PCR-DGGE-Based Study of Fecal Microbial Stability during the Long-Term Chitosan Supplementation of Humans

J. MRÁZEK, I. KOPPOVÁ, J. KOPEČNÝ, J. ŠIMŮNEK*, K. FLIEGEROVÁ

Institute of Animal Physiology and Genetics, v.v.i., Academy of Sciences of the Czech Republic, 142 00 Prague 4, Czech Republic

ABSTRACT. A feeding study was performed to monitor the effect of chitosan intake on the fecal microbiota of ten healthy human subjects. Diversity of microflora was monitored during 8 weeks including 4 weeks of chitosan supplementations. Using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons and quantitative PCR method we revealed possible changes originating in the overall bacterial composition and also in the subpopulation of Bifidobacterium group. DGGE profiles displayed high complexity and individuality for each subject. Considerable variations in the composition of band patterns were observed among different persons. A raised level of fecal Bacteroides in response to chitosan intake was found in all samples. Bifidobacterium levels following chitosan intake increased or remain unchanged. Non-significant increase was, surprisingly, found in the numbers of butyrate-producing bacteria.

The human gastrointestinal tract harbors a complex community of microorganisms that play a significant role in human health. The highest concentration of bacteria has been found in the large intestine (10^{11}–10^{12} cells per g of luminal contents) and the relevance and effect of resident microflora on a host physiology has been well documented (Guarner and Malagelada 2003). However, the colon bacteria are often considered as a potentially harmful since they may incite pathological disorders including intestinal obstruction, inflammatory bowel disease and even colon cancer. Increasing numbers of people suffering from different types of gastrointestinal tract diseases admittedly relate with unhealthy western eating habits, which promote fatty, salty and sugary foods. Diet is a major factor controlling intestinal balance and this fact stimulated the development of so-called functional food. Recently, chitosan has attracted much interest as dietary adjuvant.

Chitosan is a natural biopolyaminosaccharide, derived from crustacean or fungal chitin by alkaline deacetylation. Orally administrated chitosan is positively charged and, in theory, binds to negatively charged dietary fat in the intestine. Thus, dietary fat would be excreted in the feces rather than being absorbed (Kanauchi et al. 1995). This inhibition of fat absorption by chitosan has resulted in its widespread promotion as a nonprescription body-mass loss agent even if strong scientific evidence to support mass loss claims made for chitosan is missing (Kaats et al. 2006).

The antimicrobial activities of chitosan make this food supplement attractive as prebiotic component, however, this mode of use is not supported by sufficient experimental results. Moreover, the exact mechanism of the antimicrobial effect of chitosan is still unknown. Chitosan generally shows strong bactericidal effect for Gram-positive bacteria, while growth inhibition of Gram-negative bacteria is weaker and depends on the molar mass of chitosan (Jeon et al. 2001; No et al. 2002). The antibacterial activity of chitosan is also influenced by the degree of deacetylation (chitosan with a higher degree of deacetylation, DDA, has stronger bactericidal activity), concentration in solution and the medium pH (Yang et al. 2005). Promising results were published by Tsai and Hwang (2004), who demonstrated that probiotic bacteria Bifidobacterium and Lactobacillus are generally resistant to chitosan (95 % DDA) even if strain variations in susceptibility to chitosan were observed.

As no study has so far been done dealing with the effect of chitosan on the fecal microbiota of humans, this is the first one describing the in vivo effect of chitosan on large intestinal microbial population of healthy volunteers. Modifications of the overall bacterial composition of fecal samples were monitored by population fingerprinting using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons and quantitative PCR method.

*Corresponding author; fax +420 267 090 500, e-mail simunek@iapg.cas.cz.
MATERIAL AND METHODS

Subjects and chitosan treatment. A total of 10 healthy volunteers (5 men and 5 women) 25–55-year old were recruited for this three-period study. First, subjects entered a control 7-d period without chitosan intake, and then participants were instructed to take three chitosan (DDA 90 %; Medicol, Czech Republic) capsules thrice daily (3 g chitosan per day) before each meal for a 28-d supplement period. This treatment period was followed by no chitosan 21-d period. Volunteers maintained their usual diet and documented the type of food consumed; energy intake was not restricted. Persons involved in experiment were instructed not to take vitamins, minerals, or other dietary supplements during the study; none of the volunteers had a history of gastrointestinal or metabolic disease and none did receive antibiotic treatment or any other medical treatment influencing gastrointestinal microbiota.

This work was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of Institute of Animal Physiology and Genetics. Verbal informed consent was obtained from all subjects. Verbal consent was witnessed and formally recorded.

Sample collection and processing. Fecal samples were collected every day for the whole run time of experiment, i.e. 56 d and stored at –20 °C before analysis. Total bacterial DNA was extracted from 130 mg of human fecal samples using zirconium bead-beating ZR Fecal DNA Kit™ (Zymo Research, USA). Fecal bacterial composition was assessed by DGGE and quantified by real time PCR.

PCR and DGGE analysis. PCR amplification and DGGE analysis of total bacterial and bifidobacterial community as well as the identification of bands of interest cut from the documented stained polyacrylamide gel was done according to Mrázek et al. (2008).

Real-time PCR analysis. The quantification of total and selected bacteria was performed with MX3005P QPCR System (Stratagene, USA) using the qPCR 2x SYBR Master Mix (Top-Bio, Czech Republic). Specific primers Lm26f and Lm3r were used for PCR amplification of bifidobacteria (Kaufmann et al. 1997), Bfra-F and Bfra-R for bacteroides (Matsuki et al. 2002) and F2 and 515r for Pseudobutyrivibrio group (Mrázek et al. 2006). Temperature programs were used according to the above authors. Calibration curves were constructed using dilutions of purified genomic DNA isolated from known number of cells of control strains Bacteroides uniformis, Bifidobacterium bifidum and Pseudobutyrivibrio xylanivorans. The numbers of control bacterial cells were determined by fluorescence in-situ hybridization (FISH) method (Kleesen et al. 2002).

Statistics. The significance of differences among values was analyzed by one-way ANOVA. When interaction was significant, Student’s t-test was performed, considering p < 0.05 as significant. All statistical analyses were performed using the General Linear Model Procedure (Zoetendal et al. 1998).

RESULTS

Fecal microbiota profiling with DGGE. PCR-DGGE analyses with universal bacterial primers targeting a 200-bp region of the 16S rDNA gene were used to detect the influence of orally administered chitosan on the composition of fecal bacterial population of healthy humans. Population fingerprint profiles of each from 10 volunteers compare 3 samples of pre-treatment period (1 week), 10 samples of chitosan intake period (4 weeks) and 7 samples of post-treatment period (3 weeks). DGGE profiles (Fig. 1) were, according to a visual evaluation of bands of interest, cut out and sequenced.

DGGE profiles displayed high complexity and individuality for each subject. A considerable variation in the composition of band patterns was observed among different persons; the responses of fecal bacterial population to chitosan feeding were also subject-specific. Mostly, in the lower part some band fragments disappeared (subject I and II) or were less intensive (subject III and VI) while in the middle part of gels intensified fragments occurred (subject I, II, III, VI, VII, X). An opposite effect was observed only in the profile of subject III where new bands appeared in the lower part of gel and some fragments from the middle part disappeared.

The DGGE band fragments that intensified, newly appeared or, by contrast, disappeared after chitosan ingestion were further characterized by sequencing analysis. Fifty-nine particular bands were extracted and their sequences were compared with EMBL (GenBank) database (Table I). The matches, however, should be interpreted with caution, as only partial 16S rDNA gene sequences were determined.

Nevertheless, the obtained data showed that the chitosan intake induced two main changes in fecal microbiota composition: (1) bands related to bacteroidete clones intensified (Fig. 1, subject III – bands 16, 19; subject IV – bands 28; subject VI – bands 35, 36, 37) or newly appeared (subject I – bands 3, 4) due to chi-