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Probiotics in gnotobiotic mice: Short-chain fatty acids production *in vitro* and *in vivo*

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Introduction

Short-chain fatty acids (SCFAs) are both intermediate and end products from microbial metabolism in the gastrointestinal (GI) tract in all conventional (CV) mammalian species. The main sites of SCFAs production in man and monogastric animals are the caecum and ascending colon *(Cummings, 1981)*. They are quantitatively the major anions in the large intestine and feces of healthy humans *(Høverstad, 1989)* and of most mammalian species *(Fleming & Arce, 1986)*. SCFAs have been associated with several aspects of health and disease in ruminants and non-ruminants *(Binder et al., 1994)*. Complex carbohydrates from diet together with endogenous factors such as mucin, are the major sources for the microbial GI production of the main SCFAs. Other intermediate and anabolic/catabolic end products derived from microbial fermentation include lactic, succinic and formic acids together with hydrogen and carbon dioxide *(Cummings, 1981)*.

Many bacteria are involved in the fermentation process in the GI tract of mammals. However, the relative importance of indigenous and transient bacteria in this fermentation is not well known.

Probiotics are widely used in farm animals and humans with the aim of stabilizing the intestinal microflora. The so-called probiotics are often defined as "live microbial feed supplements which beneficially affect the host, by improving its intestinal microbial balance" *(Fuller, 1989)*. In humans, a long series of strains, mainly lactobacilli and bifidobacteria, are used in dairy commercial products. Especially during the last few years, numerous reports have appeared describing "positive" influences upon intestinal conditions in animals and man *(McFarland, 2000)*. However, the more basic mechanisms behind the possible influences on their host, are not satisfactorily elucidated.

The fermentation processes represent possible symbiotic host-microbe cross-talks. Many of these interactions have been extensively investigated by comparative studies in germfree (GF) and CV animals *(Norin & Midtvedt, 2000)*. The findings in these two groups have made it possible to define the Microflora-Associated Characteristic (MAC)/ Germfree Animal Characteristic (GAC) concept *(Midtvedt et al., 1985)*. Any anatomical structure, biochemical, immunological or physiological function in a mammal which is influenced by the microflora is described as a MAC, while the similar structure or function in the absence of the specific microorganism(s), is described as a GAC. In this context, production of SCFAs represents a MAC.

Based upon the assumption that SCFAs are factors of great importance in host-microbe cross-talks, the present investigation was undertaken in order to screen the capability of some probiotics to produce SCFAs, *in vitro* and *in vivo*, with the use of GF animals. Some of the bacterial strains commonly used in dairy industry, were selected.

Material and Methods

Animals

A total of 75 inbred NMRI-KI mice of both sexes and an average age of 80 days were allotted to

eighteen groups of 4-5 mice. Sixteen of the groups, all of them GF mice, were monoassociated with a chosen probiotic strain. The other two groups comprising 5 and 4 mice, were the GF and CV controls, respectively. The GF animals were reared in stainless steel isolators *(Gustafsson, 1959)* and the CV animals in an ordinary animal room, with artificial light between 6 a.m. to 6 p.m., temperature 24±2°C and humidity 55±10%. All the mice were fed on an autoclaved rodent diet, R36 (Lactamin, Sweden) and had free access to water. The study was approved by the local Ethical Committee for Animal Research.

Bacteria

Sixteen different probiotic bacterial strains were tested. They are presented in Table 1. The strains were purchased from international collections, received as donations or were part of the stock collection at the Laboratory of Medical Microbial Ecology. More details about the strains can be found in a recent paper *(Cardona et al, 2000)*. Thirteen of the strains were investigated *in vitro*, and all of them were investigated *in vivo*. The strains were stored in appropriate basal media.

Media

Before both the *in vitro* and the *in vivo* inoculations, all the strains except *S thermophilus* ATCC19258 were grown in de Man, Rogosa and Sharpe (MRS) broth. *S thermophilus* ATCC19258 was grown in Todd Hewitt (TH) broth.

In vitro procedure

Aliquots of 1 ml of a fresh culture of each bacterium were inoculated into their respective growth media (Table 1) and incubated anaerobically at 37°C for 72 h. Two aliquots of uninoculated MRS and TH were used as controls. All the samples were vacuum distilled prior to gas-liquid chromatography (GLC).

In vivo procedure

Aliquots of 10 ml of a fresh culture of each bacterium grown in similar conditions as in the *in vitro* investigation, were dispensed into ampoules, which were sealed, sterilized on the outside with chromsulfuric acid and taken into the respective isolator.

Each mouse group was transferred to a small stainless steel rearing isolator (SRI) containing the ampoule with the probiotic strain. The ampoule was broken inside the isolator and the bacterial suspension was spread on the bedding material and fur of the mice. The animals remained within the SRI for 11-15 days. Thereafter, they were taken out, anesthetized and killed by cervical dislocation. For testing bacterial establishment, two samples of 1 µl from each caecum were cultured in the respective medium (MRS or TH) broth and agar. From the inoculated broth, additional aliquots of 1µl and of 10 µl were plated onto the same respective agar media. All the media were incubated anaerobically at 37°C for 72 h. Bacterial counts were made on the agar plates. The total large intestinal content from each mouse was stored in closed vials at -20°C until analysis.

SCFAs analysis

All biochemical analyses were run within four weeks after sampling. The large intestinal samples were thawed, mixed and aliquots of 0.5-0.6 g were delivered into vials.

At analysis, all the samples and sterile aliquots (0.5 g) of mashed rodents food (Lactamin R36, Sweden) were acidified with 0.5 ml of H_2SO_4 (0.5 mmol/l). Before homogenization, 0.5 ml and 2 ml of a solution of distilled water containing 3 mmol/l of 2-ethylbutyric acid (internal standard), were added to the *in vitro* and the *in vivo* samples (including food), respectively. All the homogenates were vacuum distilled for 20 min as described by Zijlstra et al. *(Zijlstra et al., 1977)* with modifications by Høverstad et al. *(Høverstad et al., 1984)*. The amount of SCFAs was measured in the distillates by GLC (Perkin Elmer Autosystem XL) on a packed column of 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb at 120°C and with nitrogen as carrier gas. The chromatogram peaks were analyzed using a Turbochrom autoanalyzer system (Perkin-Elmer, USA). The total and individual SCFA concentrations were given in mmol/l culture medium, mmol/kg food and mmol/kg of large intestinal content (wet weight). A concentration of 0.1 mmol in at least 50% of the animals tested, in

Table 1. Total amount of short-chain fatty acids (SCFAs) in sterile media and in medium monoinoculated with a respective probiotic bacterial strain

Bacterial strain	Abbreviation	Test media*	SCFAs†	
			Control	Monoculture
No bacterium		MRS	16.16	
No bacterium		TH	7.67	
Bifidobacterium				
B bifidum B11	BB11	NT		NT
B bifidum B12	BB12	MRS		12.76
Lactobacillus				
L acidophilus La5	La5	MRS		9.27
L acidophilus ATCC4356	La4356	MRS		10.72
L casei strain Shirota	LC	MRS		14.33
L delbrückii subsp *bulgaricus* DSM20081	LDB	NT		NT
L fermentum ATCC14931	LF	NT		NT
L plantarum 271	Lp271	MRS		9.94
L plantarum 299	Lp299	MRS		7.24
L plantarum 299v	Lp299v	MRS		9.39
L reuteri	LR	MRS		17.72
L rhamnosus ATCC7469	Lr7469	MRS		3.92
L rhamnosus GG	LGG	MRS		14.10
Streptococcus & Enterococcus				
S thermophilus ATCC19258	St19258	TH		4.66
S thermophilus B16	StB16	MRS		7.91
E faecium	EF	MRS		8.25

*MRS, de Man Rogosa and Sharpe, TH, Todd Hewitt;
† Concentrations are expressed as mmol/l of medium;
NT not tested.

one aliquot of the food and in one aliquot of the control media investigated, was taken as a breakpoint.

Statistical analysis

Statistical evaluation of the differences between the tested groups and the control groups was performed in the log-transformed data using one way ANOVA and Dunnet's test. The results are presented with 95% confidence intervals for the mean values. STATISTICA, '99 Edition computer program (StatSoft, USA) was used to run the whole analysis.

Results

In vitro

All bacterial strains were easily cultured in the media chosen. The total amount of SCFAs in the control as well as in the inoculated tubes are presented in Table 1. As is evident from the table, none of the strains was able to increase the total amount of SCFAs. An interesting observation was that *L rhamnosus* ATCC7469 but not *L rhamnosus* GG seemed to be able to utilize some of the SCFAs present in the culture medium. However, any possible mechanism(s) for this discrepancy was not further investigated.

The total amount of SCFAs as well as the SCFAs profile i.e., individual SCFAs, found in the culture media are presented in Table 2. The SCFA profiles of the monocultures were similar to the profile in the corresponding medium (data not shown).

In vivo

All the animals remained healthy throughout the study. All the strains were established in the large intestine of the mice, and all of them except *L delbrückii* subsp *bulgaricus* DSM20081, were counted in numbers higher than 10^7. The number of *L delbrückii* subsp *bulgaricus* was 10^3.

From the data given in table 2, it is obvious that the diet contained a substantial amount of SCFAs. It is also clear that some, but not all of the individual acids could be detected in the samples from GF animals.

Fig. 1 shows the log value of total SCFAs in large intestinal content from GF, from CV and from the monoassociated animals. The lowest and highest concentrations of SCFAs correspond to the GF and CV group, respectively. Among the monoassociated groups, those inoculated with the strains: *B bifidum* B11, *L acidophilus* La5, *L casei* strain Shirota, *L plantarum* 299, *L reuteri*, *L rhamnosus* ATCC7469, *L rhamnosus* GG, *S thermophilus* ATCC19258 and *S thermophilus* B16, showed significant higher total SCFAs than the GF group. However, the CV group contained significantly more total SCFAs than all the other groups.

Data of the total amount and profile of SCFAs in monoassociated animals are presented in Table 3. The highest values were found in the animals monoassociated with *S thermophilus* ATCC19258, followed by *L reuteri* and *L plantarum* 299. As it is obvious from the table, among the individual acids, acetic is the dominant one. Propionic acid was detected only and in a very small amount in the group monoassociated with *L plantarum* 299.

The groups monoassociated with *L acidophilus* ATCC4356, *L casei* strain Shirota, *L reuteri*, *L rhamnosus* ATCC7469 and *L rhamnosus* GG, showed a tendency to increase n-butyric concentrations (>0.5 mmol). However, there was no statistically significant differences when compared to the GF group.

Discussion

SCFAs are mainly generated by bacterial fermentation *(Midtvedt, 1994)*. We found however, appreciable amount of total SCFAs in the sterile media used for culturing the probiotic strains tested. The individual SCFAs detected were mostly acetic and propionic acids. In previous studies, small amount of SCFAs have been reported in sterile media (*Høverstad et al., 1985; Høverstad and Midtvedt, 1987*). After inoculation of each bacterium, the fermentation patterns were similar to those of the respective media or showed even lower values. A possible explanation for that is that the bacteria added might have utilized the SCFAs already present in the media, thus decreasing the amount of SCFAs.

Whether and to what extent, the probiotic bacteria have produced other metabolites such as lactic acid, etc., were not investigated in the present study. Nevertheless, production of lactic acid by probiotic bacteria, warrants to be measured in the future. It is well established that fermentation profiles may vary according to the growth media used *(Turton et al., 1983)*. Beside chemical composition of the media, changes in pH, incubation time, etc., may influence upon the fermentation pattern. However, we found that under our laboratory conditions, the probiotic strains tested did not yield any substantial amount of SCFAs.

The GI tract of mammals, including man, harbors a high number of anaerobic bacteria, which obtain their energy through a fermentation process yielding SCFAs and gases. The main SCFAs are acetic, propionic and butyric acid. Previous investigations have shown measurable amounts of acetic acid in colon contents of GF mice *(Lee and Gemmell, 1972; Maier et al., 1972; Roach and Tannock, 1979; Høverstad et al., 1985; Høverstad and Midtvedt, 1986)*. In a previous study in mice, Høverstad and Midtvedt *(Høverstad and Midtvedt, 1986)*, reported differences in the concentration of SCFAs according to the diet given to the animals. Thus, it is reasonable to assume that all higher SCFAs and probably most of the acetic acid in our GF animals derive from the diet. Additionally, it

Table 2. Total amount and profile of short-chain fatty acids (SCFAs)* in sterile media†, in sterile diet‡ and in large intestinal content from germfree (GF) and from conventional (CV) mice.

Group §	Total	Acetic	Propionic	i-Butyric	n-Butyric	i-Valeric	Valeric	i-Capronic	Capronic
MRS (2)	16.16	14.94	1.06	ND	ND	ND	ND	ND	ND
	(15.2-17.1)	(14.1-15.7)	(1.0-1.2)	ND	ND	ND	ND	ND	ND
TH (2)	7.67	7.49	0.08	ND	ND	ND	ND	ND	ND
	(7.4-8.0)	(7.3-7.7)	(0-0.2)	ND	ND	ND	ND	ND	ND
Diet (2)	18.22	16.82	0.41	0.42	0.13	0.18	0.10	0.06	0.11
	(18.1-18.3)	(16.6-17.0)	(0.2-0.6)	(0.1-0.8)	(0-0.3)	(0-0.4)	(0-0.2)	(0-0.1)	(0-0.1)
GF mice (5)	16.43	15.41	ND	ND	0.22	ND	ND	ND	ND
	(15.4-18.5)	(14.9-18.3)	ND	ND	(0-2.4)	ND	ND	ND	ND
CV mice (4)	112.55	52.76	19.94	1.36	23.92	4.21	4.27	0.69	0.22
	(90.6-131.3)	(49.0-72.6)	(13.7-24.2)	(0.8-2.0)	(17.2-32.7)	(3.2-7.2)	(1.8-6.0)	(0.3-1.1)	(0.2-0.3)

*Concentrations are medians given in mmol/l medium, mmol/kg food and mmol/kg large intestinal content (wet weight).
A value of 0.1 mmol in at least 50% of the mice and in 1 of the food or the media aliquots, was taken as a breakpoint;
† MRS de Man, Rogosa and Sharpe; TH Todd Hewitt; ‡ R36 Lactamin; § figures in brackets are number of samples per group; figures in brackets below the medians are maximum and minimum; ND not detected or detected in lower values than the breakpoint established.

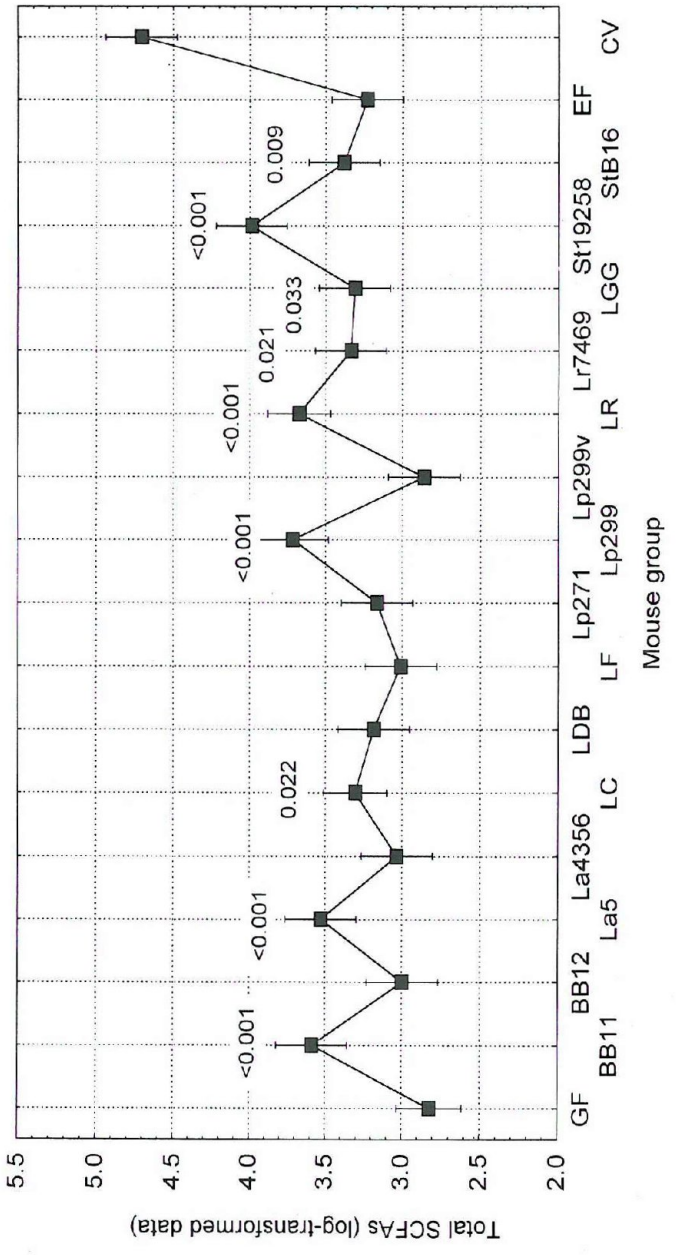

Fig. 1.
Total short-chain fatty acids (SCFAs) -log-transformed data- in large intestinal content from germfree (GF), ex-GF mice monoassociated with a probiotic strain and from conventional (CV) NMRI mice. Groups of 4-5 mice were tested. Long names for each probiotic are presented in table 1. Points are least square means; bars are 95% confidence limits for the mean values. Dunnet's test was performed to compare each monoassociated group with the GF control. The p-values indicate level of significance for statistically significant comparisons.

Table 3. Total amount and profile of short-chain fatty acids (SCFAs) * in large intestinal content from monoassociated mice.

Bacterium†	Total	Acetic	Propionic	i-Butyric	n-Butyric	i-Valeric	Valeric	i-Capronic	Capronic
BB11 (4)	33.30‡ (27.3-57.1)	32.51‡ (27.2-56.7)	ND	ND	0.29 (0-0.8)	ND	ND	ND	ND
BB12 (4)	18.77 (16.9-27.4)	18.49 (16.6-27.0)	ND	ND	0.20 (0.1-0.4)	ND	ND	ND	ND
La5 (4)	33.19‡ (30.1-41.0)	32.95‡ (29.9-40.8)	ND	ND	0.19 (0.17-0.22)	ND	ND	ND	ND
La4356 (4)	20.72 (14.8-29.7)	18.97 (14.8-21.6)	ND	ND	0.98 (0-1.2)	ND	ND	ND	ND
LC (5)	28.46‡ (19.2-36.1)	27.68‡ (18.7-32.3)	ND	ND	0.62 (0.4-1.8)	0.13 (0-2.0)	ND	ND	ND
LDB (4)	23.83 (17.9-35.6)	23.68 (17.6-35.4)	ND	ND	0.16 (0.1-0.2)	ND	ND	ND	ND
LF (4)	20.61 (18.0-22.3)	19.93 (17.0-22.1)	ND	ND	0.22 (0.1-0.4)	0.10 (0-0.5)	ND	0.12 (0-0.3)	ND
Lp271 (4)	24.53 (15.9-33.9)	24.19 (15.8-32.9)	ND	ND	0.14 (0.1-0.3)	0.07 (0-0.1)	ND	ND	0.09 (0.1-0.3)
Lp299 (4)	41.06‡ (34.5-49.1)	40.38‡ (34.2-48.8)	0.19 (0-0.2)	ND	0.15 (0-0.6)	ND	ND	ND	ND
Lp299v (4)	17.56 (15.4-19.6)	17.52 (15.4-18.7)	ND	ND	ND	ND	ND	ND	ND
LR (5)	41.08‡ (31.9-48.2)	36.98‡ (30.6-45.6)	ND	0.12 (0-0.4)	1.24 (0.9-1.3)	0.60 (0-1.9)	0.19 (0-1.4)	0.22 (0-0.4)	0.16 (0.1-0.3)
Lr7469 (4)	28.17‡ (23.8-33.5)	26.50‡ (19.7-30.7)	ND	0.15 (0.1-1.2)	0.64 (0.4-1.4)	0.62 (0.4-1.2)	ND	0.22 (0.1-0.3)	0.10 (0.08-0.1)
LGG (4)	27.59‡ (25.7-29.0)	24.83‡ (24.3-28.6)	ND	0.10 (0-0.2)	0.58 (0.2-0.8)	0.77 (0-1.4)	ND	0.28 (0.2-0.4)	0.10 (0.1-0.2)
St19258 (4)	59.16‡ (34.6-73.7)	57.50‡ (33.8-72.0)	ND	ND	0.44 (0.3-1.4)	0.11 (0.1-0.3)	ND	0.14 (0-0.7)	0.17 (0.2-0.5)
StB16 (4)	30.04‡ (20.2-42.4)	29.79‡ (20.0-38.5)	ND	ND	0.23 (0.1-0.8)	ND	ND	ND	ND
FF (4)	24.87 (21.0-32.1)	23.61 (20.0-31.9)	ND	0.11 (0-0.2)	0.37 (0.1-0.6)	0.16 (0-0.2)	ND	0.23 (0-0.3)	0.11 (0.1-0.2)

*Figures are medians given in mmol/kg of large intestinal content (wet weight), taking 0.1 as a breakpoint in at least 50% of the animals tested; † long names of the strains are presented in Table 1; figures in parenthesis are number of animals tested; figures in parenthesis below each median, are maximum and minimum; ‡ statistically significant differences between the group and the germfree, performed with ANOVA and Dunnet's test; ND not detected or detected in lower values than the breakpoint established.

has to be mentioned that the analytical technique used in the present investigation was slightly different from those used in earlier investigations, which could have contributed to minor divergences from previous results.

As shown in the results, increased values of SCFAs could be demonstrated in more than half of the monoassociated group. However, none of the probiotics tested was able to induce levels of SCFAs coming into the neighborhood of those found in the CV group. Whether and to what extent, these increased levels of SCFAs represent a true *de novo* synthesis of SCFAs or alterations in absorption of dietary derived SCFAs, were not evaluated.

Nevertheless, whatever the mechanisms might be, increased levels of total as well as individual SCFAs represent important functional factors in intestinal ecosystems. Thus, it has been assumed that short-chain organic acids such as acetic acid may have an inhibitory effect on *Shigella flexneri (Maier et al., 1972)* as well as on *Salmonella typhimurium (Hudault et al., 1997)*. Interestingly, it has also been shown low concentration of acetic acid in feces of children with acute salmonellosis and shigellosis *(Siigur et al., 1996)*.

Because of the important role that n-butyrate plays in the welfare of the colonic mucosa *(Roediger, 1980; Bugaut and Bentejac, 1993; Cook and Sellin, 1998)* of mammals, one of the effects that might be expected from probiotics consumption is an increase in that specific acid. However, we found a very small increase in n-butyric acid only in a few of the monoassociated groups and not increase at all in the group associated with *L plantarum* 299v. This finding is in agreement with a previous study in humans *(Johansson et al., 1998)* where no changes in fecal n-butyric acid were reported after consumption of a probiotic preparation containing *L plantarum* 299v. In contrast to us, they found higher amount of total fecal SCFAs.

Taken together, it can be concluded that the probiotics tested had minor influence upon the SCFAs parameter. However, absence of SCFAs formation certainly does not exclude an effect when a bacterial strain is acting in concert with other bacteria in the intestinal ecosystem, as reported earlier *(Gustafsson et al., 1968; Gustafsson et al., 1998)*.

When the complex intestinal host-microbe interactions are worked out, the importance of working with different models as one host/one microbial species and one host/several microbial species, should be underlined. Gnotobiotic animals, i. e., animals with a known flora, represent a "must" in these types of studies.

Summary

Several bacterial strains are currently used as probiotics. Sixteen of them belonging to the genera: *Bifidobacterium*, *Enterococcus*, *Lactobacillus* and *Streptococcus*, were selected to test short-chain fatty acids (SCFAs) production *in vitro* and/or *in vivo*. The probiotic strains were monocultivated in specific media and/or monoassociated with NMRI-KI germfree (GF) mice. The individual and total amounts of SCFAs were measured in the media and in the large intestinal content of the ex-GF mice. All the samples were assayed by gas-liquid chromatography.

We found that commercially available media contain detectable amounts of acetic and propionic acids. When cultivated *in vitro*, none of the probiotic strains was able to increase the amounts of SCFAs present in the medium. Rather, a tendency to lowering the concentration of SCFAs following cultivation, was observed.

We also found that commercially available laboratory rodents chow contained detectable amount of all SCFAs. When the probiotics were monoinoculated to GF animals, nine out of sixteen groups of mice showed higher amount of intestinal SCFAs than in the GF control group. Acetic acid was the dominant one. In all cases, however, the values of the SCFAs were far from those found in conventional mice.

The results clearly underline the importance of working with laboratory animals with a known flora, i. e. gnotobiotic animals, when the biochemical "profile" of a probiotic is worked out.

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