Original Contribution

ASSESSMENT OF LACTOBACILLUS BULGARICUS STRAIN IN HUMAN INTESTINAL SAMPLES BY DENATURATION GRADIENT GEL ELECTROPHORESIS

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ABSTRACT

PURPOSE. The purpose of this work was to evaluate the content of *Lactobacillus bulgaricus* in faecal samples of volunteers after consumption of yoghurt produced with selected *L. bulgaricus* strain.

METHODS. Using Denaturation Gradient Gel Electrophoresis (DGGE) the DNA band corresponding to *L. bulgaricus* was well separated from the DNA bands of other lactobacilli.

RESULTS. Faecal samples of all four volunteers consumed yoghurt contained a major DNA band indicating the presence of *L. bulgaricus*, unlike the faecal samples from the three volunteers no consumed yoghurt where the specific for *L. bulgaricus* DNA band was absent. The differences in the intensity of the electrophoretic band of *L. bulgaricus* are influenced by the individuality of the volunteers and the duration of yoghurt consumption. By help of DGGE it was possible to assay the presence of *L. bulgaricus* in faecal samples and on the base of the intensity of the specific DNA fragment to evaluate the dynamics of *L. bulgaricus* content during 10 days consumption of yoghurt. **CONCLUSIONS.** DGGE proved to be an applicable tool to monitor the presence of *L. bulgaricus* strains in human intestinal samples.

Key words: faecal microflora, microbiota, microbial communities

INTRODUCTION

The gastrointestinal tract in humans contains complicated microbial communities consisting of hundreds of bacterial species. Much of the intestinal bacteria are difficult to cultivate or non-cultivated, and it is therefore appropriate to use molecular techniques such as denaturing gradient gel electrophoresis (DGGE) to characterize microbial societies because it does not require prior cultivation of strains by the bacterial community (1). By using DGGE, information on species diversity in a given bacterial community can be obtained by analysing PCR fragments of the same or similar size but with different sequences (2). Because of the sequential differences, the fragments denature in the polyacrylamide gel with increasing towards the anode concentration of urea and formamide at a different distance from the electrophoretic start (3). Initially, total DNA is

obtained from a given bacterial community, and the hypervariable regions most often within the 16S rDNA gene are amplified by PCR. One primer contains the so-called GC-tail and this results in a site of the PCR products with a reduced denaturation at one end. The resulting PCR products denature according to their sequence as the GC-terminus remains non-denatured. At the denaturation point, the molecular volume is greatly increased because of the separation of the two DNA strands (without the GC-terminus) and the movement of the fragments practically stops. With proper selection of the analytical conditions, and if the molecular weight of PCR products is in the range of 200-600 bp, it is possible to separate fragments that differ even with one nucleotide base (4).

By using a DGGE effects of probiotic and prebiotic products on the composition of the intestinal microbiota were monitored (5, 6). DGGE was applied to study the link between

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the compositional changes within gut microbiota and obesity (7), as well as the composition of vaginal microflora (8). The relation between the development of hypercholesterolemia and gut microbiota alterations was evaluated by help of DGGE (9). Scanlan *et al* (10) evaluated using DGGE the variation in targeted bacterial subgroups of the dominant faecal microbiota in Crohn's Disease patients. A number of new strains with beneficial properties have been detected by DGGE (11, 12).

Bacterial species of the genus *Lactobacillus* are common in human intestinal microflora, and their detection by DGGE would allow a rapid evaluation of the species composition. *Lactobacillus bulgaricus* is an irreplaceable part of the Bulgarian yoghurt. Typically, strains of this species do not adhere to the intestinal epithelium and do not persist for longer in the intestinal microflora. However, with regular consumption of fermented dairy products, produced with selected *L. bulgaricus* strains, such strains can reach alive the colon and be present in the faecal microflora (13). DGGE is a valuable tool in assessing the presence of *L. bulgaricus* strains in complex microbial communities, such as intestinal samples. The main purpose of this study is to demonstrate the applicability of the DGGE non-culture method for detecting the presence of selected *L. bulgaricus* strain in faecal samples of volunteers after consumption of Bulgarian yoghurt. The present study is the first one where a selected strain of *L. bulgaricus* is detected and traced in the intestinal microflora by DGGE.

MATERIALS AND METHODS

Bacterial strains

The strain *L. bulgaricus* C3/2 is of plant origin (Cornel-tree) and belongs to the collection of microorganisms of LB-Bulgaricum Plc. This strain, together with the *S. thermophilus* t12 strain, were used to produce Bulgarian yoghurt according to the established Bulgarian state standard (14), which yoghurt is consumed by volunteers in this study. The standard strains were purchased from the American Type Culture Collection (ATCC): *L. plantarum* No14917, *L. johnsonii* No33200, *L. gasseri* No33323, *L. fermentum* No14931, *L. brevis* No14869, *L. acidophilus* No4356, *L. helveticus* No15009, *L. bulgaricus* No11842, *L. reuteri* No23272, *L. casei* No393, *L. rhamnosus* No7469. All lactobacilli were anaerobically propagated in MRS broth (Oxoid, UK) for 18 h at 37°C.

Treatment of faecal samples

Four healthy volunteers took part in this experiment. At least two weeks before the trial, the volunteers had not consumed any fermented dairy product. Faecal samples were taken from the volunteers at the start of the trial, on the 5th day and on the 10th day of consumption of the product. Human faecal samples were diluted tenfold (10 g to 100 ml) in pre-reduced saline (0.9 % w/v NaCl), homogenized with a sterile hand homogenizer and filtered through a 50 micron pores filter (Millipore, USA). Sample homogenization and serial dilutions were performed in an anaerobic glove box (M.D.H., Intermed, Hampshire UK) under an atmosphere of 85 % v/v N₂, 10 % v/v CO₂ and 5 % v/v H₂.

DNA extraction

Faecal suspensions (10 ml) prepared as described above were washed twice with PBS buffer pH 7.5 and treated with lysozyme and mutanolysin at final concentrations of 10 mg/ml and 50 U/ml, respectively, in 20 mM Tris-HCl, pH 8.0 and 20 mM EDTA for 2 h at 37°C. To decompose the cells, an equal volume of solution was added: 1 % Triton X100, 0.1 N NaOH, 1.0 mM NaCl, and 100 mM Tris-HCl pH 9.0. The mixture was incubated at 90°C for 5 min. DNA extraction was carried out initially with 25:24:1 phenol: chloroform: isoamyl alcohol, and the next extraction was with chloroform: isoamyl alcohol in a 24:1 ratio. The extracted DNA was precipitated with 50 % v/v iso-propanol, washed with 70 % v/v ethanol and dissolved in 20 mM TE buffer, pH 8.0.

PCR-DGGE

For the DGGE the method of Walter *et al* (15) was used with some modifications. Briefly, after collecting faecal samples and isolating DNA from them, a *Lactobacillus*-specific PCR amplification of a part of the 16S ribosomal gene was performed with the primers 5'-AGAGTTTGATCTATGGCTCAG-3' and the genus-specific primer 5'-CACCGCTACACATGGAG-3'. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen). Following the nested PCR, PCR-DGGE was performed with primers 5'-GGAAACAGATGCTAATACCG-3' and 5'-ATCGTATTACCGCGGCTGCTGGCA-3', to the 5'-end of which was attached the GC fragment 5'-CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGCACGGGGG-3'. The PCR conditions were: initial denaturation 95°C/5min; 35 cycles at 95°C/30 s, 6°C/30 s and 72°C/40 s; final elongation at 72°C/7min. Using these primer combinations, the V2-V3 region of

the 16S rDNA of the strains was amplified. The gradient polyacrylamide gel contained 8% total acrylamide and 2.7 % bisacrylamide in 0.5X TBE buffer. The denaturation percentage of 100 % is equivalent to the mixture of 7 M urea and 40 % formamide. In this connection, the gradient gel is from 35 % to 55 % denaturation percentage. The 0.75 mm thick gel is prepared using a gradient gel pouring tool (Scie-Plas, UK). The electrophoretic process was carried out on an apparatus TV400-DGGE (Scie-Plas, UK) for 16 h at 80 V and 60°C in 0.5X TBE buffer. After the end of the electrophoretic process, the gels were subjected to silver staining, as instructed by the manufacturer of the stain kit (Promega, USA).

RESULTS AND DISCUSSION

In this study, we evaluated the influence of consumption of yoghurt produced with a selected strain of *L. bulgaricus* on the content of this species in the faecal microflora of volunteers for a 10-day period of consumption. The *L. bulgaricus* strain C3/2 was selected for this study because it proved to be resistant to low pH and intestinal juices (13). In an experiment with two groups of volunteers consuming and non-consuming fermented milk with *L. bulgaricus* C3/2, DGGE was applied to demonstrate the presence of the strain in faecal samples of volunteers who consumed this product. Isolation of DNA from faecal samples, genus-specific *Lactobacillus* PCR amplification of 16S rDNA, nested PCR

in DGGE format, and DGGE electrophoretic process were performed as indicated in Materials and Methods. To verify the good separation of the amplified DNA fragment of *L. bulgaricus* from the DNA fragments of other lactobacilli, DGGE of various bacterial species of the genus *Lactobacillus* was carried out. **Figure 1** shows the good separation of the DNA fragment from V2-V3 regions of 16S rDNA of *L. bulgaricus* from the other fragments corresponding to other species of the genus *Lactobacillus*. It is necessary to note the successful separation between DNA fragments of *L. bulgaricus*, *L. helveticus*, *L. casei*, *S. thermophilus* and *Lc. lactis*, which are often present in fermented dairy products. From **Figure 1**, it appears that there is no obstacle by DGGE to monitor the bacterial balance of microbial communities with an emphasis on *Lactobacillus* species. DGGE is a method of separating fragments of equal or close molecular weight based on their sequence. According to their sequence, the DNA fragments denature at different concentrations of urea and formamide in the polyacrylamide gel. In DGGE, the gels contain an increasing concentration of urea and formamide from the cathode to anode, at the same acrylamide concentration. It has been assumed that a 100 % denaturation rate corresponds to a mixture of 7 M urea and 40 % formamide. To obtain good separation of DNA fragments of *Lactobacillus* species, a gradient in the range 35 % - 55 % was used.

Figure 1. PCR-DGGE of V2-V3 regions of 16S rDNA of standard *Lactobacillus* strains. Lanes: 1 – *L. plantarum*, 2 – *L. johnsonii*, 3 – *L. gasseri*, 4 – *L. fermentum*, 5 – *L. brevis*, 6 – *L. acidophilus*, 7 – *L. helveticus*, 8 – *L. bulgaricus*, 9 – *L. reuteri*, 10 – *L. casei*, 11 – *L. rhamnosus*.

Another important factor for the denaturation of DNA fragments, besides urea and formamide, is the temperature. The maintained temperature in the electrophoretic cell is 60°C. Maintaining a constant temperature is important for the reproducibility of the results. Since denaturation occurs at a different gradient percentage for fragments with different sequence, their position in the gel differs. With proper selected DGGE conditions, it is possible to separate fragments that even have only one nucleotide pair in their sequence. DGGE has the great advantage over a number of other methods as the analysis is not preceded by cultivation of single bacterial colonies. Therefore, the presence of bacterial species in complex microbial communities, such as in the intestinal samples, can be demonstrated by DGGE.

Figure 2 shows a picture of DGGE of faecal samples of volunteers consuming and non-consuming yoghurt with the selected *L. bulgaricus* C3/2 strain. With an arrow in **Figure 2**, the line of the *L. bulgaricus* DNA fragments is pointed. The picture clearly shows that the samples from volunteers who had not been consumed the product with *L. bulgaricus* C3/2 did not contain the electrophoretic band corresponding to *L. bulgaricus*. The electrophoretic lines of volunteers consumed yoghurt contained the specific band for *L. bulgaricus*. Comparing the intensities of the *L. bulgaricus* bands, it is found that it increases in three (numbers 1, 2 and 4) of the four volunteers 5 and 10 days after commencement of consumption of the product, and in volunteer number 3 it does not change. In addition, it can be seen that the concentration of the *L. bulgaricus* C3/2 strain is also dependent on the volunteer's individuality.

Figure 2. Effect of consumption of yoghurt containing the *L. bulgaricus* C3/2 strain on volunteers. Lanes 1a, 2a, 3a and 4a: Volunteers 1, 2, 3, and 4 - 5 days after commencement of consumption. Lanes 1b, 2b, 3b and 4b: volunteers 1, 2, 3, and 4 - 10 days after commencement of consumption. Lines 5, 6, and 7: volunteers not consuming yoghurt.

The amplified by PCR DNA fragments originate from the V2-V3 regions of the 16S rDNA of the *Lactobacillus* strains. The presence of non-degraded *Lactobacillus* strains DNA in faecal samples means that the bacterial cells are not lysed in the gastrointestinal tract, as claimed by Drouault *et al* (16). Otherwise, after the disintegration of the bacterial cells, the DNA would rapidly degrade under the action of nucleases of the pancreatic juice (17). The presence of probiotic strains of other *Lactobacillus* species was evaluated by DGGE in faecal samples (15, 18), in the sour dough (19) as well as in naturally fermented lactic

acid products (20). Using DGGE three lactobacilli were found able to modify intestinal microbiota in piglets (21). In the present study, the presence of selected *L. bulgaricus* strain in intestinal samples of volunteers consumed yoghurt containing this strain was assessed for the first time.

CONCLUSION

From the results obtained, it can be concluded that DGGE can successfully be used to monitor the presence of a *L. bulgaricus* strain in the intestinal samples. It is possible also to track the dynamics of the species population. DGGE is a method by

which the influence of consumption of fermented dairy products on the bacterial composition in intestinal samples can be evaluated. In particular, DGGE helps to monitor the content of *Lactobacillus* species in intestinal samples without preliminary cultivation.

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