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Are probiotics detectable in human feces after oral uptake by healthy volunteers?

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Sind Probiotika nach der oralen Aufnahme auch im Stuhl vorhanden?

Zusammenfassung. *Ziel:* Analyse der Anwesenheit von kommerziellen Probiotika in Stuhl nach oraler Aufnahme.

Hintergrund: Probiotika wird häufig ein günstiger Effekt auf die Gesundheit nachgesagt. Eine Bedingung für jeglichen Effekt ist dabei das Überleben von Bakterien während der gastrointestinalen Passage.

Studie: Nach einer einwöchigen oralen Aufnahme von sechs kommerziellen Probiotikapräparaten [E.coli Nissle 0.5–5 x 109 (Mutaflor®), Enterococcus faecium SF 68 7.5 x 107 (Bioflorin®), Lactobacillus acidophilus and Bifidobacterium infantis both 1 x 109 (Infloran®), Lactobacillus gasseri 108 and Bifidobacterium longum 108 (Omniflora®), Lactobacillus casei rhamnosus 109 (Antibiophilus®), und Yoghurt welches mittels Lactobacillus casei „immunitass" 1010 angereichert wurde (Actimel®)] wurde die An- bzw. Abwesenheit der ausgewiesenen Keime im Stuhl untersucht. Dabei wurden von jeder Stuhlprobe 10 Kolonien untersucht. Nach der DNA Extraktion kam die randomisierte Amplifikation polymorpher DNA zum Einsatz (RAPD). Danach wurden die RAPD Ergebnisse der Probiotikakeime direkt aus der kommerziellen Präparation mit den gewonnenen Stuhlisolaten verglichen.

Resultate: Identische RAPD Ergebnisse zu den aufgenommenen Probiotika fand man bei den Stuhlproben von 4/7 Personen nach einer Woche Mutaflor®, bei 4/6 nach Bioflorin®, bei 1/6 nach Infloran®. Nach der Einahme von Antibiophilus®, Omniflora® oder Actimel® war eine Kultur von Bakterien der selben Species in Stuhlproben nicht möglich.

Schlussfolgerung: Nach der oralen Einnahme von probiotischen E. coli und Enterokokken können die selben Erreger in Stuhlproben in 57 bzw 67% der studierten Population gefunden werden. Im Gegensatz dazu können oral aufgenommene Laktobazillen oder Bifidobakterien im Stuhl nicht gefunden werden.

Summary. *Goals:* Assessment of the presence of probiotic bacteria in feces after oral ingestion.

Background: Probiotic bacteria are said to have beneficial effects on the host. As a precondition for any effect, probiotic strains must survive passage through the gastrointestinal tract.

Study: The feces of seven volunteers were analyzed for the presence of probiotic strains after one week's oral ingestion of each of six commercially available products: *E. coli* Nissle 0.5–5 x 10^9 cells (Mutaflor®), *Enterococcus faecium* SF 68 7.5 x 10^7 cells (Bioflorin®), *Lactobacillus acidophilus* and *Bifidobacterium infantis* both 1 x 10^9 cells (Infloran®), *Lactobacillus gasseri* and *Bifidobacterium longum* both 1 x 10^8 cells (Omniflora®), *Lactobacillus casei rhamnosus* 1 x 10^9 cells (Antibiophilus®), and yoghurt enriched with *Lactobacillus casei* Immunitas 1 x 10^{10} cells (Actimel®). Ten colonies were selected from each stool sample, and DNA was extracted and typed using random amplification of polymorphic DNA (RAPD). Typing patterns of the ingested probiotics and the fecal isolates were compared.

Results: Fingerprints identical to the ingested probiotic strains were recovered from fecal samples of 4/7 volunteers after one week of Mutaflor®, from 4/6 after taking Bioflorin®, and from 1/6 after Infloran®. Cultivation of strains of the same species from fecal specimens was negative after consumption of Antibiophilus®, Omniflora® and Actimel®.

Conclusions: After oral consumption of probiotics, *E. coli* and enterococci could be detected in stool samples (57% and 67%, respectively). In contrast, with only one exception, ingested lactobacilli and bifidobacteria could not be detected in human feces.

Key words: Probiotics, intestinal colonization, random amplification of polymorphic DNA.

Introduction

At the beginning of the last century Metchnikoff suggested that the use of live bacteria in fermented milk products such as yogurt could increase longevity and improve health by detoxifying putrefactive substances

[1]. In the more recent past, interest in the potential to improve human health through modifications of the intestinal microflora has re-emerged and various commercially available dairy products claim such effects. However, it has been difficult to establish the existence of associations between specific microbes and health benefits [2].

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [3]. Postulated health advantages associated with probiotic intake are the alleviation of symptoms of lactose malabsorption and inflammatory bowel disease [4], prevention of antibiotic-associated diarrhea [5], eradication of *C. difficile* infection [6] and enhancement of intestinal immunity [7–9]. Further health effects described are suppression of recurrence of cancer, reduction of serum cholesterol concentrations and improved digestion [10]. Preliminary data support the concept that probiotics may be useful in conditions such as irritable bowel syndrome, vaginal and urinary tract infections in women, kidney stone disease, malabsorption and infection caused by *Helicobacter pylori* [11]. Oral probiotics have also been given for adverse effects of abdominal radiotherapy, constipation and food allergy [12, 13]. It has also been reported that consumption of probiotics can reduce the frequency of atopic disease in infants and the frequency and severity of diarrhea [14–16]. However, when tested for its therapeutic effect on acute diarrhea, results with the probiotic strain *Enterococcus faecium* SF68 were inconclusive [17, 18], and in rotavirus infection, *Lactobacillus* GG shortened the duration of diarrhea by only one day [5,19–24]. Current evidence suggests that probiotic effects are strain-specific; that is, other strains belonging to the same species may have no probiotic effect [25].

The increasing commercial interest in probiotics leads to the requirement for methods of precisely monitoring survival of probiotic strains during passage through the human gastrointestinal tract. Nevertheless, there have been very few studies evaluating the ability of probiotic bacteria to colonize the human intestine. One such study using PCR with specific primers showed that even after a bacterial intake of 10^{17} cells the ingested bacteria could not be detected in feces [26].

The aim of the present study was to apply a molecular biology-based method, random amplification of polymorphic DNA (RAPD) typing, for discrimination of viable probiotic strains in the feces of human volunteers after oral intake of a variety of probiotic products. Fecal samples were cultured before and after consumption of the probiotics, on the assumption that the composition of fecal microorganisms reflects the flora in the large intestine. RAPD typing was used to determine whether the probiotic strains and the strains isolated from feces were identical. The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in patterns of diagnostic value [27, 28].

The ability of six commercially available probiotics to survive gastrointestinal passage was tested in healthy volunteers.

Materials and methods

Protocol, human volunteers and probiotic preparations

The trial required oral consumption of each of six probiotic compounds in six courses over a total period of 18 weeks as follows: an initial 7-day intake period was followed by a washout period of 14 days; this was followed by the second course and washout period, and then followed by four more courses and washout periods.

Seven healthy volunteers were the study personnel (five physicians and two laboratory technicians); they maintained their usual lifestyles and dietary intakes throughout the 18 weeks of the study period. The volunteers were between 20 and 50 years of age; five women and two men. None of the subjects received antimicrobial agents during the investigation period and none had a history of chronic gastrointestinal disease, including chronic constipation, or any episode of diarrhea during the previous month and did not present any current sign or symptom of gastrointestinal disorder or infection.

Six commercially available probiotics were tested: *E. coli* Nissle $0.5–5 \times 10^9$ cells (Mutaflor® capsules), *Enterococcus faecium* SF 68 7.5×10^7 cells (Bioflorin® capsules), *Lactobacillus acidophilus* and *Bifidobacterium infantis* both 1×10^9 cells (Infloran® capsules), *Lactobacillus gasseri* and *Bifidobacterium longum* both 1×10^8 cells (Omniflora® capsules), *Lactobacillus casei rhamnosus* 1×10^9 cells (Antibiophilus® capsules), and yoghurt enriched with *Lactobacillus casei* Immunitas 1×10^{10} cells (Actimel® suspension). Each subject was instructed to take the respective supplement three times a day (before breakfast, lunch and dinner) for seven days (total bacterial uptake $1.5 \times 10^9–2.1 \times 10^{11}$).

Stool samples were obtained for each probiotic at baseline prior to probiotic ingestion (control sample) and after one week's ingestion of the probiotic (test sample).

All the volunteers gave their informed consent before the experiment, and the protocol was approved by the ethics committee of the Regional Health Authority.

Sampling and microbiological processing

Fecal samples were collected in sterile disposable containers and delivered to the microbiology laboratory within six hours after collection. Samples were processed within 12 hours after collection. All samples were homogenized in saline and diluted from 1:1 up to 1:10 000 000, then seeded on appropriate agar plates (*E. coli*: Endo agar; *E. faecium*: CNA; lactobacilli and bifidobacteria: GV, MRS and Schaedler agar); samples were routinely incubated aerobically for 24 h at 37 °C, and anaerobically with 10% CO_2. All pre-assigned agar plates supported cultivation of the individual probiotic strains; these were seeded directly from the commercial preparation onto the appropriate agar plates.

Identification of genera was made on the basis of colony morphology, Gram stain and cell morphology, in conjunction with the ability of the isolate to grow on the appropriate selective medium [29, 30]. In addition, genera were identified with VITEK 2 (bioMerieux) automated microbiology systems, using the respective updated colorimetric identification cards. Lactobacilli were identified with the negative catalase test.

Selection of colonies

Following incubation, 10 colonies were randomly selected from primary dilution plates of each sample that contained discrete colonies (30 to 300) and were then subcultured onto

blood agar media for future genetic fingerprinting. The number of isolates selected from each fecal sample was chosen on the basis of results obtained in previous studies where it was shown that 10 randomly selected colonies gave good coverage of the numerically predominant strains cultured on a selective medium [29, 30, 33].

Isolation of genomic DNA

DNA extracts of each sample were prepared using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the protocol provided. The resulting solutions of chromosomal DNA of the fecal bacteria were used as templates for RAPD-PCR. Probiotic strains were included for comparison.

Typing of random amplified polymorphism DNA

Probiotic strains and fecal isolates recovered from the volunteers were analyzed using RAPD PCR [27, 34, 35]. The RAPD primers were synthesized by VBC-Genomics (Vienna, Austria) and were as follows: primer P15, 5'AATGGCGCAG3'; primer 272, 5'AGCGGGCCAA3'; primer PL1, 5'-ACGCGCCCT-3'. Primer P15 was used by Maroye et al. [36] to type *Ralstonia pickettii* isolates and was used in the present study to type *E. coli* and *E. faecium* isolates after proving its discriminatory power for these pathogens. We also used primer 272 to type *E. coli* isolates [37]. Thus, primer P15 was used to type the *E. coli* isolates initially; primer 272 was used to confirm the ability of the primers to produce discriminatory polymorphisms with these organisms. Primer PL1 had been previously used in PCR amplification of lactic acid bacteria [35, 38, 39].

Reaction mixtures comprised 2 µl of primer (25 pmol/µl), 3 µl of template DNA (approximately 90 ng) and 20 µl of sterile distilled water with reaction beads (Ready-to-go RAPD analysis beads, Amersham Bioscience, Buckinghamshire, UK), making a total volume of 25µl. The mixtures were amplified in an Eppendorf Mastercycler using the following program: 1 cycle at 95 °C for 5 min, then 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min, completed by a final extension step at 72 °C for 10 min. RAPD products were separated by electrophoresis in 2% agarose gels at 75V for 150 min; a 100 base-pair ladder (Amersham Bioscience, Buckinghamshire, UK) was included on all gels as the molecular size standard. Gels were stained with ethidium bromide and photographed under UV illumination.

Analysis of RAPD data, detection of probiotics in fecal samples

RAPD fingerprints were analyzed visually. Polymorphisms that differed by two or more bands were considered distinct [37]. The probiotic strains were traced by comparing the banding pattern of each isolate with that of the reference strain on the gel, as previously described by Gardiner et al. [35].

Results

Control samples

At baseline, none of the volunteers was colonized with strains having the same genotype (identical RAPD pattern) as the probiotic strain ingested thereafter. *E. coli* and enterococci were cultured consistently (in control and test samples); however, microbiological culture failed to obtain lactobacilli or bifidobacteria in two volunteers prior to courses 4 and 6, and in three volunteers prior to courses 4 and 5.

Course 1: E. coli Nissle 1917, Mutaflor®

The presence of *E. coli* strain Nissle 1917 in fecal samples was assessed with RAPD-PCR twice, using primers P15 and 272 in succession. The same results were obtained with both primers: the probiotic strain was detected in stool samples of four volunteers (4/7; 57%) (Table 1, Fig. 1). One volunteer experienced meteorism during intake of this strain, the symptom disappearing spontaneously at the end of the ingestion period.

Table 1. Results of ingestion of different probiotics and their subsequent detection in stool samples

Substance	Mutaflor	Bioflorin	Infloran	Omniflora	Antibiophilus	Actimel
Probiotic strains	*Escherichia coli* strain Nissle 1917	*Enterococcus faecium* SF68	*Lactobacillus acidophilus* and *Bifidobacterium infantis*	*Lactobacillus gasseri* and *Bifidobacterium longum*	*Lactobacillus casei var. rhamnosus*	*Lactobacillus casei*
Primers	P15, 272	P15	PL1	PL1	PL1	PL1
Growth of test bacteria (species) in stool sample (growth/number of tested samples [%])	7/7 (100%)	6/6 (100%)	4/6 (66%)	3/6 (50%)	4/5 (80%)	3/5 (60%)
Probiotic culture and stool culture*: identical RAPD pattern (number of identical RAPD pattern/number of test persons [%])	4/7 (57%)	4/6 (67%)	1/4 (25%)	0/3 0%	0/4 0%	0/3 0%

*After 7 days of probiotic strain ingestion.

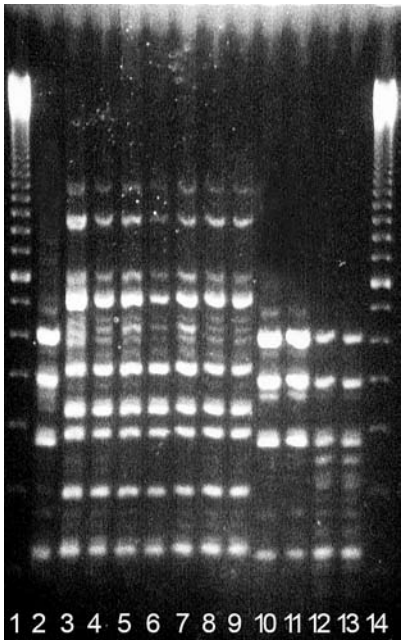

Fig. 1. RAPD fingerprinting profile: Lane 1/14 base-pair-ladder, Lane 2/13 probiotic strain *Escherichia coli* Nissle (Mutaflor®), Lane 3–12 fecal isolates of one volunteer after 7-day intake of Mutaflor®. RAPD pattern of Lane 10–12 are identical to those of the probiotic strain (Lane 2, 13)

Course 2: Enterococcus faecium SF 68, Bioflorin®

RAPD-PCR with primer P15 was used to detect *E. faecium*. Probiotic strain SF 68 was found in the feces of four volunteers (4/6; 67%) (Table 1, Fig. 2). One volunteer (a physician) suffered meteorism and painless watery diarrhea on day 3; this stopped on discontinuation of the probiotic. No test sample was available from this volunteer since he refused to continue to take any more of this, or any other, probiotic strain.

Course 3: Lactobacillus acidophilus and Bifidobacterium infantis, Infloran®

Isolates of lactobacilli and bifidobacteria were analyzed with RAPD-PCR and primer PL1. Following the 7-day ingestion of Infloran®, stool samples were positive in of 4/6 (66%) volunteers; however, an identical RAPD pattern in the stool isolates and direct culture of the Infloran® capsule was seen in only one volunteer (Table 1). Two subjects had mild self-limited borborygmi while they were taking Infloran® but this did not result in their discontinuation of the study.

Course 4: Lactobacillus gasseri and Bifidobacterium longum, Omniflora®

Lactobacilli and bifidobacteria were recovered from the feces of three volunteers after 7-day consumption of Omniflora® (3/6; 50%); however, the probiotic strains *L. gasseri* and *B. longum* in the Omniflora® were not observed in any of the fecal samples (0/6; 0%) when analyzed by RAPD PCR (no identical pattern) (Table 1). One volunteer suffered massive metorism and mild diarrhea but nevertheless completed the intake of Omniflora®. This volunteer refused to continue with courses 5 and 6 *(L. casei* var. *rhamnosus* and *L. casei* Immunitas*).*

Course 5: Lactobacillus casei var. rhamnosus, Antibiophilus®

Lactobacilli were cultured from fecal samples of four of the five remaining volunteers after intake of Antibiophilus® for a week (4/5; 80%). However, the probiotic strain was not found in any of the fecal samples (0/5; 0%) (different RAPD pattern) (Table 1). No adverse effects were seen.

Course 6: Lactobacillus casei Immunitas, Actimel®

After intake of Actimel®, fecal samples from three volunteers contained culturable lactobacilli (3/5; 60%) but the probiotic strain *L. casei* could not be detected in feces of any of the five participants (0/5; 0%) (dissimilar RAPD pattern) (Table 1). No adverse effects were seen.

Discussion

Fingerprints identical to the ingested probiotic strains were recovered in the fecal samples of 4/7 volunteers after one week of taking Mutaflor®, of 4/6 taking Bioflorin®, and of 1/6 taking Infloran®. Cultivation of probiotic strains from fecal specimens was not possible after consumption of Antibiophilus®, Omniflora® and Actimel®. Lactic acid bacteria are common inhabitants of the large intestine of humans but constitute less than 1% of the

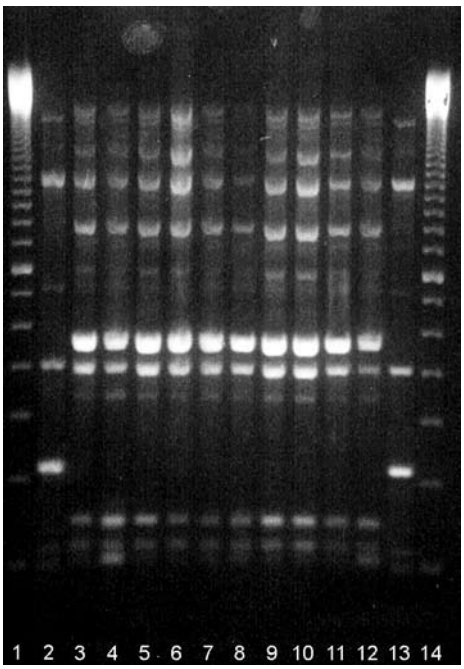

Fig. 2. RAPD fingerprinting profile: Lane 1/14 base-pair-ladder, Lane 2/13 probiotic strain *Enterococcus faecium* SF 68 (Bioflorin®), Lane 3–12 fecal isolates of one volunteer after 7-day intake of Bioflorin®. RAPD pattern of Lane 3–12 are different to the probiotic strain (Lane 2, 13)

total bacterial community [40]. They are outnumbered by the cells of anaerobic species and in a study in the USA were detected in approximately 27% of fecal samples collected [30]. In contrast, several studies have shown temporary colonization of the gastrointestinal tract by some lactic acid bacteria after oral intake [9, 12, 13, 41–49].

There are many possible reasons why, after oral consumption, probiotic strains are not present in feces. De Champs et al. [12] found that the greatest increases of *L. casei* subsp. *rhamnosus* Lcr35 were observed in subjects harboring the least abundant indigenous population of Lcr35-like bacteria. Alander et al. [13] added that high counts of endogenous lactic acid bacteria mean that a probiotic strain faces strong competition when establishing itself. Morelli et al. [9] showed that a marked increase of *L. paracasei*-like bacteria was found in subjects who had low counts of this group of lactobacilli at baseline. Nevertheless, we found that even in subjects where no lactobacilli were present at baseline, establishment of the probiotic lactobacilli did not occur. In the large intestine, the bacteria must compete effectively with a complex and metabolically active indigenous flora [10, 49]. Whether an existing disorder of the gut flora could prevent or facilitate the establishment of probiotic strain in the intestinal ecosystem remains to be shown.

The minimum effective dose of a probiotic is not precisely known, but the usual recommended oral dose is in excess of 10^9 CFU/day [12]. Furthermore, many factors, such as the capacity to adhere, growth rate and antimicrobial activity, may be important for establishment on the intestinal mucous membrane [42, 50]. As described by Jacobsen et al. [49], lactobacillus strains with adhesion properties (adhesion to Caco-2 cells) and tolerance to pH 2.5 survive passage through the intestinal tract at higher rates than those without adhesion properties. However, even strong adhesion properties and pronounced pH tolerance seemed not to result in colonization and persistence of the lactobacilli for any length of time after discontinuation of administration [49]. Alander et al. demonstrated that the study of fecal samples alone may underestimate colonization by probiotic strains [13]. Although some studies have shown differences between the composition of fecal microflora and the kinds of bacteria that are present at other anatomical sites, including bacteria in the cecum [51] and those associated with the mucosa [52], differences in the microflora at various anatomical sites are not well documented. It can be assumed that, although the proportions and activities of the microflora change with passage through the intestinal tract, most viable intestinal bacteria in feces will be detectable with molecular methods [53].

Lactic acid bacteria are difficult to detect in human feces when using bacteriological culture methods, because these bacteria constitute only a minor part of the microflora. *L. rhamnosus* was below detectable limits in the feces when using bacteriological culture in a probiotic trial [41], and it is well known that a major proportion of microflora detected by microscopy cannot be cultivated [54]. In addition, the random selection of 10 isolates from a given sample could in theory miss some of the strains present; however, several studies have shown that 10 randomly selected colonies gives good coverage of the dominant strains [29, 30, 55].

Complete analysis of the intestinal microflora was not the aim of the present study; nevertheless, some interesting findings arose regarding indigenous microflora. For example, when baseline samples were typed, predominance of certain strains of *E. coli* and *Enterococcus faecium* was noted. However, after probiotic intake, considerable variation among these bacteria was observed; the uniformity of these strains was displaced (data not shown).

Lactobacilli vary greatly in different people and even among samples collected from the same individual [29]. In the present study, alterations in lactobacilli and bifidobacteria were seen when test and baseline samples were compared. These bacterial populations appeared to be dynamic with regard to strain composition. Thus, the changes that were observed cannot be exclusively attributed to probiotic consumption.

When using live microbes as dietary adjuncts, an inherent problem is the difficulty of detecting and enumerating the specific probiotic in the gut or feces. In particular, for assessment of viability it is essential to distinguish between the probiotic and indigenous strains of the same species within the host. Several molecular diagnostic approaches, such as pulsed-field gel electrophoresis, ribotyping, denaturing gradient gel electrophoresis, analysis with DNA probes and RAPD analysis, can be used to address this problem [10, 27, 54, 56]. 16S rRNA probing strategies [47] and 16S rDNA restriction fragment length polymorphism analysis [57] have already been used to monitor feces for the presence of ingested bifidobacteria.

This comparative assessment allows identical RAPD patterns of probiotic strains to be separated from the indigenous flora before and after probiotic intake. RAPD typing has been used to identify lactobacilli in the vagina after insertion of a probiotic preparation: vaginal isolates were subjected to RAPD-PCR and the resulting profiles were compared with those of lactobacilli recovered from the probiotic capsules [35]. To our knowledge, the present report is the first to use RAPD analysis in the detection of probiotic strains in feces. The results of the investigation support the concept that RAPD analysis is efficiently capable of detecting genomic polymorphisms among various microbial species, without requiring previous knowledge of the nucleotide sequence of the target DNA, and can be used to qualitatively monitor the presence of probiotic strains in feces. Indigenous microflora and potentially clinically important probiotic strains were differentiated by this method, which uses arbitrarily chosen PCR primers to amplify randomly sized DNA fragments, separation of which results in a characteristic DNA fingerprint [27].

MacFarlane et al. [58] reviewed the most recent contributions to this rapidly developing area and came to the conclusion that probiotics invariably do not work and that study of mechanisms is urgently needed. We believe that probiotic strains must be capable of colonizing the intestine [59], at least transiently, to effectively fulfill a beneficial or prophylactic role. This should also be necessary for the production of metabolic products by probiotic strains, for which health-promoting effects have been claimed. In this study we show that some probiotic bacteria are able to survive passage through gastrointestinal

tract. After oral consumption of various probiotic preparations, *E. coli* Nissle [60] and enterococci can be consistently detected in stool samples (57%, 67%, respectively). In contrast, with one exception, ingested lactobacilli or bifidobacteria were not found in human feces in this study. However, the study group was too small to permit general conclusions.

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