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Monoassociation with *Lactobacillus acidophilus* UFV-H2b20 stimulates the immune defense mechanisms of germfree mice

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Abstract

Probiotics are formulations containing live microorganisms or microbial stimulants that have some beneficial influence on the maintenance of a balanced intestinal microbiota and on the resistance to infections. The search for probiotics to be used in prevention or treatment of enteric infections, as an alternative to antibiotic therapy, has gained significant impulse in the last few years. Several studies have demonstrated the beneficial effects of lactic acid bacteria in controlling infection by intestinal pathogens and in boosting the host's nonspecific immune response. Here, we studied the use of *Lactobacillus acidophilus* UFV-H2b20, a lactic acid bacterium isolated from a human newborn from Viçosa, Minas Gerais, Brazil, as a probiotic. A suspension containing 10^8 cells of *Lactobacillus acidophilus* UFV-H2b20 was inoculated into groups of at least five conventional and germfree Swiss mice to determine its capacity to stimulate the host mononuclear phagocytic activity. We demonstrate that this strain can survive the stressing conditions of the intestinal tract *in vivo*. Moreover, the monoassociation of germfree mice with this strain for seven days improved the host's macrophage phagocytic capacity, as demonstrated by the clearance of a Gram-negative bacterium inoculated intravenously. Monoassociated mice showed an undetectable number of circulating *E. coli*, while 0.1% of the original inoculum was still present in germfree animals. Mice treated with viable or heat-killed *Lactobacillus acidophilus* UFV-H2b20 presented similarly improved clearance capacity when compared with germfree controls. In addition, monoassociated mice had twice the amount of Kupffer cells, which are responsible for the clearance of circulating bacteria, compared to germfree controls. These results suggest that the *L. acidophilus* strain used here stimulates a nonspecific immune response and is a strong candidate to be used as a probiotic.

Key words
- *Lactobacillus*
- Macrophages
- Kupffer cells
- Probiotics

Introduction

The adverse effect of toxic metabolites from the intestinal microbiota was first claimed by Elie Metchnickoff at the begining of this century. According to Gilliland (1), Metchnickoff postulated that the consumption of milk fermented with lactic acid bacteria could control this “intoxication” by regulating the number and types of microorganisms present in the intestinal tract. Since then, lactic acid bacteria, mainly those of the genus *Lactobacillus*, have been present in food and drugs for human and animal consumption. These formulations containing live microorganisms or microbial stimulants that have some beneficial influence on the maintenance of a balanced intestinal microbiota and on the improvement of the host immune system are called probiotics (2).

In order to be used as probiotics, microorganisms must have the following characteristics: 1) to be normal inhabitants of the host intestinal tract, 2) resist stressing conditions of the intestinal tract and establish in this environment, 3) exert beneficial effects on the host, 4) stay viable in food and drug formulations (3). Lactic acid bacteria have all these characteristics, are not enteropathogenic or enterotoxigenic and are known for their ability to suppress the growth of species of enteric bacteria *in vitro* (4-6). Although physiological conditions *in vivo* are vastly different from *in vitro* model systems used for most studies, the presence of lactic acid bacteria in the intestine is postulated to limit the growth, metabolism or survival of other enteric bacteria, which may in turn limit pathogenic or toxigenic effects (7-9). Many other biological effects have been attributed to lactic acid bacteria, such as bile salt deconjugation (10), induction of higher tolerance to lactose when given to lactose-intolerant subjects (11,12), anticholesterolemic effects (13-15) and anticarcinogenic properties (16-18). The latter effect seems to be related to macrophage activity and recent studies have demonstrated that *Lactobacillus* and other lactic acid bacteria can increase the phagocytic capacity and enzymatic activity of mouse peritoneal cells (19-23). Moreover, Sato (24) has demonstrated that intravenous injection and ingestion of *L. casei* increase the survival of mice to infection with *Listeria monocytogenes*.

L. acidophilus UFV-H2b20 was isolated at the Universidade Federal de Viçosa, Minas Gerais, Brazil, from the feces of a newborn child, with the objective of isolating a new probiotic for human use in pathological states. The work described here was designed to investigate the capacity of this strain to survive and establish in the intestinal tract and affect the host phagocytic capacity. We found that *L. acidophilus* UFV-H2b20 passes through the intestinal tract of Swiss mice without suffering any damage, and colonizes germfree mice successfully. Mice receiving viable or heat-killed *L. acidophilus* UFV-H2b20 were more efficient in clearing an enteropathogenic bacterium from the blood stream and had a higher number of Kupffer cells in the liver. These results demonstrate that *L. acidophilus* UFV-H2b20 is a candidate to be an effective probiotic.

Material and Methods

Animals and microorganisms

Swiss/NIH mice were maintained at the Gnotobiology Laboratory of the Department of Biochemistry and Immunology, Federal University of Minas Gerais, MG, Brazil, according to the procedures described by Pleasants (25) and were used shortly after weaning (21-23 days). Conventional animals of the same age and of both sexes were obtained from our colony. *Lactobacillus acidophilus* UFV-H2b20, a strain of human origin, was isolated at the Federal University of Viçosa, Minas Gerais, Brazil, and maintained at -70°C in non-fat reconstituted dry milk containing 20% glycerol. The strain

was grown in MRS broth (De Man, Rogosa & Sharpe, Merck, São Paulo, Brazil) for 18 h at 37°C just before use. *Escherichia coli* B_{41} ($K99^+$, F41, kind gift from Dr. Moon, NADL, Ames, IA, USA) was maintained at 4°C on Lignières agar (Merck). Before use, cultures were grown overnight in BHI broth (Brain and Heart Infusion, Merck) at 37°C.

Monoassociation with *L. acidophilus* and treatment with killed organisms

The activated culture of *L. acidophilus* UFV-H2b20 was centrifuged at 2000 *g* at 4°C and resuspended in phosphate-buffered saline (PBS) in order to obtain 10^9 colony-forming units (CFU)/ml. One hundred microliters of this suspension was inoculated intragastrically into germfree mice. The success of monoassociation was checked by investigating the number of microorganisms per gram of feces and by determining the number of bacteria in several parts of the gastrointestinal tract. Heat-killed *L. acidophilus* UFV-H2b20 was obtained by heating a suspension containing 10^9 CFU/ml of saline at 121°C for 15 min in a hospital-type autoclave. One hundred microliters of this suspension was inoculated daily intragastrically for 7 days. Control groups received the same amount of saline solution.

Clearance of *Escherichia coli* B_{41}

Germfree Swiss mice were monoassociated with *L. acidophilus* UFV-H2b20 by single inoculation. After 7 days, mice were tested for their capacity to clear a Gram-negative bacterium injected systemically. An 18-h culture of *E. coli* B_{41} was centrifuged (2000 *g*) and resuspended in sterile saline at 10^8 CFU/ml. This number of bacteria was estimated by turbidity (λ = 550 nm) and the absorbance was adjusted to 50 units of transmittance. Mice were injected with 0.2 ml of this suspension into the tail vein after ether anesthesia. Blood samples were collected from the retro-orbital plexus of anesthetized animals immediately after injection of *E. coli* B_{41} (time 0) and at 15, 30, 60 and 90 min after injection. After the last blood collection, mice were sacrificed. Ten-fold serial dilutions of blood samples were plated and cultured on Petri dishes containing MacConkey medium (Merck). Dishes were incubated overnight at 37°C and colonies counted. Results are reported as percent CFU of the counts obtained for time 0. Means were compared by the Student *t*-test.

Clearance of colloidal carbon

A 2:1 dilution of colloidal carbon (India ink, Pelikan, São Paulo, Brazil) in sterile PBS, pH 7.2, was injected into the tail vein of monoassociated and control mice. Twenty microliters of blood was collected from the retroorbital plexus at 0, 5, 10 and 15 min after injection and hemolyzed in 4 ml 0.1% Na_2CO_3 and absorbance was measured at 650 nm. The results are reported as percent absorbance of the value obtained for time 0 (26).

Macrophage ablation by silica injection

In order to impair the phagocytic activity of macrophages, conventional Swiss mice were inoculated with 100 µl of a suspension containing 4 mg of silica dust (particle size <5 µm) in PBS. After 4 h, the animals were injected with either *E. coli* B_{41} or colloidal carbon as described above.

Histological analysis of organs and small intestine

Tissues (small intestine, liver and spleen) were fixed in 10% formalin in PBS, embedded in paraffin, cut into 5-µm thick sections and stained with hematoxylin-eosin. Preparations were coded and examined by one individual who had no access to the codes. The morphometry for crypts and villi was

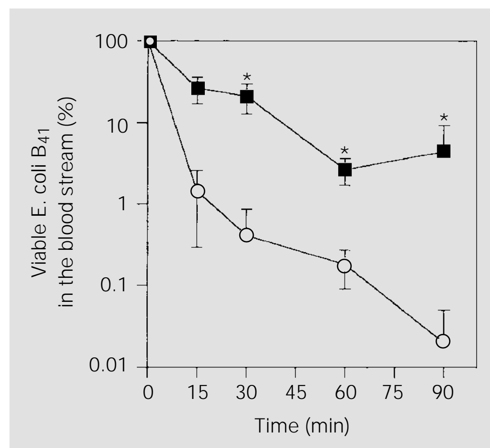


Figure 1 - Clearance of E. coli B_{41} in germfree (squares) and conventional (circles) Swiss mice. E. coli B_{41} was injected into the tail vein and blood samples were taken from the retro-orbital plexus at the time of injection and 15, 30, 60 and 90 min after injection. Ten-fold serial dilutions of blood were plated and cultured on Petri dishes containing MacConkey medium. Dishes were incubated overnight at 37°C and colonies counted. The results are reported as percent viable cells per ml blood in relation to time 0. Each point is the mean for 5 mice of one representative experiment of three performed. Vertical bars indicate standard deviation of the means. *$P \leq 0.05$ compared to conventional mice (Student t-test).

performed using an image analysis program (KS 300-Zeiss) running on an IBM 486 computer. Six vertically arranged villi were analyzed for each animal.

Results

In order to determine the effect of *L. acidophilus* on the capacity of mice to clear a systemic infection, conventional Swiss mice were inoculated daily intragastrically with 0.1 ml of a suspension containing 10^9 CFU/ml of *L. acidophilus* UFV-H2b20 for 7 days and their capacity to clear *E. coli* B_{41} from the blood stream was determined. Both the control group (treated with saline solution) and the *Lactobacillus*-treated group showed efficient clearance within 90 min, with the levels of bacteria in the bloodstream being below the detection limit (data not shown). Similar results were observed when colloidal carbon was used (data not shown). In both experimental models, the control group showed a very efficient clearance capacity, therefore masking the effect of ingestion of *Lactobacillus*. Thus, these models were not appropriate for demonstrating the effect of *Lactobacillus* on host clearance capacity.

Macrophages of germfree animals have a deficient phagocytic power (27). In addition, the lack of normal microflora makes these animals good models to test the ability of a microorganism to survive in the gastrointestinal tract and to determine the effect of association with a single microorganism on the system. Monoassociation of germfree mice with *L. acidophilus* UFV-H2b20 was successfully obtained in the present study. The high level of *L. acidophilus* UFV-H2b20 in feces 7 days after inoculation of a single dose of the bacterium (up to 10^{10} CFU/g of feces) suggests that this microorganism is capable of passing through the intestinal tract without being damaged by intestinal secretions or peristaltism, and of colonizing the gut. In fact, up to 10^8 CFU/g of organ were found even in the stomach, where lactobacilli are exposed to very low pH (data not shown). We then proceeded to compare the kinetics of *E. coli* B_{41} clearance from the bloodstream in conventional and germfree mice. Germfree animals had an impaired clearance capacity when compared to their conventional counterparts (Figure 1). Thus, while conventional animals had reduced the number of bacteria by a factor of five log cycles within 90 min, germfree mice brought the number of bacteria down only one log cycle. Interestingly, treatment of conventional mice with silica dust 4 h before the clearance test, although impairing clearance of *E. coli* B_{41} (Figure 2A), did not reduce the clearance to the low level observed in germfree animals. Silica dust also diminished the clearance of colloidal carbon (Figure 2B). Monoassociation of germfree mice with *L. acidophilus* UFV-H2b20 for 7 days improved the capacity of these animals to clear *E. coli* B_{41} injected systemically (Figure 3). In fact, the clearance capacity of monoassociated mice was quite comparable to that of conventional mice (Figures 1 and 2A). Even more interestingly, germfree mice who received a high dose (10^8 CFU) of heat-killed *L. acidophilus* UFV-H2b20 daily for one week also showed improved clearance when compared to untreated germfree mice (Figure 4). These results suggest that there may be a thermostable, systemic stimulating factor in *Lactobacillus* cells.

Hepatosplenomegaly could be an undesirable side effect of association with *L. acidophilus* UFV-H2b20 which would result in an improved clearance of bacteria from the bloodstream. However, there was no difference in the organ weight/body weight ratio in the tested groups (data not shown). Nevertheless, the number of Kupffer cells in the livers of monoassociated mice was higher than in germfree controls (Figure 5). These results suggest that Kupffer cells might be responsible for the improved clearance in monoassociated mice.

Morphometric analysis of the small intestine showed that monoassociation with *L. acidophilus* UFV-H2b20 significantly reduced the height of the villi in the majority of animals tested (Figure 6). There was no variation in the number of intraepithelial leucocytes in monoassociated animals when compared with germfree controls. This number was three-fold higher in conventional animals than in germfree controls (data not shown).

One mechanism that could explain the systemic effect of ingestion of *L. acidophilus* would be the translocation of these microorganisms to mesenteric lymph nodes, where they could be taken up by macrophages or dendritic cells which, in turn, could release pro-inflammatory and phagocyte-activating cytokines. Mesenteric lymph nodes from mice associated for 7 days with *L. acidophilus* were mostly positive for the bacterium, which, however, was present in variable numbers (between zero and 10^5 CFU/g organ).

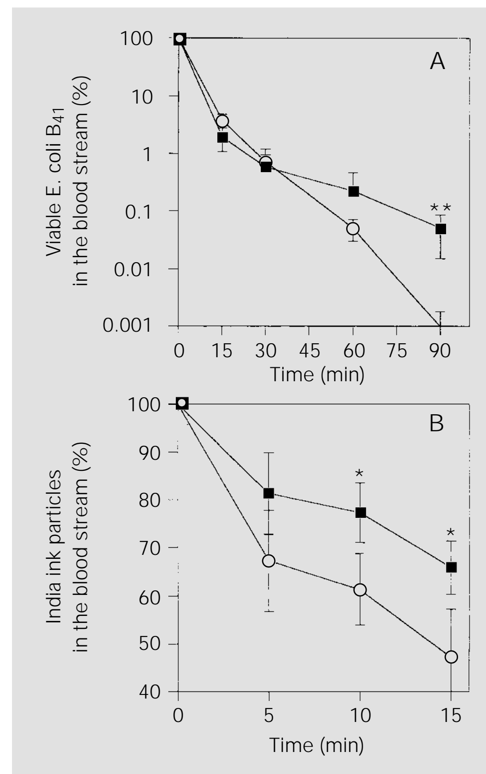


Figure 2 - Effect of treatment with silica on the clearance of E. coli B_{41} (A) and India ink (B). E. coli B_{41} or India ink suspension was injected into the tail vein of conventional (circles) or silica-treated conventional (squares) mice and blood samples were taken from the retro-orbital plexus at the time of injection and 15, 30, 60 and 90 min after injection and treated as described in Material and Methods. The results are reported as percent viable cells per ml blood determined as described in the legend to Figure 1 (A) or as absorbance (λ = 650 nm) (B) in relation to time 0. Each point is the mean for 5 mice of one representative experiment of two performed. Vertical bars indicate standard deviation of the means. *$P \leq 0.05$, **$P \leq 0.01$ compared to conventional mice (Student t-test).

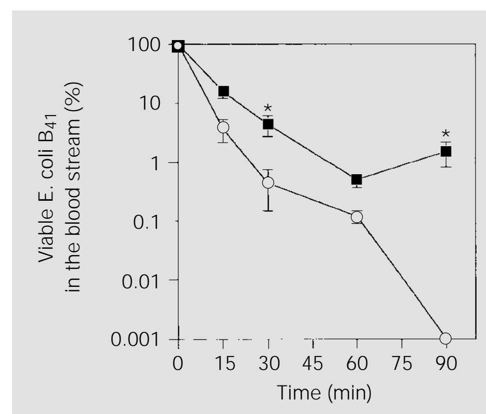


Figure 3 - Clearance of E. coli B_{41} in germfree (squares) and L. acidophilus UFV-H2b20-monoassociated (circles) mice. E. coli B_{41} was injected into the tail vein and blood samples were taken from the retro-orbital plexus at the time of injection and 15, 30, 60 and 90 min after injection. Ten-fold serial dilutions of blood were plated and cultured on Petri dishes containing MacConkey medium. Dishes were incubated overnight at 37ºC and colonies counted. The results are reported as percent viable cells per ml blood in relation to time 0. Each point is the mean for 5 mice of one representative experiment of three performed. Vertical bars indicate standard deviation of the means. *$P \leq 0.05$ compared to monoassociated mice (Student t-test).

Discussion

The search for microorganisms to be used as probiotics has gained a significant impulse in the last few years (2). Increasing emphasis has been placed on the therapeutic and nutritional effect of foods fermented with lactic acid bacteria, mainly lactobacilli and bifidobacteria (17). For this reason, efforts have been made to isolate strains of lactobacilli that can become established in the host intestinal tract and effectively promote their beneficial effects.

The strain studied here, *L. acidophilus* UFV-H2b20, is a strong candidate for a probiotic since it survived the conditions of the intestinal tract in germfree mice *in vivo*. These data are in accordance with *in vitro*

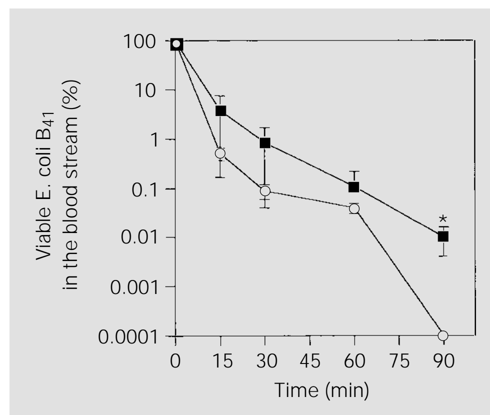
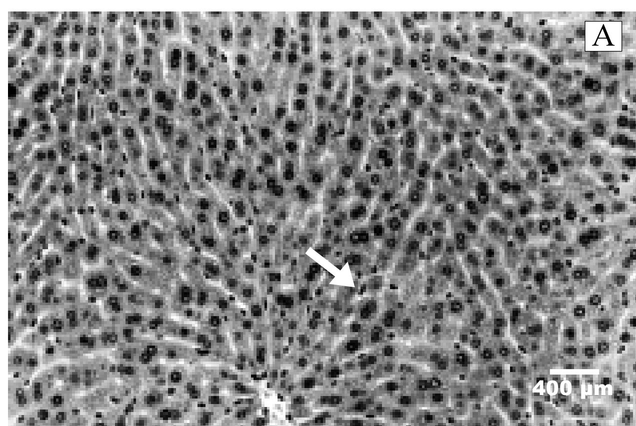
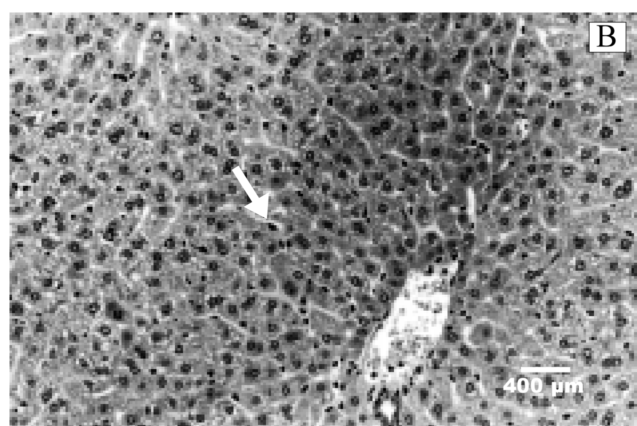
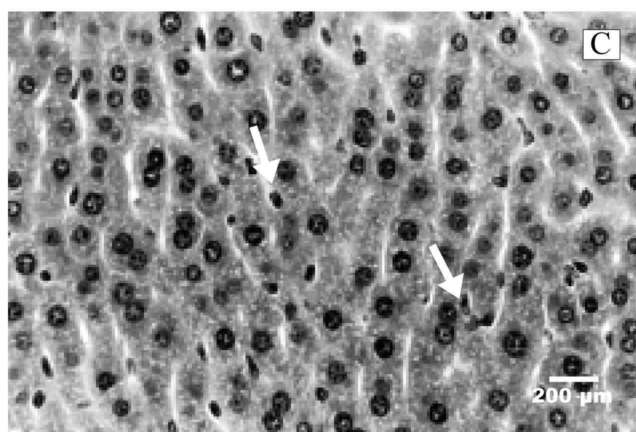


Figure 4 - Clearance of E. coli B_{41} in germfree (squares) or heat-killed L. acidophilus UFV-H2b20-treated Swiss mice (circles). Germfree mice received 10^8 cells of L. acidophilus intragastrically daily for 7 days. E. coli B_{41} was injected into the tail vein and blood samples were taken from the retro-orbital plexus at 0, 15, 30, 60 and 90 min after injection. Ten-fold serial dilutions of blood were plated and cultured on Petri dishes containing MacConkey medium. Dishes were incubated overnight at 37°C and colonies counted. The results are reported as percent viable cells per ml blood in relation to time 0. Each point is the average of data for 5 mice from one representative experiment of two performed. Vertical bars indicate standard deviation of the means. *$P \leq 0.05$ compared to monoassociated mice (Student t-test).







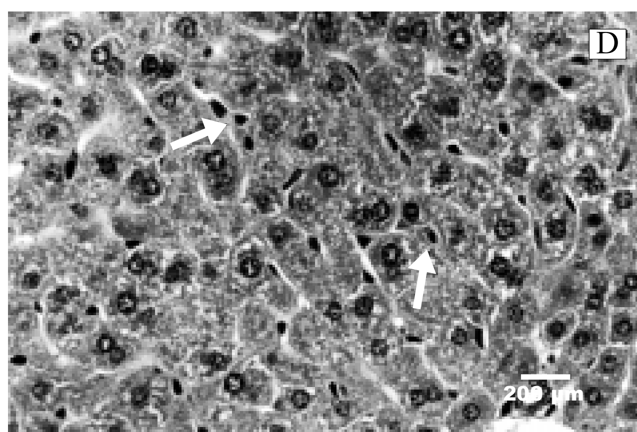


Figure 5 - Liver histology of germfree (A,C) and L. acidophilus UFV-H2b20-monoassociated Swiss mice (B,D). Note the increase in the number of Kupffer cells (arrows) in liver of monoassociated mice. Stained histological samples of liver were examined using a computerized image analyzer system composed of a standard binocular microscope (Zeiss) attached to an IBM compatible computer using the KS 300 program to count cells. Hepatocytes and Kupffer cells were counted in 5 fields (53334.4 µm^2/field) for each sample. Germfree animals had 107.5 ± 16.5 hepatocytes/field, 21.5 ± 4.9 Kupffer cells/field and a ratio of 0.20 Kupffer cells/hepatocyte. Monoassociated mice had 86.3 ± 17.6 hepatocytes/field, 36.0 ± 5.57 Kupffer cells/field and a Kupffer cell/hepatocyte ratio of 0.42. Hematoxylin-eosin staining. Magnification bars: A and B, 400 µm; C and D, 200 µm.

tests of resistance of *L. acidophilus* UFV-H2b20 to intestinal conditions (28). According to Gilliland (1), one of the most important characteristics of a candidate for a probiotic is its capacity to resist the stressing conditions in the gut. The presence of *L. acidophilus* UFV-H2b20 in the stomach of germfree mice is not surprising since mice normally present lactobacilli in the stomach (29). However, it is interesting to note that the *Lactobacillus* strain used in our studies is of human origin, and was not necessarily expected to survive at high concentrations in the stomach and intestine.

The present results suggest that this strain is capable of stimulating the phagocytic capacity of the host macrophages. The same effect has been shown by other authors for another species of *Lactobacillus*. Kato et al. (19) demonstrated that intraperitoneal injection of *L. casei* improved the cytotoxic activity of phagocytes against tumoral cells. Perdigon et al. (20) showed the increased phagocytic capacity of mice who received high doses of *L. casei*. However, the latter authors tested the phagocytic capacity by measuring the clearance of an inert particle from the host bloodstream (colloidal carbon). We did not observe this effect of our *L. acidophilus* strain using the same system. Moreover, we demonstrated that there was no effect of *L. acidophilus* on the host phagocytic capacity by measuring the clearance of a complex agent (a Gram-negative bacterium) which could more faithfully reproduce a pathological situation. That Perdigon et al. (20) found an effect on the clearance of particles by conventional mice that we did not observe may be explained by the fact that, as demonstrated by that group, different strains of lactobacilli differ as to their effect in conventional animals. It is also possible that in our study *L. acidophilus* H2b20 was not present in high enough number in conventional animals, since the normal microbiota would be an efficient barrier against the colonization by foreign organisms in a healthy animal (9). However, we demonstrated here that an effect was observed when the animal does have an impaired clearance capacity. Moreover, our preliminary observations suggest that, although immunosuppressed conventional mice show a diminished clearance capacity, treatment of these mice with *L. acidophilus* H2b20 improved clearance (data not shown).

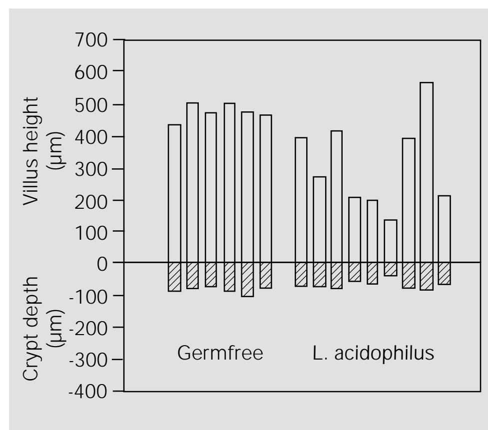


Figure 6 - Small intestine morphometry of germfree and L. acidophilus UFV-H2b20-monoassociated mice. After 7 days of monoassociation, mice were sacrificed and the small intestine was collected in phosphate-buffered formalin. Each bar represents one animal. Villus height was significantly smaller in monoassociated animals (Student t-test, P = 0.013). Crypt depth was also significantly smaller in monoassociated mice (Student t-test, P = 0.03).

Germfree mice are good as models to overcome the barrier effects of the normal microbiota and to demonstrate the effects of a single species of microorganism on the host (30). The clearance of *E. coli* from the bloodstream of germfree mice was inefficient when compared with that observed for monoassociated and conventional animals (Figure 1). These results confirm the putative involvement of the microbiota in the systemic stimulation of the immune response (31). *L. acidophilus*-monoassociated mice showed as efficient a clearance as conventional mice (Figures 1 and 3), which means that this strain of *Lactobacillus per se* has the capacity of stimulating a systemic response, as was demonstrated for other lactic bacteria (19,20,32). More interesting was the fact that heat-killed *L. acidophilus* also stimulates the clearance capacity (Figure 4), suggesting that a thermostable stimulating factor is present. This observation is important for the formulation of a probiotic and speaks for the stability of preparations made with the particular strain we used in the present

study. Accordingly, Chauvière et al. (29) showed the effect of heat-killed *L. acidophilus* on inhibition of enterotoxigenic *E. coli* adhesion onto the brush border of the polarized epithelial human intestinal cells in culture. Furthermore, Coconnier et al. (33) showed the same effect of heat-killed *L. acidophilus* against *Salmonella typhimurium*, enteropathogenic *E. coli*, *Yersinia pseudotuberculosis* and *Listeria monocytogenes*. Several other investigators have demonstrated that components of Gram-positive bacterial membranes could stimulate nonspecific host resistance against *E. coli* (34), produce acute joint inflammation (35) and stimulate synthesis of TNF-α and IL-6 by human monocytes (36). Taken together, these data suggest that pro-inflammatory and phagocyte-activating factors may be the mediators triggered by Gram-positive probiotics, producing a systemic effect which is independent of the more obvious local effects of inhibition of adhesion, inhibition of growth or competition for nutrients in the gut.

The role of Kupffer cells in the clearance of some pathogens was studied by Friedman and Moon (37) who demonstrated that destruction of Kupffer cells by silica treatment enhanced susceptibility to infection with *Salmonella typhimurium* in mice. The same laboratory (38) also demonstrated that the bactericidal effect of Kupffer cells depends on complement activation. Interestingly, Conlan and North (39) showed that Kupffer cells are involved in different ways in early defense strategies against three different intracellular bacteria. The lower number of Kupffer cells in germfree animals than in conventional animals has been previously shown (40). In this study, we observed an increase in the number of Kupffer cells in livers of monoassociated mice (Figure 5) which could explain the improved clearance capacity of these mice. This increase brought the number of Kupffer cells in monoassociated mice to values similar to the ones found in conventional mice. Furthermore, we demonstrated that silica-treated mice had less *E. coli* and colloidal carbon clearance than normal conventional animals (Figure 2).

Finally, the intestinal wall of monoassociated mice was altered. Monoassociated mice showed smaller villi (Figure 6), which could increase translocation of bacteria and cause the exposure of distant lymphoid organs to the probiotic. Accordingly, viable lactobacilli were detected in the mesenteric lymph nodes in several individuals. The present results demonstrate that lactic bacteria or one of their constituents can be in direct contact with the host systemic immune milieu and stimulate the host natural immune response. Other experiments are being performed in our laboratory in order to elucidate the mechanism of action of these bacteria.

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