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PROTECTION BY *LACTOBACILLUS ACIDOPHILUS* UFV-H2B20 AGAINST EXPERIMENTAL ORAL INFECTION WITH *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SER. TYPHIMURIUM IN GNOTOBIOTIC AND CONVENTIONAL MICE

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ABSTRACT

The ability of *Lactobacillus acidophilus* UFV-H2B20 to antagonize *Salmonella enterica* subsp. *enterica* ser. Typhimurium and to reduce the pathological consequences for the host was determining using conventional and gnotobiotic animals. Conventional NIH mice received daily by gavage a 0.1 ml suspension containing about 10^8 cfu *L. acidophilus* UFV-H2B20 and germfree animals received a single 0.1 ml dose. The gnotobiotic and conventional groups were infected orally with 10^2 and 10^5 cfu of *S.* Typhimurium, respectively, 7 days after the beginning of treatment. Control groups were treated with sterile saline instead of *Lactobacillus*. Survival data showed a protective effect against the pathogenic bacteria in both conventional and gnotobiotic *Lactobacillus*-treated mice. *L. acidophilus* UFV-H2B20 colonized the digestive tract of gnotobiotic mice and the number of viable cells ranged from 10^9 to 10^{10} cfu/g of faeces. In both experimental and control gnotobiotic animals, *S.* Typhimurium became rapidly established at a level ranging from 10^8 to 10^{10} cfu/g of faeces and remained at high levels until the animals died or were sacrificed. In conclusion, the previous treatment of mice with *L. acidophilus* UFV-H2B20 protects the animals against the experimental infection with *S.* Typhimurium but this protection was not due to the reduction of the pathogenic populations in the intestines.

Key words: *Lactobacillus*, *Salmonella*, probiotic, gnotobiotic mice

INTRODUCTION

Challenges in the control and treatment of infectious diseases include the development of antibiotic resistance, increased frequency of opportunistic infections in immunocompromised patients, and emergence of new types of pathogens. These trends result in an increase in antibiotic exposure in the population, which in turn selects for emergence of resistant pathogen strains. The World Health Organization (WHO) held a conference (12) to discuss the increase in resistance to antibiotics, which today is "a major public health problem in both developed and developing countries throughout the world". With this in mind, the WHO recommends global programmes to reduce the use of antibiotics and increased efforts to prevent disease through the development of newer, more effective and safer therapies. In addition, several older form of therapies, such as bacterial interference, may be worth reconsidering. Microorganisms that have been used to achieve this goal have been called probiotics and defined as: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (4). Species of lactic acid bacteria (*Lactobacillus*, *Bifidobacterium*, *Streptococcus*) and yeast (*Saccharomyces*) constitute a significant proportion of probiotic cultures used in the world.

The potential mechanisms by which probiotic agents might exert their protective or therapeutic effect against infections include competition for nutrients or adhesion receptors (1), production of inhibitory metabolites or antimicrobial compounds against pathogens (6), immunomodulation (5), and modulation of toxin production or action (2,3). The first three mechanisms were suggested for lactobacilli. The likely contribution of each of these mechanisms is difficult to determine in the presence of a broad and complex gastrointestinal ecosystem. The use of a gnotobiotic

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animal model with a simplified intestinal microbial status allows the *in vivo* observation of interactions between microorganisms such as a probiotic and a pathogenic bacterium. In a recent report, Neumann *et al.* (7) showed that *Lactobacillus acidophilus* UFV-H2B20, a lactic acid bacterium isolated from a human newborn, stimulates a nonspecific immune response in mice.

As *L. acidophilus* UFV-H2B20 has been shown to be capable of stimulating the immune defense mechanisms, the objective of the present study was to determine whether this lactic acid bacterium was also capable of antagonizing *Salmonella enterica* subsp. *enterica* ser. Typhimurium *in vivo*, and consequently of reducing the pathological consequences for the host, using conventional and gnotobiotic mice.

MATERIALS AND METHODS

Animals

Germfree 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 (8). 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., Zevenar, The Netherlands). The conventional animals were originally derived from the germfree colony and kept in a conventional animal room for at least two generations before use.

Bacteria

Lactobacillus acidophilus UFV-H2B20, a strain of human origin, was isolated at the Federal University of Viçosa (UFV, Viçosa, Brazil) and maintained at -70ºC in non-fat reconstituted dry milk containing 20% glycerol.

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 enterica* subsp. *enterica* ser. Typhimurium. The isolated bacterium was maintained at –70ºC in medium containing 20% glycerol.

Probiotic treatment

The *Lactobacillus* was grown in de Mann, Rogosa and Sharp (MRS) broth (Merck) for 18 h at 37ºC. This activated culture was centrifuged at 2000 *g* at 4ºC and resuspended in phosphate-buffered saline in order to obtain 10^9 colony forming units (cfu)/ml. A single dose of 0.1 ml of this suspension was administered to gnotobiotic mice by gavage, 7 days before the challenge with the pathogenic bacteria. The same dose was administered daily to the conventional animals 7 days before the challenge, and then throughout the remaining experimental period. The control conventional and gnotobiotic groups were treated with 0.9% saline according to the same schedule as the corresponding experimental groups.

Bacterial challenge

Salmonella Typhimurium was grown in liquid brain heart infusion (BHI) medium (Difco) for 18 h at 37ºC. Mice were challenged by the oro-gastric route with 0.1 ml of the bacterial suspension containing about 10^2 and 10^5 cfu for the gnotobiotic and conventional animals, respectively.

Experimental design

For the experiments using conventional animals, each experimental or control group consisted of 25 mice. For the experiments with gnotobiotic animals, 12 germfree mice were used for each of these groups. The population levels of the pathogenic and lactic bacteria in the faeces were determined only in gnotobiotic mice. Weight gain was measured daily only in conventional mice. Cumulative mortality was recorded during the experiments with both gnotobiotic and conventional animals. At the end of the experiments, all remaining mice were sacrificed by ether inhalation.

Bacterial counts in gnotobiotic mice

Freshly collected faeces were diluted 100-fold in sterile buffered saline and homogenized by hand. Serial 10-fold dilutions were obtained and 0.1 ml amounts were plated onto MacConkey agar and MRS agar (Merck) for *Salmonella* and *Lactobacillus* counts, respectively. The Petri dishes were incubated at 37ºC for 24 h after which colonies were counted.

Statistical analysis

Data for faecal population levels, cumulative mortality and mean survival time of mice were evaluated by analysis of variance, Fisher's exact test and Student's t test. 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

Fig. 1 shows the ponderal curves for the experimental and control conventional groups challenged with *S.* Typhimurium and for the control group treated with *Lactobacillus* only. A weight loss was observed for both experimental and control groups beginning 5 days after the pathogenic challenge and with a maximum by day 10. The apparent increase in weight gain observed later in the control group was due to a single mouse which resisted until day 20 and then died. In the experimental group, after a critical phase a weight gain was noted for the surviving animals. There was no weight loss in the group only treated with the probiotic.

Fig. 2 shows that survival on day 22 after the oral challenge with *S.* Typhimurium was significantly higher ($P < 0.05$) in the experimental conventional group (34.6% survival) when compared with the control group (0% survival). All the mice in the control group died until day 19 after the oral infection. There was no mortality in the group only treated with the probiotic.

Fig. 3 shows that all the animals in the experimental and control gnotobiotic groups died after the oral challenge with *S.* Typhimurium. However, the mean survival time for the experimental group (14.00 ± 2.25 days) was significantly higher ($P < 0.05$) than for the control group (12.00 ± 0.30 days).

Fig. 4 shows that *L. acidophilus* UFV-H2B20 became established in the digestive tract of the experimental gnotobiotic group and the number of cfu was about 10^{10}/g of faeces. After the challenge with the bacterial pathogen, the faecal *Lactobacillus* population decreased to levels of 10^9 cfu/g of faeces. The kinetics leading to the establishment of *S.* Typhimurium in the experimental and control groups are also shown in Fig. 4. In the experimental group, harbouring *L. acidophilus* UFV-H2B20, the pathogenic bacteria became established at levels ranging from 10^8 to 10^{10} cfu/g 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.

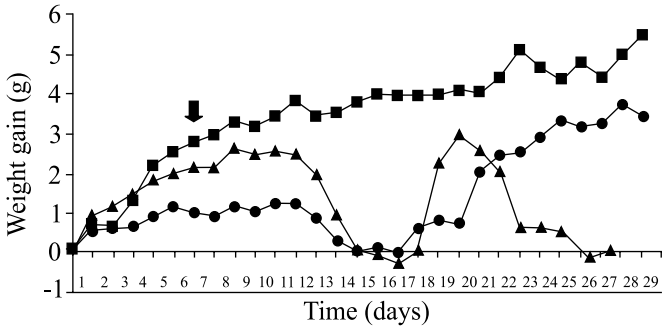

Figure 1. Weight gain of conventional NIH mice treated (●) or not (▲) with *Lactobacillus acidophilus* UFV-H2b20 and orally infected with *Salmonella Typhimurium*. The control group was only treated with *Lactobacillus acidophilus* (■). The arrow shows the oral infection.

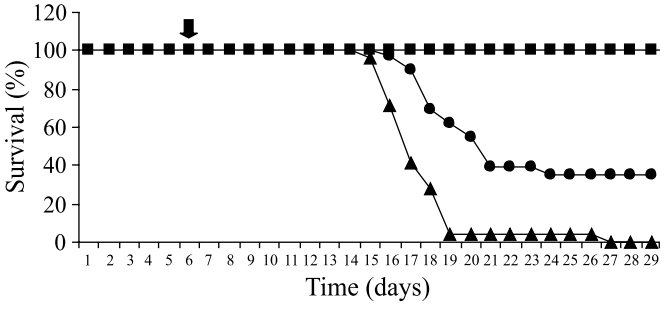

Figure 2. Survival of conventional NIH mice treated (●) or not (▲) with *Lactobacillus acidophilus* UFV-H2b20 and orally infected with *Salmonella Typhimurium*. The control group was only treated with *Lactobacillus acidophilus* (■). The arrow shows the oral infection.

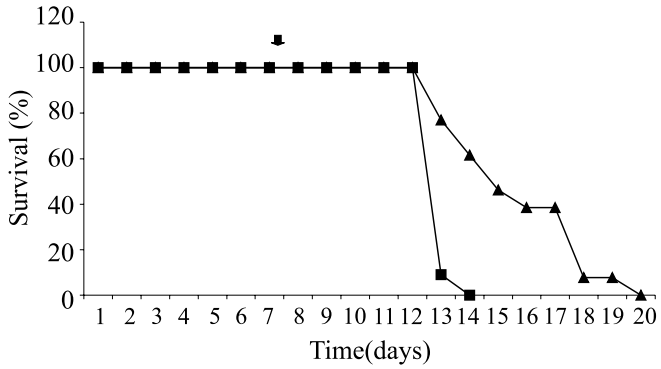

Figure 3. Survival of gnotobiotic NIH mice treated (▲) or not (■) with *Lactobacillus acidophilus* UFV-H2b20 and orally infected with *Salmonella Typhimurium*. The arrow shows the oral infection.

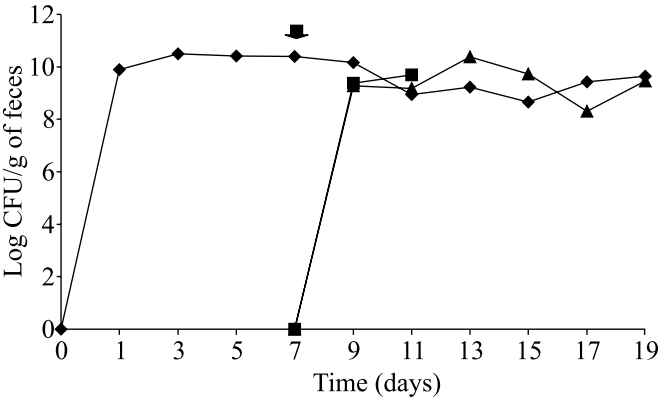

Figure 4. Fecal population levels of *Salmonella Typhimurium* in gnotobiotic NIH mice treated (▲) or not (■) with *Lactobacillus acidophilus* UFV-H2b20 and orally infected with the patogenic bacteria. Faecal population level of *Lactobacillus acidophilus* (◆). The arrow shows the oral infection

DISCUSSION

Lactobacilli have the longest history of use as probiotics and are still the most common ingredients of preparations intended for consumption by human beings and farm animals (10). While many *Lactobacillus* strains have been promoted as good probiotics for human or animal use, substantial supporting *in vitro* and *in vivo* data are available for only a few. Biotherapeutic properties and mechanisms of action are too often unknown, and arguably too many human studies are undertaken or contemplated with strains which have not been thoroughly characterized in these aspects. *Lactobacillus acidophilus* UFV-H2B20 was isolated at the Federal University Federal of Viçosa, Minas Gerais, Brazil, from the faeces of a newborn child, with the objective of isolating a new probiotic for human use. The

present study was designed to investigate some properties and mechanisms of action of this possible biotherapeutic agent.

Like other probiotics, such as *Saccharomyces boulardii* and *Bifidobacterium longum* (9,11), *L. acidophilus* UFV-H2B20 is drastically eliminated from the digestive tract of mammals harbouring a complex intestinal microbiota and for this reason daily ingestion of these microorganisms is necessary to maintain high artificial levels in the gastrointestinal tract of conventional mice. On the other hand, its implantation was possible in germfree animals using a single dose. Gnotobiotic mice were used in this study to obtain a simplified *in vivo* ecosystem allowing the observation of ecological interactions between the probiotic and a bacterial pathogen.

As expected and observed with other probiotics (9,11), the protection offered by *L. acidophilus* UFV-H2B20 against the pathogenic challenge was higher in conventional than in gnotobiotic mice due to the complementary protective mechanisms from the normal intestinal microbiota and from the biotherapeutic agent present in the first animal model.

The protection observed above, was not due to the reduction of the *Salmonella* populations in the feces as shown in Fig. 4. However, inhibition of this pathogenic bacteria could occur in higher regions of the digestive tract than colon such as in the small intestine. Similar results were observed with *S. boulardii* and *B. longum* (9,11). Various other properties could explain the protective effect of *Lactobacillus* against enteropathogenic bacteria such as the competition for adhesion sites in the presence of the probiotic and/or the previously demonstrated stimulation of a host nonspecific immune response (7). Experiments based on these hypotheses are being carried out in our laboratory.

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RESUMO

Proteção por *Lactobacillus acidophilus* UFV-H2B20 contra o desafio oral experimental com *Salmonella enterica* subsp. *enterica* ser. Typhimurium em camundongos gnotobióticos e convencionais

A capacidade de *Lactobacillus acidophilus* UFV-H2B20 de antagonizar *Salmonella enterica* subsp. *enterica* ser. Typhimurium, e de reduzir as conseqüências patológicas para o hospedeiro foram determinadas em animais convencionais e gnotobióticos. Camundongos NIH convencionais receberam diariamente, por via oral, 0,1 ml de uma suspensão contendo em torno de 10^8 ufc de *L. acidophilus* UFV-H2B20 e os animais sem germes receberam uma única dose de 0,1 ml. Os grupos gnotobióticos e convencionais foram desafiados oralmente com, respectivamente, 10^2 e 10^5 ufc de *S.* Typhimurium 7 dias após o início do tratamento. Os grupos controles foram tratados com salina estéril em vez do *Lactobacillus*. Dados de sobrevida mostraram um efeito protetor contra a bactéria patogênica em ambos os grupos convencional e gnotobiótico tratados com o *Lactobacillus. L. acidophilus* UFV-H2B20 colonizou o trato digestivo dos camundongos gnotobióticos e o número de células víaveis flutuou entre 10^9 e 10^{10} ufc/g de fezes. Em ambos os grupos experimental e controle, *S.* Typhimurium se estabeleceu rapidamente numa faixa de 10^8 a 10^{10} ufc/g de fezes e se manteve em níveis elevados até os animais morreram ou serem sacrificados. Em conclusão, o tratamento prévio de camundongos com *L. acidophilus* UFV-H2B20 protege os animais contra a infecção experimental com *S.* Typhimurium mas essa proteção não se deve à uma redução das populações patogênicas nos intestinos.

Palavras-chave: *Lactobacillus*, *Salmonella*, probiótico, camundongo gnotobiótico

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