnotobiotic than in conventional mice. The results obtained in the present study confirm these data and the established protective effect of the normal microbiota against enteropathogenic bacteria (Berg 1996). Bifidobacterium bifidum was not capable of protecting the mice against Salmonella completely when the normal microbiota was absent, and this protection was obtained more rapidly (5 d in conventional mice instead of 10 d in gnotobiotic animals) when there was a synergism between the microbiota and the biotherapeutic agent (Fig. 2). Nevertheless, as generally expected for a probiotic, B. bifidum may contribute to a complementary protection in hosts with a perturbed or impaired digestive microbiota. On the other hand, the protection against Salm. enteritidis subsp. typhimurium shown by the survival (Fig. 1) and histopathological data (Fig. 2) obtained for mice previously associated or treated with B. bifidum is not due to an antagonism against the pathogenic bacteria in the gastrointestinal ecosystem, as shown in Fig. 4. Other factors are required to explain the protective effect of bifidus milk against Salm. enteritidis subsp. typhimurium, such as host immunomodulation (Hatcher and Lambrecht 1993). Wagner et al. (1997) showed that of four probiotic bacterial species, B. animalis provided the best overall protection against mucosal and systemic candidiasis in immunodeficient mice. The probiotic apparently stimulated host resistance to candidiasis via thymus and mucosal tissue-associated lymphoid tissues. An increased humoral immune response after ingestion of bifidobacteria (Yasui et al. 1989), and increased macrophage phagocytic activity in rats supplemented with B. longum and Lactobacillus acidophilus, has been also documented (Hatcher and Lambrecht 1993). The most probable mechanism to explain this phenomenon is the passage of Bifidobacterium soluble substances or whole cells into the Peyer’s patches present at intervals along the intestinal wall. The patches are separated from the intestinal lumen by M cells that appear to have an antigen-sampling role (Neutra et al. 1996). Alternatively, the bifidobacteria may be able to stimulate the immune system via the enterocytes which are now recognized as immunocompetent cells and which produce cytokines involved in the regulation of the immune system. When exposed to certain bacterial cells or their products, enterocytes have been shown to increase the expression of genes encoding interleukin 8, tumour necrosis factor x, monocyte chemotactic protein 1 and granulocyte-macrophage colony-stimulating factor (Eckmann et al. 1995). These substances have well established roles in the attraction and activation of polymorphonuclear leucocytes and macrophages. On the other hand, Corthier et al. (1985) demonstrated a higher survival and a lower faecal cytotoxin titre in gnotobiotic mice associated with B. bifidum isolated from the neonatal faecal microbiota and later challenged with Clostridium difficile. The modulation of cytotoxin production occurred without any strong bacterial antagonism against Cl. difficile. The mechanisms of this modulation have not been elucidated. Experiments based on these hypotheses are being carried out in our laboratory.

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