Effect of probiotics on five biochemical microflora-associated characteristics, *in vitro* and *in vivo*

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Abstract

Background: Several bacterial strains, mainly those belonging to the genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium*, are currently used as probiotics.

Objective: The aim of this study was to investigate the influence of probiotics on five biochemical microbial-related functions *in vitro* and *in vivo*, in gnotobiotic mice, by applying the GAC/MAC (germ-free animal characteristic/microflora-associated characteristic) concept.

Design: Sixteen probiotics were monoinoculated *in vitro* and/or monoassociated to germ-free mice to investigate the following biochemical parameters in large intestinal samples: inactivation of tryptic activity, degradation of β -aspartylglycine and of mucin, conversion of bilirubin to urobilinogen and β -glucuronidase activity.

Results: *Lactobacillus reuteri* 2010, *L. rhamnosus* strain 271, *L. rhamnosus* ATCC7469 and *L. rhamnosus* GG ATCC53103 expressed β -glucuronidase activity. The other parameters tested were not influenced by any of the probiotics.

Conclusions: Bacterial β -glucuronidases have an important intestinal function. As many microbial–host and microbe–diet interactions occur in the gastrointestinal tract of mammals, microbial-related functions should be studied in greater detail in probiotics.

Keywords: *Germ-free, germ-free animal characteristic, β -glucuronidases, gnotobiotic mice, microflora-associated characteristic, probiotics.*

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Introduction

Several bacterial strains, mainly those belonging to the genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium*, are currently used as probiotics, defined as “live microbial feed supplements which beneficially affect the host, by improving its intestinal microbial balance” (1).

Over the years, many health claims have been related to intake of probiotics (for review see Refs 2–4). However, the mechanisms of action in the gastrointestinal tract are not well known and relatively little is known about their biochemical performance. Comparisons of conventionally raised (CV) mammals with their germ-free (GF) counterparts have revealed a series of anatomical, biochemical, immunological and physiological phenotypes collectively known as microflora-as-

sociated characteristics (MACs). In the absence of the functionally active micro-organisms, the phenotype observed is defined as germ-free animal characteristic (GAC) (5). The microflora exerts an influence on faecal tryptic activity (FTA), β -aspartylglycine, intestinal mucin and bilirubin metabolism, among other biochemical parameters.

FTA represents the net sum of several interactions involving the secretion of trypsinogen and trypsin inactivators from the pancreas, activation of trypsinogen to trypsin, and the intestinal presence of microbial- and diet-derived compounds that may inactivate trypsin. GF rodents always excrete high levels of FTA, whereas CV rodents excrete little or no FTA (6). Inactivation of FTA was followed in this investigation to study pancreatic/intestinal flora interactions.

β -Aspartylglycine is a member of a group of β -carboxyl dipeptides in the intestinal tract, formed from dietary proteins by host-derived proteolytic enzymes. The β -carboxyl bonds are assumed to be cleaved only by proteases derived from microbes. β -Aspartylglycine was initially detected in caecal contents from GF mice, whereas it was not present in the CV controls. Further studies showed that association of GF mice with an increasing number of anaerobic intestinal bacterial strains leads to a gradual reduction of β -aspartylglycine in faeces. β -Aspartylglycine has been suggested as an indicator of “colonization resistance” (7). Therefore, the presence of β -aspartylglycine in the faeces of humans or CV mammals indicates that the normal intestinal microbial ecosystem is markedly altered.

The gastrointestinal mucin, produced by goblet cells and glandular mucous cells, comprises a polypeptide core with oligosaccharide side-chains linked by *o*-glycosidic bonds. Mucin plays many physiological and pathophysiological roles in the intestine of mammals (8). Mucin is degraded in CV animals, while it is undegraded in GF animals. Carlstedt-Duke et al. (9) isolated a *Peptostreptococcus* strain able to obliterate all gel electrophoretic mucin bands completely, both *in vitro* and *in vivo*. Degradation of mucin was followed to study an intestinal function of major importance in preserving the integrity of the intestinal mucosa.

Bilirubin is derived from the catabolism of haemoglobin and some haem-containing compounds. In the liver, bilirubin is conjugated to glucuronate and the conjugates are secreted into the bile. Following secretion into the intestine, the bilirubin glucuronides undergo deconjugation and transformation by microbial enzymes to a series of urobilinogens and related products, collectively termed urobilinogen. GF animals do not transform bilirubin to urobilinogen, whereas CV animals do (10). Until recently only one bacterium had been implicated in that transformation (10, 11).

The deconjugation of bilirubin by microbial β -glucuronidases represents another MAC. Many xenobiotics are eliminated from the body by conjugation with glucuronic acid, forming glucuronides. Deconjugation of glucuronides in the intestine is performed by β -glucuronidases. Some intestinal β -glucuronidases may be derived from endogenous sources, but most are of microbial origin (12). GF animals show much lower levels of β -glucuronidase activity in their faeces than do CV animals.

Conversion of bilirubin to urobilinogen and β -glucuronidase activity were followed to investigate hepatic/intestinal flora interactions.

The aim of this study was to investigate the influence of some probiotic strains on the MACs described above, *in vitro* and *in vivo*, in monoassociated mice. Sixteen probiotic strains, most of them used commercially in Europe, were analysed.

Material and methods

Bacterial strains

The strains investigated in this study are listed in Table 1. Most of them were either purchased from international collections or kindly provided as free gifts from various sources (13). Two of the strains, *Clostridium ramosum* G62 and *Escherichia coli* X7, were used as positive controls for conversion of bilirubin to urobilinogen and β -glucuronidase activity, respectively.

Before the *in vitro* and the *in vivo* inoculations, aliquots of each bacterium were cultured in their respective growth media (Table 1) and incubated anaerobically at 37°C for 72 h. Then, for the *in vivo* inoculations, aliquots of 10 ml were dispensed into ampoules, which were sealed and sterilized on the outside with chromsulfuric acid, before being transferred to the GF isolator.

Animals

Eighty GF and nine CV mice, of the strain NMRI-KI, around 75 days old of both sexes, were used. From the total GF animals, 71 were allotted to 18 groups comprising four or five mice each. Sixteen groups were monoassociated with the respective probiotic and one group with *C. ramosum* G62. The remaining nine GF mice and the CV animals were used as controls.

The GF mice were reared in lightweight stainless-steel isolators and the CV mice in an ordinary animal room with artificial light between 06:00 and 18:00, temperature $24 \pm 2^\circ\text{C}$ and humidity $55 \pm 10\%$. All animals were fed an autoclaved rodent diet (R36 Lactamin, Sweden) and had free access to water. The study was approved by the local ethics committee for animal research, Sweden.

Monoassociation procedure

Each mouse group was transferred into a small stainless-steel rearing isolator (SRI) together with the ampoule containing the bacterium. The ampoule was broken inside the SRI and the bacterial

Table 1. Bacterial strains tested *in vitro* and/or *in vivo*, as monoassociates in gnotobiotic mice, and their corresponding β -glucuronidase activity *in vitro*

Bacterial strain ^a	Obtained from	β -Glucuronidase <i>in vitro</i> ^b		
		4 h	24 h	48 h
No bacterium (control)		N	N	N
<i>Escherichia coli</i> X7 (control)	MME ^c stock	+++	+++	+++
<i>Bifidobacterium bifidum</i> B11	Tine, Norway	N	N	N
<i>Bifidobacterium bifidum</i> B12	Ch. Hansen, Denmark	N	N	N
<i>Lactobacillus acidophilus</i> La5	Ch. Hansen, Denmark	N	N	N
<i>Lactobacillus acidophilus</i> ATCC4356	Arla, Sweden	N	N	N
<i>Lactobacillus casei</i> Shirota YIT9018		N	N	N
<i>L. delbrückii</i> subsp. <i>bulgaricus</i> DSM20081	Ch. Hansen, Denmark	N	N	N
<i>Lactobacillus fermentum</i> ATCC14931		N	N	N
<i>Lactobacillus plantarum</i> 299	Probi, Sweden	N	N	N
<i>Lactobacillus plantarum</i> 299v	Probi, Sweden	N	N	N
<i>Lactobacillus reuteri</i> strain 2010	BioGaia	+	++	++
<i>Lactobacillus rhamnosus</i> strain 271 ^c	Probi, Sweden	N	N	+
<i>Lactobacillus rhamnosus</i> ATCC7469		N	+	++
<i>Lactobacillus rhamnosus</i> GG ATCC53103	Valio, Finland	N	+	++
<i>Streptococcus thermophilus</i> ATCC19258	Ch. Hansen, Denmark	N	N	N
<i>Streptococcus thermophilus</i> B16	Ch. Hansen, Denmark	N	N	N
<i>Enterococcus faecium</i>	Gaio, Denmark	N	N	N
<i>Clostridium ramosum</i> G62 (control)	MME ^d stock	NT	NT	NT

^a *E. coli* X7, *S. thermophilus* ATCC19258 and *C. ramosum* G62 were grown in Glucose (Difco, USA), Todd Hewitt (Difco, USA) broth and in Reinforced Clostridial medium (Merck, Germany), respectively. The other strains were grown in MRS broth (Merck, Germany).

^b β -Glucuronidase activity was assayed qualitatively by the p-nitrophenyl method (PGUA tablets). Results after 4, 24 and 48 h incubation are given as N: no enzyme activity (colourless); +: weak positive reaction; ++: clear positive reaction; +++: strong positive reaction (strong yellow colour); NT: not tested.

^c The strain was labelled as *L. plantarum* strain 271 on receipt.

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suspension was spread on the bedding material and fur of the mice. The animals remained in the SRI for 10–15 days. Then, they were taken out and killed by cervical dislocation. Establishment and number of bacteria were ensured by taking samples from the caecum into appropriate broth media and spreading on to agar plates, followed by anaerobic incubation at 37°C. Growth was inspected for at least 72 h. The total caecum and colon content from each mouse was stored in a closed vial at –20°C for biochemical analyses.

Biochemical analyses

Except for differences in preparing the aliquots to be tested, the methods were performed similarly in the *in vitro* and in the *in vivo* part of this investigation.

In vitro

Medium. A medium to analyse both FTA and β -aspartylglycine was prepared. GF AGUS rat faeces plus sterile Todd Hewitt medium (1:5) were thoroughly mixed and filtered. This proce-

dures was carried out in a GF isolator. The mixture was then dispensed in 10 ml tubes and brought out of the isolator. Thereafter, these media were inoculated with aliquots of 0.5 ml from each probiotic culture. These preparations, together with two inoculated controls, were incubated at 37°C for 24 h. In addition, two tubes containing the prepared medium were stored at –20°C and used as uninoculated and unincubated controls. The samples were assayed as follows.

Faecal tryptic activity. The assay was performed at room temperature (20°C). An aliquot of 0.1 ml from the inoculated medium (above) was added to 2.9 ml of Tris buffer (pH 8.2) containing 4.4 g of calcium chloride per litre. The reaction was initiated by adding 0.6 ml of 0.003 M *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) and terminated after 10 min by adding 0.6 ml of 5 M acetic acid. Bovine pancreas trypsin type III diluted in 2 mM hydrochloric acid was used for the construction of a

standard curve. All samples and standards were analysed in parallel with blanks at 405 nm on a spectrophotometer (Hitachi 150-20, Tokyo, Japan). Results are given as mg FTA kg⁻¹ faeces.

Degradation of β -aspartylglycine. Aliquots of 30 μ l from the inoculated media (above) and from a commercial standard dilution of β -aspartylglycine (2 mg ml⁻¹) were applied on Whatmann 3MM chromatography paper. Then, high-voltage paper electrophoresis was performed in the buffer pyridine:acetic acid:water (1:10:89), pH 3.5, giving a current of 150 mA and 100 W for 1 h. After electrophoresis, the paper was dried at 80°C and sprayed with ninhydrin. Heating afterwards at 150°C for 10 min gives a clear blue spot for β -aspartylglycine. The intensity of the colour of the samples was compared visually with that of the standard and reported as present or absent.

β -Glucuronidase activity. The activity of β -glucuronidase was assayed qualitatively by the *p*-nitrophenyl method, using diagnostic PGUA tablets (Rosco Diagnostica, Denmark). A dense bacterial suspension of each probiotic, approx. McFarland No. 4, was prepared in 0.25 ml of phosphate buffer at pH 6.5 and at pH 8.0. A similar suspension of *E. coli* X7 and a solution of phosphate buffer without any bacterium were used. A tablet of substrate, *p*-nitrophenyl- β -D-glucuronide, was added to each tube, followed by incubation at 37°C. β -Glucuronidase activity was recorded by the degree of *p*-nitrophenol coloration from negative (colourless) to + + + (strong yellow).

In vivo

The total caecum and colon content from the monoassociated mice, above, was thawed and homogenized. An aliquot of 0.7 g was taken for determination of urobilinogen. A second aliquot of 0.5–1 g was diluted with two volumes of saline, homogenized and placed at 4°C for 2 h. Thereafter, the homogenate was centrifuged at 4500 rpm, 4°C, for 30 min. The supernatant was separately used for the determination of inactivation of FTA, and degradation of β -aspartylglycine and of mucin.

Faecal tryptic activity. Aliquots of 0.1 ml from the supernatants were assayed as for the *in vitro* test. Results are expressed as mg of FTA kg⁻¹ large intestinal content.

Degradation of β -aspartylglycine. Aliquots of 30 μ l from the supernatants were assayed as for the *in vitro* test. The results are given as presence or absence. β -Aspartylglycine is present in faeces from GF animals, but absent in CV animals.

Degradation of mucin. Aliquots of 5 μ l from the supernatants were assayed by agar gel electrophoresis on glass plates, using 1% agarose in sodium barbiturate buffer, pH 8.6. The electrophoretic separation was run for 30 min with a potential gradient of 20 V cm⁻¹, giving a current of 160 mA. The plate was immediately transferred to a fixing solution containing ethanol:chloroform:acetic acid (6:3:1). After 30 min, it was dried in a warm-air stream. The staining procedures were Toluidine blue (TB) 0.05% w/v in acetate buffer, pH 5.0, periodic acid–Schiff (PAS) and Coomassie brilliant blue R (CB) 0.05% w/v in 12% trichloroacetic acid. Faecal samples extracts from GF and from CV rats were run on each gel as controls. The samples were compared with the controls and evaluated as follows. An electrophoretic pattern similar to that found in the CV control (no bands = 0) is presented as degraded; an electrophoretic pattern similar to that found in GF control (presence of bands = 3) is presented as undegraded; and electrophoretic patterns with some bands are scored 1 and 2.

Conversion of bilirubin to urobilinogen. Urobilinogen was assayed by applying the Ehrlich's aldehyde reaction with *p*-dimethylaminobenzaldehyde directly to an aqueous homogenate of the large intestinal content, after reduction of urobilin to urobilinogen with ferrous sulfate. The procedure was as follows. An aliquot of 0.7 g from the large intestinal content was homogenized with 4.2 ml of distilled water. Then, 4.7 ml of 1.3 M ferrous sulfate was added and mixed. Thereafter, 4.7 ml of 2.5 M sodium hydroxide were added and shaken vigorously. The mixture was left in darkness at room temperature. After 2 h, it was filtered and centrifuged at 4500 rpm, 4°C, for 20 min. Two aliquots of 1 ml from the supernatant were run as test and blank, and read against distilled water on a spectrophotometer (Hitachi 150-20). Phenolsulfonphthalein was used as an artificial standard for "urobilinogen-aldehyde". The values are converted and given as mmol urobilinogen kg⁻¹ large intestinal content (wet weight).

Results

In vitro

As is evident from Table 2, all of the incubations of probiotic strains for inactivation of FTA and degradation of β -aspartylglycine gave similar results as uninoculated but incubated controls.

Lactobacillus reuteri 2010, *L. rhamnosus* strain 271, *L. rhamnosus* ATCC7469 and *L. rhamnosus* GG ATCC53103 expressed β -glucuronidase activity (Table 1). They showed either a weak positive or a clear positive reaction as the incubation increased. A yellowish colour was observed in the initial suspension of *L. reuteri* 2010. However, the colour also became stronger as the incubation increased. To confirm its enzyme activity, *L. reuteri* 2010 was retested using the same amount of substrate (*p*-nitrophenyl- β -D-glucuronide, Sigma) contained in the diagnostic tablet previously used. The absorbance of the sample was read at 400 nm on a spectrophotometer (Hitachi 150-20) and compared with that of *E. coli* X7 strain. A slight positive reaction was observed. No differences were found with respect to pH 6.5 and pH 8.0 (data not given).

In vivo

All animals remained healthy throughout the study. All of the bacterial strains were established as monoassociates in the intestine of the mice, and all of them except for *L. delbrückii* subsp. *bulgari-*

cus were counted in numbers higher than 10^7 ; the number of *L. delbrückii* subsp. *bulgaricus* was 10^3 . In none of the monoassociated animals was contamination observed.

The median value of FTA in all groups of mice monoassociated with a probiotic was similar to that of the GF mice (Table 2). β -Aspartylglycine was present in all samples from monoassociated mice (Table 2).

In all monoassociated animals the mucin patterns were qualitatively and quantitatively similar to the patterns found in the GF control.

There was no transformation of bilirubin to urobilinogen in any of the mice monoassociated with a probiotic.

Discussion

In the present study, none of the probiotic bacterial strains was able to inactivate FTA or to degrade β -aspartylglycine *in vitro* or *in vivo*. As stated before, these capabilities seem to be a rare event among intestinal micro-organisms (6, 14, 15).

All probiotic lactobacilli and bifidobacteria have saccharolytic action. Therefore, it might be reasonable to assume that they may participate in the breakdown of mucin *in vivo*. However, as stated in the Results section, the band patterns in monoassociated and GF mice were similar. In the present study, three different staining methods were used for the visualization of bands. TB is commonly used for the expression of glycoproteins, PAS is a staining method for neutral glycoproteins, whereas CB is a staining method for proteins. The methods do not allow the conclusion to be drawn that the probiotic strains have not removed certain sugar residues from major molecules. However, the parallelism in the stainings in GF and monoassociated animals indicates that a great part of the mucin is electrophoretically intact.

None of the probiotics showed an ability to convert bilirubin to urobilinogen *in vivo*. The microbial species that are performing this reaction *in vivo*, to the authors' knowledge, are virtually unknown.

Bilirubin as well as some endogenous and many foreign compounds (xenobiotics) are conjugated with glucuronic acid in the liver, to facilitate their elimination. The glucuronides formed are then secreted into the intestine and may undergo hydrolysis. In general, the free compounds are more easily absorbed from the intestinal tract than the conjugated ones. As stressed by Kroemer and Klotz (16), glucuronidation can be viewed as a

Table 2. Biochemical microflora-associated characteristics (MACs) *in vitro* and *in vivo*, in germ-free (GF) and conventional (CV) mice and in ex-GF mice monoassociated with a probiotic

Group ^a	FTA ^b		β -Aspartylglycine ^c	
	In vitro	In vivo	In vitro	In vivo
Control ^d	45	—	Present	—
Probiotic ^e (4–5)	48 (40–65)	1007 (695–1297)	Present	Present
GF mice (9)	—	1091 (696–1398)	—	Present
CV mice (9)	—	18 (0–27)	—	Absent

^a Figures in parentheses are number of animals used.

^b Figures are medians (maximum and minimum) of milligrams of faecal tryptic activity (FTA) either per litre of medium (*in vitro*) or per kilogram of large intestinal content (*in vivo*).

^c An electrophoretic pattern showing bands similar to that of the positive control and one showing no bands are given as present and as absent, respectively.

^d GF AGUS rat faeces plus sterile Todd Hewitt medium.

^e The probiotic strains listed in Table 1, except for *L. casei* and *L. reuteri*, were investigated.

first-line detoxification mechanism. The importance of knowing the presence of β -glucuronidases in probiotics, can be exemplified as follows. Very recently, several conjugated and unconjugated IX α and IX β isomers of bilirubin were found to be present, in various levels, in meconium and the stools from newborns (17). Depending on the type of isomers and degree of conjugation, they might be excreted either by the mother (before delivery) or stored in the meconium (until delivery). At birth, establishment of an intestinal flora capable of deconjugating and/or further transforming the bilirubin derivatives may influence the enterohepatic circulation and the faecal excretion of these compounds. Therefore, when giving a β -glucuronidase-producing probiotic strain to a newborn child, this function has to be evaluated. It is well known that bilirubin and some of its derivatives may cause damage to the newborn child.

In the present investigation, β -glucuronidase activity was expressed in four out of 16 probiotic strains, when tested *in vitro*. These results are in agreement with previous investigations showing deconjugating activity in some lactobacilli (18): "With the exception of a *L. reuteri* strain which was weakly positive, all other strains that produced β -glucuronidase belonged to the *L. rhamnosus* species. Whether β -glucuronidase production is a common trait in this species or not, remains to be determined". Previous studies on β -glucuronidase enzyme, comparing its activity *in vitro*, and *in vivo* in monoassociated animals, have clearly shown that the activity is expressed *in vivo* when screened to be present *in vitro* (19, 20). Therefore, it seems reasonable to assume that the strains used in this study will express *in vivo* activity as well.

Alteration in levels of intestinal microbial β -glucuronidases may, in addition to bilirubin, influence the metabolism of other glucuronides, such as several procarcinogens and other xenobiotics (21).

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