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Barrier effect of *Bifidobacterium longum* on a pathogenic *Escherichia coli* strain by gut colonization in the germ-free rat

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Summary

The predominance of Bifidobacteria in normal breast-fed babies is well established. Even under unfavourable hygienic conditions of delivery and during the breast-feeding period Bifidobacteria develop and colonize the intestinal tract at high concentrations. In the present study we investigated the interaction between Bifidobacterium longum and a pathogenic E. coli strain in the germ-free rat. Sequential counts of the two bacterial strains allowed their proliferation to be followed. Electron microscopic as well as light microscopic examinations of selected intestinal mucosa segments revealed minor morphological changes.

Bifidobacterium completely protected the rats against mortality from a consecutive infection with E. coli. Repeated inoculation of Bifidobacteria even decreased and kept down the initial E. coli population.

Thus it appears that the germ-free rat is an appropriate model to study the development and interaction of both bacterial species and that the sequence of inoculation is most important.

Zusammenfassung

Das Vorherrschen von Bifidobakterien in normalen, mit Muttermilch ernährten Säuglingen ist gut dokumentiert. Sogar unter ungünstigen hygienischen Bedingungen bei der Geburt und während der Stillperiode entwickeln sich die Bifidobakterien und kolonisieren den Darmtrakt in großer Anzahl. In dieser Studie untersuchten wir die Wechselwirkung zwischen Bifidusbacterium longum und einem pathogenen E.-coli-Stamm in der keimfreien Ratte.

Die Proliferation der beiden Bakterienstämme wurde durch sequenzielle Probeentnahme verfolgt. Die licht-mikroskopische und elektronen-mikroskopische Untersuchung von ausgewählten Darmabschnitten zeigte geringgradige morphologische Veränderungen.

Die Bifidobakterien schützen die Ratten vollständig gegen Mortalität durch nachfolgende Infektion durch E. coli. Wiederholte Verabreichung von Bifidobakterien verminderte darüber hinaus die E.-coli-Population und hielt diese auf einem niedrigen Niveau. Daher erscheint die keimfreie Ratte als ein angemessenes Modell zum Studium der Entwicklung und Wechselwirkung von beiden Bakterienstämmen. Die Sequenz der Inokulation ist dabei von großer Bedeutung.

Key words: germ-free rat, intestinal tract colonization, interaction, protection, Bifidobacterium, E. coli

Introduction

As early as 1900, Tissier (18) discovered and characterized, in the intestinal tract of newborns, the protective effect exerted by Bifidobacterium against pathogenic microorganisms. Thereafter this subject received intensive and permanent attention although some facets have remained unsolved.

The predominance of Bifidobacterium (10^9/g of feces) in normally born, breast-fed babies is well established (3, 13, 11, 8, 12). It is, however, much less developed in babies delivered by Caesarian section, in those whose mothers had undergone antibiotic therapy, in prematures or in babies fed infant milk formulas. In all these cases, a multiple mixed flora mainly composed of Escherichia coli, Clostridium and Bacteroides rapidly predominates and under unfavourable hygienic, nutritional or immunological conditions, serious life-threatening infections may occur (17, 1, 15, 16, 19).

For this reason, it is of primary importance to further investigate the role of Bifidobacterium and to establish definitively whether this species really exerts a protective effect in the newborn gastrointestinal tract.

Several hypotheses of the possible protection mechanism have already been advanced (17, 1, 6). Firstly, some evidence suggests that Bifidobacteria may lower the intestinal pH to 5, thus selectively inhibiting other ecological competitors. This condition is favoured by the composition of human breast milk, which is high in lactose and works as a specific substrate for Bifidobacteria. Furthermore, the low protein and low phosphate content of breast milk may contribute to the low buffering capacity in comparison to cow's milk (4). It was also postulated that the continuous supply of a specific but yet undefined growth factor for Bifidobacteria might be at least partially responsible for the predominance of this species in breast-fed babies (9). In addition, there is unequivocal evidence that human colostrum contains variable amounts of certain immunoglobulins which protect against common pathogens. However, screening of common non pathogenic microorganisms, particularly of the Streptococcus and Lactobacillus species, revealed only in S. faecalis and L. bulgaricus some cell-bound and cell-free factors, which developed a protective mechanism against E. coli (10).

Our objective in the present study was to test the proposed protective effect of Bifidobacterium against pathogenic Escherichia coli and to establish the short-term evolution of these two species in the germ-free rat. In this context, we were interested in studying the influence of the inoculation sequence as well to follow up, by light and scanning electron microscopy, the possible changes of intestinal morphology subsequent to colonization and interaction by both bacterial species.

Material and methods

Germ-free rats, 4–6 weeks old, obtained from Iffa Credo (Saint-Germain-sur-l'Arbresle, France) were kept in plastic isolators of the Trexler type. The feed, supplied by Usines Alimentations Rationnelles (U.A.R., Villemoisson-sur-Orge, France), was irradiated at 4 MR. This sterile standard rat chow as well as water was given ad libitum. The air locks were sterilized with an aqueous solution of 2 % to

35 % peracetic acid (Air Liquide Champigny, France), and all other material was autoclaved at 120 °C for 30 minutes.

The first experiment consisted of a single oral administration of the E. coli and B. longum. In the second experiment repeated inoculations took place. The E. coli strain of human origin 0111B 75-44 was obtained from Dr. H. Hilpert, Nestlé Products Technical Assistance, Co. Ltd., Research Dep., La Tour-de-Peilz, Switzerland.

The Bifidobacterium strain B1990, classified as Bifidobacterium longum, was obtained from Dr. T. Sozzi, Nestlé Products Technical Assistance Co. Ltd., LINOR, Food Products Development Centre, Orbe, Switzerland, from the collection of Prof. Scardovi, Bologna, Italy.

Experiment 1

The rats were divided into 4 groups of 6 animals. One group served as B. longum control, another as a germ-free control while in the two other groups animals received cross-over inoculation either with E. coli or B. longum first followed by the other strain after 20 days.

After one week of adaptation and controls for the germ-free status all groups received the first inoculation of 1 ml of culture by gavage: broth + 3×10^8 B. longum, or broth + 35×10^6 E. coli. After 20 days the animals of the cross-over groups received a 2nd inoculation with the other strain. Microbial counts in the faeces were made 24–48 hours later, again after 72–96 hours as well as once a week thereafter.

The experiment lasted 8 weeks after which all animals were killed by chloroform narcosis and weighed. Samples of ileum, caecum and colon were removed for examination by light and scanning electron microscopy. The culture media used for sterility tests were Eugon Agar plates, 10 ml tubes of nutritional peptone broth, 10 ml tubes of Schaedler medium, a broth of heart and brain and agar plates of Sabouraud and Fungiphil (B. D. Mérieux, Lyon, France). For E. coli, MacConkey broth and agar plates of the same medium were used for detection and counting. B. longum were grown in a specific agar broth (hydrolysate of lactalbumin 16 g/l – lactose 20 g/l – sodium acetate 20 g/l – meat extract 12 g/l – Agar Difco 6 g/l), steam-sterilized for 20 minutes at 120 °C. This deep gel technique allows verification of the respiratory type in the absence of gaseous emissions.

Experiment 2

This experiment was designed to investigate multiple inoculations of both E. coli and B. longum. Three groups of 16 rats each received at the start (To) of the experiment either E. coli alone or E. coli followed by B. longum or B. longum followed by E. coli. Cross-over inoculation was initiated 20 days after the first inoculation, E. coli alone inoculated rats received only broth at this time. Administrations were continued with the respective strains at days 34, 36 and 37. At day 54, all rats were killed (chloroform anaesthesia) and samples of intestine were removed as in the first experiment.

Scanning electron microscopy

The intestinal samples were severed 2 cm above and 2 cm below the caecal junction. Catheters were inserted into the cut ends and secured with surgical thread. Fixative solution (2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2) was injected with a syringe attached to the catheters via a Y-coupling until the intestine and caecum were slightly but obviously inflated (14). The exterior of the tissue was bathed with fixative solution during the procedure. The tissue was fixed for two hours and stored in phosphate buffer. Cross-sectioned pieces of tissue approximately 7 mm in width were dehydrated through a graded series of water-ethanol solutions and dried by the critical point method, using CO_2. After mounting

on stubs with conductive silver paint, the specimens were coated with gold in a sputtering device. Examination and photography were performed with a Cambridge Stereoscan 600 or a Cambridge S4-10 SEM.

Optical microscopy

Sections of caecum, colon and ileum were opened, applied to a piece of cardboard and plunged into liquid nitrogen. The frozen pieces of tissue were cut on a cryostat microtome, at −17 °C and mounted onto a warm microscope slide. The sections were cut to 10 µm and left to dry at room temperature. They were stained with New Gram stain (5), Acridine Orange (C.I. Basic Orange 14) and Phosphine 3R (C.I. Basic Orange 15). Immunofluorescence labelling of E. coli was performed with Bacto-FA E. coli poly A (Difco).

Results

Microbial counts were carried out on the feces of each animal and the results are expressed as means of 6 to 16 rats.

1. *Single inoculations* (experiments 1 and 2, figs. 1A/1B)

When given alone to germ-free rats, B. longum or E. coli developed to a stable population of 10^9–10^{10} live bacteria per gram of feces, which corresponds to the usual 5.10^8–10^{10} found for other microorganisms.

2. *Crossed inoculations* (experiment 1, figs. 2A/2B)

A) When given 20 days after a first inoculation of B. longum, E. coli grew only to 10^8 which is 10 % of the level attained in 1. The established

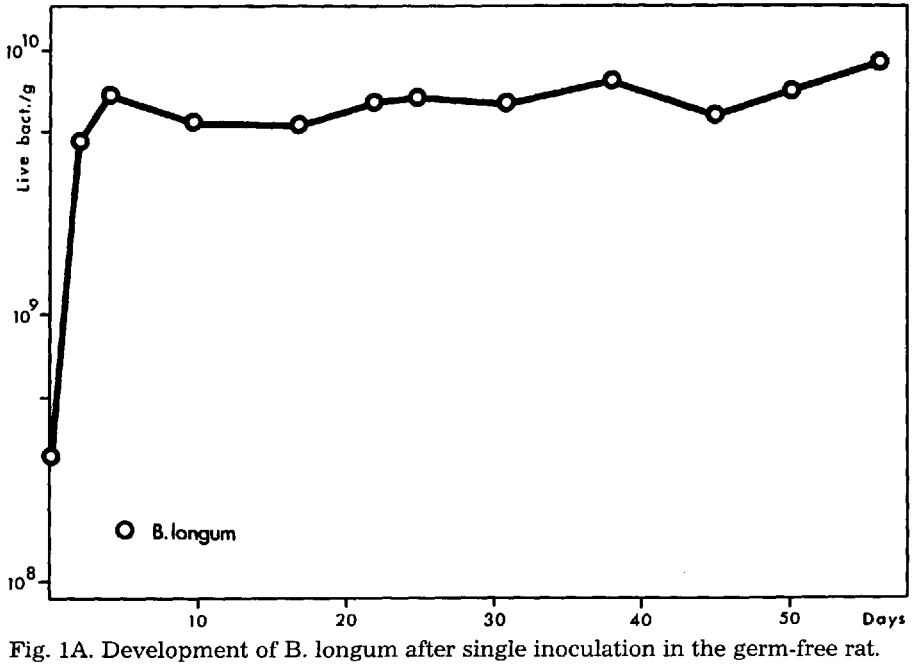

Fig. 1A. Development of B. longum after single inoculation in the germ-free rat.

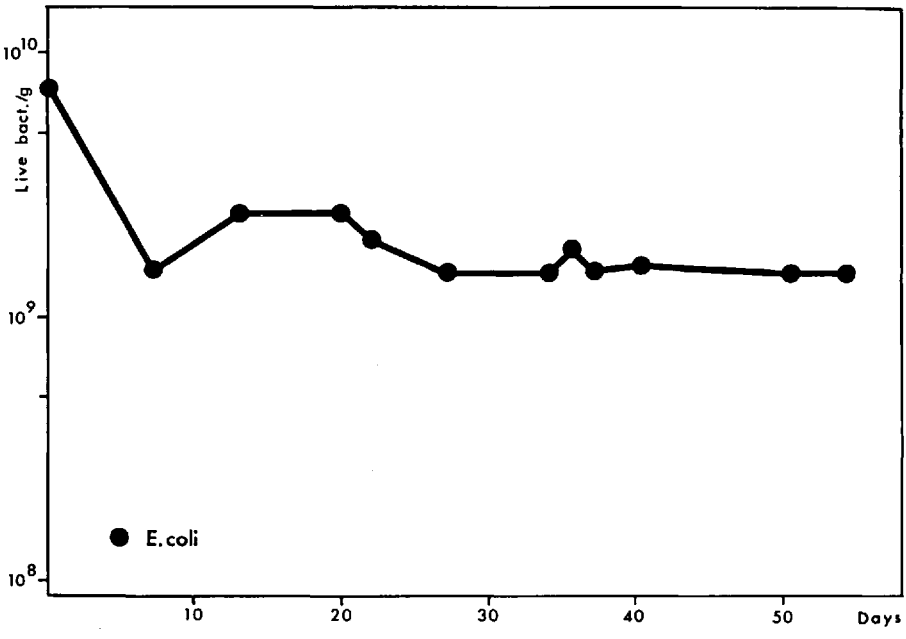

Fig. 1B. Development of E. coli after single inoculation in the germ-free rat.

population of B. longum (5.10^9) was not altered by subsequent inoculation of E. coli.

B) The established population of E. coli dropped from 5.10^9 to 5.10^8 when B. longum was introduced after 20 days; while B. longum established a normal population level. After 15 days, E. coli recovered and its population increased to previous levels.

3. Crossed inoculation followed by repeated inoculations with the second strain, (experiment 2, figs. 3A/3B)

A) The inhibiting effect of previously established B. longum population on E. coli was not abolished by repeated inoculations with E. coli.

B) On the contrary, repeated inoculations with B. longum inhibited the recovery of E. coli as established in 2B.

Pathogenic effects

On all occasions, when B. longum was given first, no clinical signs or other pathogenic effects were observed. However, when E. coli was the first inoculant, we observed, in the first experiment, 3 deaths out of 6 rats and, in the second experiment, 6 deaths out of 32 rats. This shows that the pathogenic effect of E. coli was effective in the germ-free rats. Deaths occurred always within 48 hours of inoculation.

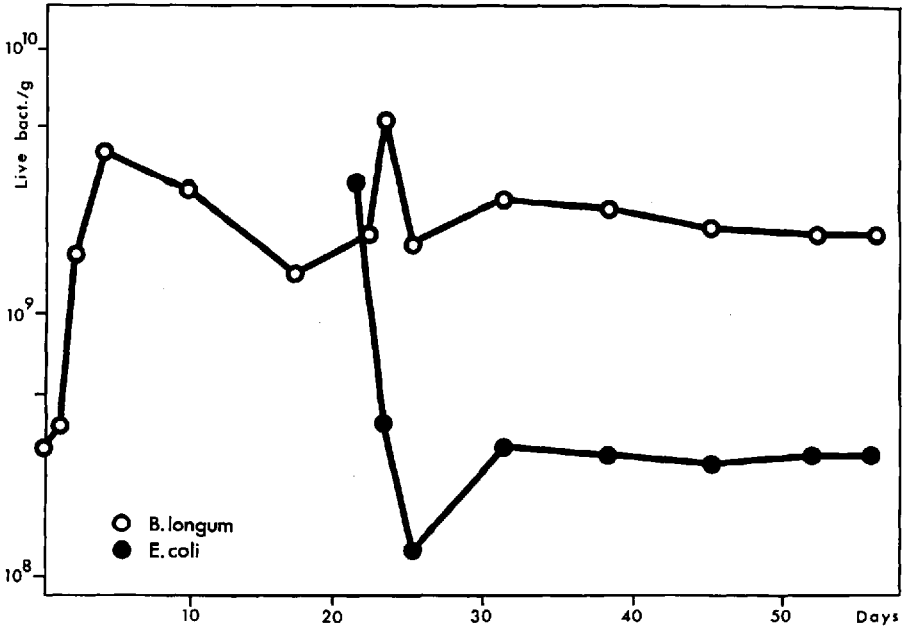

Fig. 2A. Development of B. longum and E. coli after single cross inoculation in the germ-free rat.

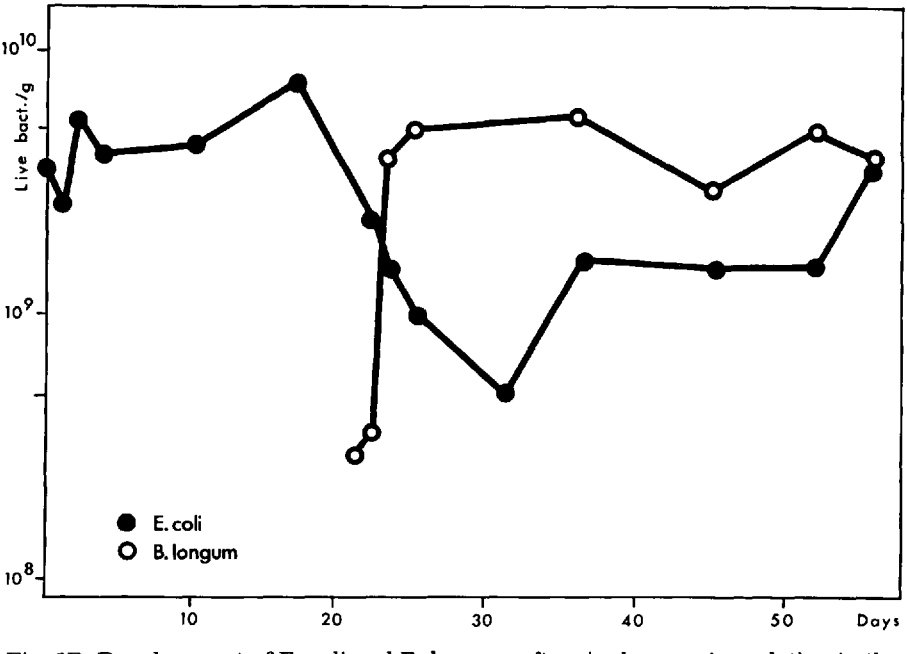

Fig. 2B. Development of E. coli and B. longum after single cross inoculation in the germ-free rat.

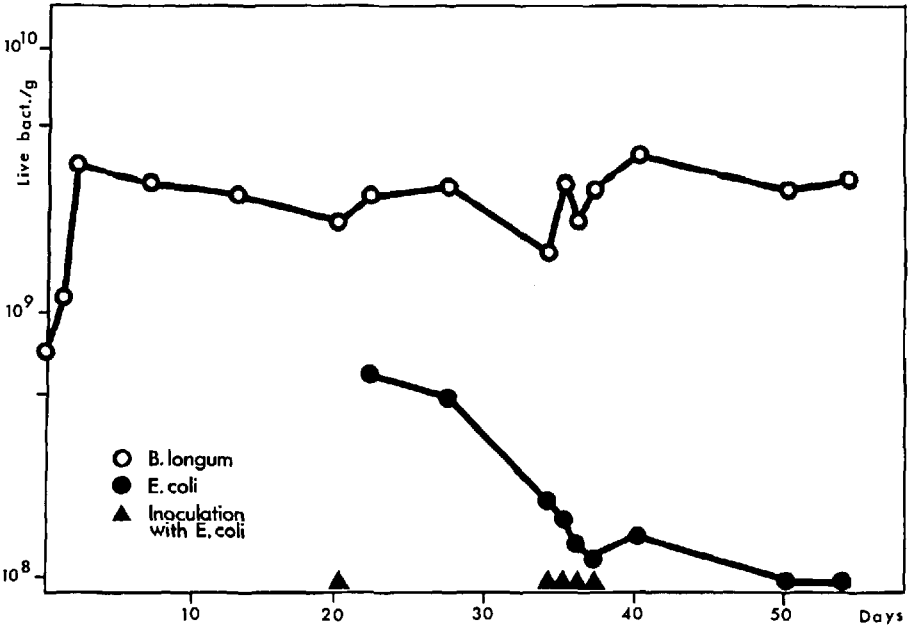

Fig. 3A. Development of B. longum and E. coli after crossed and repeated inoculation with E. coli in the germ-free rat.

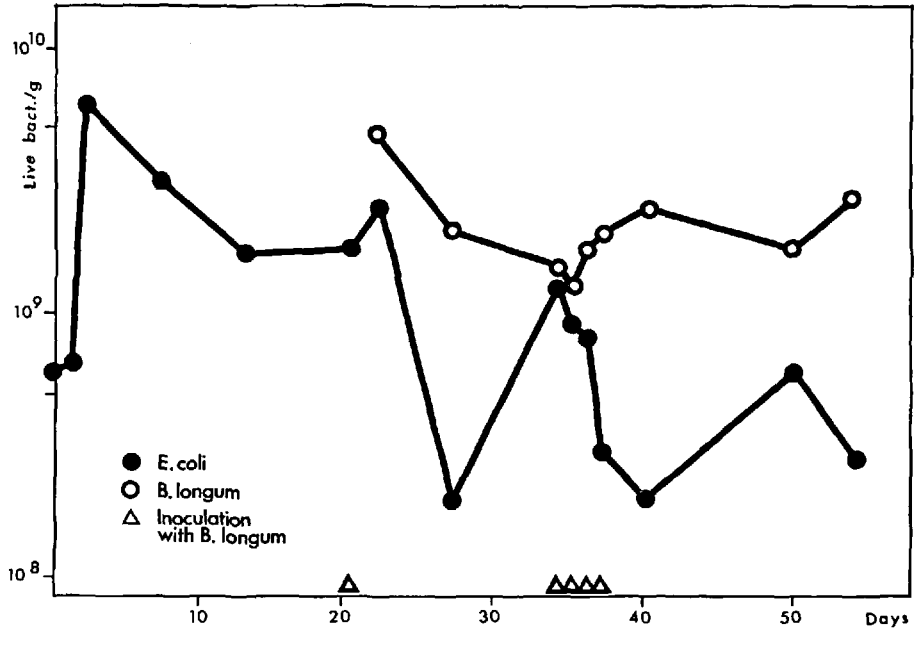

Fig. 3B. Development of E. coli and B. longum after crossed and repeated inoculation with B. longum in the germ-free rat.

Scanning electron microscopy

A striking observation was that mucus production closely related to the presence of bacteria. In germ-free controls, only some smears of mucus were seen and the activity of the goblet cells was very low; when inoculated either by E. coli or B. longum, the intestine showed a higher content of mucus; the highest amount being observed in rats inoculated with both species.

Scanning of the intestinal mucosa surface did not show any changes of its morphology. Thus it appeared that the different microorganisms did not induce morphological modification of the mucosal surface.

In the inoculated animals, the bacteria colonized the lower part of the ileum, the caecum and the colon. Most of them were entrapped in the mucus. Others were situated close to the surface of the mucosa. The B. longum adhered by means of filamentous strands extending to the mucosal surface (fig. 4). E. coli were sporadic and were seen in places where microvilli were destroyed (fig. 5).

Optical microscopy

B. longum stained well with New Gram, Acridine Orange and Phosphine 3R. This species was found on the mucosal surface or in clusters of

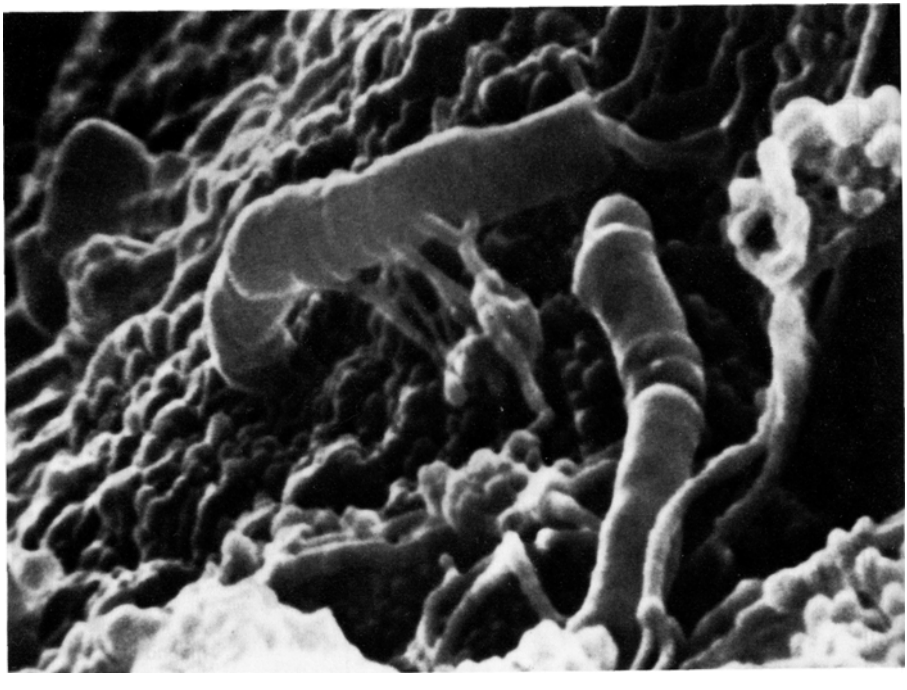

Fig. 4. Scanning electron microscopy of the ileum of a B. longum inoculated rat. × 40,000. This picture shows the mucosal surface. The tips of the microvilli appear tightly packed. The B. longum adhere closely to this surface, and filamentous strands extending from the bacteria reinforce this adhesion.

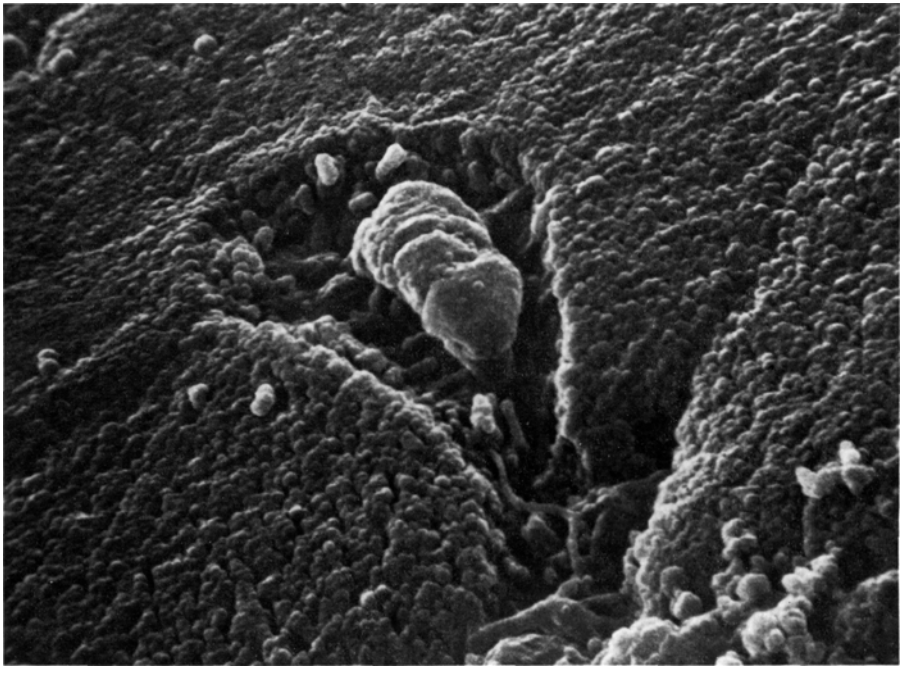

Fig. 5. Scanning electron microscopy of the ileum of an E. coli inoculated rat. × 17,000. The microvilli of the mucosal surface are destroyed in the region where the bacteria (E. coli) adheres to the membrane.

mucus. In contrast, E. coli, identified by immunofluorescence, was seen to penetrate the epithelium of the mucosa and to form colonies in deeper layers of the gut wall.

Discussion and conclusion

This study provides evidence that inoculation of Bifidus bacterium longum protects germ-free rats from the pathogenic effects of a subsequent E. coli infection. In fact, no deaths were observed in the experiments wherein germ-free rats received B. longum prior to E. coli. However, with the reverse sequence of inoculation after single oral administration of E. coli alone or B. longum after several days, mortalities occurred always within the first 48 hours. Thus there were 3 deaths out of 6 rats and 6 deaths out of 32 rats respectively. As no other pathological symptoms were observed over the following 50 days, it is assumed that the survivors developed a resistance to the pathogenicity. It cannot be excluded that in certain cases the acute death was attributable to a rapid bacteriaemia, since immunofluorescence revealed disseminated nests of E. coli in the submucosa of killed animals.

The mechanism by which B. longum protected the rats against the pathogenic effects of E. coli under our experimental conditions are still unclear.

In mice, it was shown (6, 7) that Shigella flexneri was eliminated by E. coli in short term experiments and that a strain of Ristella was eliminated by a strain of Lactobacillus when lactose was added to the diet.

We can probably exclude such a nutritional substrate effect in our trials since in the present investigations an unspecific commercial standard diet was fed to the rats, and no attempts were made to boost the growth of B. longum *in vivo*.

Likewise in our studies the involvement of lactoferrin (2) or specific growth factors such as present in breast milk (1) or immunoglobulins of the IgA type can be excluded from the explanation of the protective mechanism of B. longum against E. coli. The possibility must therefore be retained that B. longum stimulated the immuno-competence of the germ-free rats in such a way that subsequent infection with E. coli after twenty days abolished the pathogenic effects of the latter. This possibility should be further investigated including use of specific immunological techniques to verify this hypothesis.

If either B. longum or E. coli are administered alone to germ-free rats both strains develop to a stable population in the gut. The level of this population being set by the conditions encountered by the microorganisms in this particular ecological niche such as nutrients, aerobic conditions and space. E. coli when preceded by B. longum could develop only to a much inhibited population of about 10% the unrestricted level. This clearly demonstrates a barrier effect of B. longum in this condition.

The inverse sequence of inoculations indicates that E. coli developed first to the expected uninhibited level but, after cross inoculation with B. longum, had to compete with this new population which temporarily reduced E. coli in a significant manner. The inhibiting effect, however, is not permanent and E. coli again attain their previous level of 10^9 live bacteria/g faeces.

Surprisingly the B. longum population is completely unaffected by the increase of E. coli. Thus there is a marked difference dependent on which strain is developing first. Therefore, although the barrier effect of B. longum against E. coli is evident in both conditions, the duration of the effect is affected by the order of inoculation.

Furthermore repeated booster inoculation with B. longum kept down the E. coli population for the rest of the observation period.

These investigations suggest that it might be worthwhile to study further interactions with other pathogenic microorganisms currently being isolated from rat intestines. In addition we arbitrarily chose a B. longum strain without ascertaining whether other strains of this species were more efficient in inhibiting the E. coli chosen.

In conclusion, we demonstrated that in the frame of our experimental conditions B. longum protects germ-free rats against mortality caused by the pathogenic effects of E. coli.

The order of inoculation is of importance for the preventive effect and for a durable protection. Multiple booster inoculations change the developmental profile of both species in a specific manner.

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References

1. Beerens H, Romond C, Neut C (1980) Influence of breast-feeding on the bifid flora of the newborn intestine. Amer J Clinical Nutr 33:2434–2439
2. Bullen JJ, Rogers HJ, Leigh L (1972) Iron binding proteins in milk and resistance to Escherichia coli infection in infants. Br med J 1:69
3. Bullen CL, Tearle PV, Steward MG (1977) The effect of "humanised milks and supplemented breast feeding on the faecal flora of infants". J Med Microbiol 10:403
4. Bullen L, Wills AT (1971) Resistance of breast-fed infant to gastroenteritis. Brit med J 3:338–343
5. Davis J (1976) A gram stain for smears of blood culture, body fluids and tissues. Amer J techn 42:II.
6. Ducluzeau R, Dubos F, Raibaud P (1971) Effet antagoniste d'une souche de Lactobacillus sur une souche de Ristella sp. dans le tube digestif de souris "Gnotoxéniques" absorbant du lactose. Ann Inst Pasteur 121:777–794
7. Ducluzeau R, Raibaud P (1974) Interaction between Escherichia coli and Shigella flexneri in the digestive tract of "gnotobiotic" mice. Infection and Immunity Apr 730–733
8. Finegold SM et al (1977) fecal microbial flora in seventh day adventist populations and control subjects. Amer J Clin Nutr 30:1781–1792
9. Kehagias C, Jao WC, Mikolajcik EM, Hansen PMT (1977) Growth response of B. bifidum to a hydrolytic product isolated from bovine casein. J Food Sci 42:146
10. Mitchell IG, Kenworthy R (1976) Investigation on a metabolite from Lacatobacillus bulgaricus which neutralizes the effect of enterotoxin from Escherichia coli pathogenic for pigs. J Bacteriol 41:163–174
11. Mitsuoka T, Hayakawa K, Kimura N (1974) Die Fäkalflora bei Menschen. II. Mitteilung: Die Zusammensetzung der Bifidobakterienflora der verschiedenen Altersgruppen. Zbl Bakt I Abt Orig A 226:469
12. Nelson DP, Mata JL (1970) Bacterial flora associated with the human gastrointestinal mucosa. Gastroenterology 58:56–61
13. Reuter G (1963) Vergleichende Untersuchungen über die Bifidus flora in Säuglings- und Erwachsenenstuhl. Zbl Bakt I Abt Orig A 191:486
14. Schellenberg D, Pangborn J (1980) Preservation of the microbial association to intestinal mucosal epithelium in rats for scanning electron microscopy. Scanning 3:3
15. Schmittbuhl J, Thèse (1965) Strasbourg, Fac. Médecine. Essais d'implantation de Bifidobacterium bifidum chez le nourrisson: son intérêt dans l'éradication d'Escherichia coli des gastro-entérites infantiles
16. Schneegans E, Haarscher A, Lutz A, Lévy-Silagy J, Schmittbühl J (1966) Contribution à l'étude de Bifidobacterium bifidum: essais d'implantation chez le nourrisson sain et chez le porteur d'Escherichia coli pathogènes. Ann Pédiatr (Paris) 26–27:1665–1670
17. Shahani KM, Ayebo AD (1980) Role of dietary lactobacilli in gastrointestinal microecology. Amer J Clin Nutr 33:2448–2457
18. Tissier H, Thèse (1960) Paris. Etude sur la flore intestinale normale et pathologique du nourrisson
19. Mata LJ, Urrutiu JJ (1971) Intestinal colonization of breast-fed children in a rural area of low socioeconomic level. Ann NY Acad Sci 176:93–109

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