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Biochemical Functions of *Bacillus licheniformis* in Gnotobiotic Mice

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A probiotic bacterial strain, *Bacillus licheniformis* NCTC 13123, has been mono-associated with germ-free mice. After colonization, the following biochemical microflora-associated characteristics (MACs) were analyzed in the large intestinal contents: excretion of short-chain fatty acids (SCFAs), inactivation of tryptic activity, degradation of β-aspartylglycine, breakdown of mucin, conversion of cholesterol to coprostanol and transformation of bilirubin to urobilins. An additional MAC, β-glucuronidase activity, was tested in vitro. β-Glucuronidase activity was not expressed by the bacterium. The amount of total SCFAs was lower than that previously found in germ-free mice. Utilization of individual SCFAs rather than production by the strain may be assumed. The other MACs remained similar to the basal values in germ-free mice. We conclude that *B. licheniformis* NCTC 13123 is not able to alter any of the microbial functions investigated in vitro or in the gastrointestinal tract of germ-free mice. Whether and to what extent the strain has the capability to interfere with these functions in the presence of other micro-organisms remains to be investigated. *Key words*: *Bacillus licheniformis*, probiotics, microflora-associated characteristics, germ-free mice.

INTRODUCTION

The use of different micro-organisms as feed additives referred to as probiotics (1) has been promoted in raising domestic animals in an attempt to improve their growth and also to avoid the consequences of the use of antibiotics. The latter compounds have been added to the diet of farm animals mainly during weaning and post-weaning periods to prevent digestive disorders or infections (2–4).

Among probiotics, strains of *Bacillus* spp. have been used as growth and health promoters in piglets (5, 6). *Bacillus licheniformis*, a Gram-positive rod included in the 'subtilis group', has been used in industry for the production of the well-known antibiotic bacitracin. This bacterial strain has also been investigated as a probiotic in pigs (7, 8).

Although several beneficial effects of the use of probiotics have been claimed (9), the mechanisms of action and the biochemical performance of the strains used have not been satisfactorily investigated. Basic knowledge of the biochemical actions of individual strains is necessary to understand their effects within the gastrointestinal ecosystem.

Comparative studies in animals harboring a normal flora – i.e. conventional (CV) animals and those harboring no microflora, i.e. germ-free (GF) animals – have demonstrated series of anatomical structures, biochemical, immunological and physiological functions performed by members of the intestinal flora and they are referred to as microflora-associated characteristics or MACs (10). Some of the MACs include production of short-chain fatty acids (SCFAs), inactivation of tryptic activity, degradation of β-aspartylglycine, breakdown of mucin, conversion of cholesterol to coprostanol and conversion of bilirubin to urobilins. GF animals, on the other hand, do not show these features. Any of these characteristics in the absence of the functional micro-organism(s) has been defined as a germ-free animal characteristic or GAC.

The aim of this study was to investigate the influence of the probiotic *B. licheniformis* on the GACs referred to above in gnotobiotic mice. An additional MAC, i.e. β-glucuronidase activity of the strain, was tested in vitro.

MATERIALS AND METHODS

Bacteria

B. licheniformis NCTC 13123 was kindly provided by Alpharma Inc. (Oslo, Norway). *Escherichia coli* X7, part of the collection of stock cultures at the Laboratory of Medical Microbial Ecology, was used as a positive control

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for β-glucuronidase activity. The strains were grown on thioglycollate and glucose broth, respectively. The cultures were incubated aerobically at 37°C for 24 h.

Mice

Eight GF NMRI-KI female mice (average age 142 days) were included in the study. The animals were reared according to the Gustafsson technique (11), fed a sterilized diet (R36, Lactamin, Sweden) and had free access to water.

The animals were mono-associated as described previously (12) and remained within a stainless steel rearing isolator (SRI) for 12–13 days. Thereafter, the mice were taken out of the SRI and killed by cervical dislocation. Bacterial establishment was verified by culturing samples from the cecum. The large intestinal content of each mouse was sampled and stored at −20°C until the biochemical analyses were performed.

The study was approved by the local ethical committee for animal research, Sweden.

Biochemical analyses

The samples were thawed and homogenized. Aliquots of 0.5 g were separated to investigate the amount of SCFAs and of 0.7 g to measure urobilins. Additional aliquots of 0.5–1.0 g were diluted in saline solution (1:2), placed at 4°C for 2 h and centrifuged at 4000 *g*, 4°C for 30 min. Then, the supernatants were separated to assay tryptic activity, degradation of β-aspartylglycine and breakdown of mucin. The remaining supernatant plus the sediment was used to measure coprostanol.

In vitro assays

The activity of β-glucuronidase was assayed qualitatively using *p*-nitrophenyl-β-D-glucuronide (PNG) as substrate (PGUA tablets, Rosco Diagnostica, Denmark). Dense suspensions from overnight cultures of *B. licheniformis* and *E. coli* X7 were prepared in 0.25 ml of phosphate buffer at pH 6.5 and pH 8.0. A tablet of PNG was added to each of these suspensions and to buffer with no bacterium. Liberation of *p*-nitrophenol (revealed by the development of a yellow color) was determined by inspection after incubation for 4, 24 and 48 h at 37°C. β-Glucuronidase activity was recorded as +, ++, +++ or negative.

In vivo assays

The amount of SCFAs was assayed by gas-liquid chromatography as previously described (12). The total and individual concentrations of SCFAs were reported as mmol of SCFA/kg sample (wet weight).

The other parameters investigated, i.e. inactivation of tryptic activity, degradation of β-aspartylglycine, breakdown of mucin, formation of coprostanol and of urobilins, were determined as described previously (13, 14).

RESULTS

B. licheniformis did not express β-glucuronidase activity after incubation for 48 h.

All the animals remained healthy throughout the experimental period. *B. licheniformis* NCTC 13123 was present in the cecal content of the animals (10^7 cfu/g).

Total and individual concentrations of SCFAs found in the large intestinal content of the mice are presented in Table I.

Values for tryptic activity, degradation of β-aspartylglycine, breakdown of mucin, formation of coprostanol and of urobilins were as found in GF animals.

DISCUSSION

Fecal SCFAs represent dietary and bacterial fermentation in the intestinal ecosystem. As most probiotics have saccharolytic properties, we expected induction of production of SCFAs from *B. licheniformis*. In the present study however, total and individual SCFAs in the large intestinal contents from mice monoassociated with *B. licheniformis* NCTC 13123 were found in smaller amounts than those previously reported in GF mice (11). Even more, some individual SCFAs such as proprionic acid were detected only and in a very small amount in one of the samples investigated. As *B. licheniformis* is able to utilize acetic acid and propionic acid as a sole carbon source (15), the result suggests that the bacterium might have utilized the acids present rather than produced them. However, the utilization of these acids by the strain was not investigated. Other factors that should be taken into consideration are the content of SCFAs in the animal's diet as well as the degree of SCFA absorption from the gut.

The results of the present study show that *B. licheniformis* by itself does not interfere with any of the other functions investigated: inactivation of tryptic activity, degradation of β-aspartylglycine, breakdown of mucin, formation of coprostanol and urobilins, and β-glucuronidase activity. More detailed information about the micro-organisms involved in these functions is described elsewhere (14).

Although *B. licheniformis* on its own did not perform any of the functions investigated, it may interfere with any of them when acting within the gastrointestinal tract in a CV animal. Gustafsson et al. (16) showed an enzyme-enhancing effect of an oral suspension containing two bacterial strains, on 7 α-dehydroxylation. Interestingly, none of the strains was able to perform that function by itself.

As the gastrointestinal tract of mammals is a highly complex ecosystem where myriads of micro-organisms interact not only with the host but also with each other, further research is needed in order to understand the probiotic action.

Table I

Total and individual amounts of short-chain fatty acids (SCFAs) in the large intestinal contents from mice mono-associated with Bacillus licheniformis NCTC 13123

Group[a]	Total SCFAs[b]	Acetic acid	Propionic acid	n-Butyric acid	Other SCFAs[c]
B. licheniformis	12.35 (2.47)	11.65 (2.38)	0.15 (0.34)	0.08 (0.01)	0.47
GF[d]	16.89 (1.51)	16.05 (1.37)	0.25 (0.56)	0.59 (1.01)	ND
CV[d]	111.73 (17.29)	56.79 (10.70)	19.45 (4.66)	24.43 (6.33)	11.06

[a] *B. licheniformis*; GF, germ-free; CV, conventional; eight, five and four animals were used, respectively.
[b] Figures are means (SD) of mmol SCFAs/kg of large intestinal content (wet weight).
[c] Other SCFAs included iso-butyric, iso-valeric and capronic acid.
[d] Data from Cardona et al, 2001 (12); ND, not detected.

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