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SCIENCE DIRECT
Food Research International 39 (2006) 530–535
FOOD RESEARCH INTERNATIONAL
www.elsevier.com/locate/foodres

Molecular detection of *Bifidobacterium animalis* DN-173010 in human feces during fermented milk administration

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Received 17 October 2005; accepted 21 October 2005

Abstract

We tested different techniques to detect exogenous bifidobacteria (DN-173010) in feces; genus- and species-specific PCR technique; amplified ribosomal DNA restriction analysis (ARDRA) and fluorescent in situ hybridization (FISH) technique. A significant increase in the number of bifidobacteria in feces was observed during ingestion of fermented milk, and also, we detected a decrease in this number when the ingestion stopped. The number of bifidobacteria enumerated by culturing was 10–100-fold lower than by FISH technique. *Bifidobacterium animalis* DN-173010 can survive passage through the gastrointestinal tract and was detected viable in human feces. Combination of ARDRA and FISH was a powerful tool to detect exogenous bifidobacteria. The aim of this study is to demonstrate that *Bifidobacterium* DN-173010 can survive passage through the gastrointestinal tract and be recovered live in human feces. For this purpose, we have assessed different techniques to detect and quantify the number of bifidobacteria following fermented milk supplemented with *B. animalis* subsp. *lactis* DN-173010 administration and to confirm that this strain can survive passage through the gastrointestinal tract by recovering viable cells in human feces.
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Keywords: *Bifidobacterium*; Fermented milk; ARDRA; FISH; Fecal samples

1. Introduction

The use of live microbes as dietary adjuncts or "probiotics" is a subject of intense and growing interest. Probiotics have been defined as living organisms that, when included in the diet, have a favorable effect on the host (Fuller, 1991). Bifidobacteria are particularly important since they represent up to 91% of the total population of the intestinal tract in newborns and between 3% and 7% in adults (Biavati & Mattarelli, 2001). The contribution of these bacteriato good health has been recognized for quite some time and has led to widespread use of bifidobacteria as probiotics for maintaining or improving human and animal health (Stanton et al., 2001). This growing interest in the health benefits of bifidobacteria has prompted inclusion of these organisms in many dairy foods and led to increased consumption. This development requires control of the quantity of probiotics that the product contains, their capacity for survival in gastrointestinal conditions in order to arrive live at the end of the intestine in enough quantities (10^6–10^7 microorganisms per ml) (Bouhnik et al., 1992). For this reason, methods for specific identification of probiotic strains are necessary. Accurate identification techniques could serve in monitoring the progress of the population of specific probiotics through the gastrointestinal tract. Since there are inherent difficulties in obtaining definitive evidence for the suggested beneficial effects of bifidobacteria consumption, there is a great deal of speculation about the possible prophylactic and therapeutic properties of foods containing bifidobacteria. One difficulty is the presence of endogenous bifidobacteria in the gastrointestinal tract and human feces, which complicates the

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doi:10.1016/j.foodres.2005.10.011

task of differentiating these bifidobacteria from ingested bifidobacteria and unequivocally demonstrating the survival of ingested bifidobacteria through the gastrointestinal tract. Molecular based techniques can be used to improve the efficiency of detection and enumeration methods. Thus, PCR genus-specific, RAPD-PCR, amplified ribosomal DNA restriction analysis (ARDRA) and hybridization with specific DNA probes have been used to compare and identify bifidobacteria isolated from dairy products and human feces (Gueimonde et al., 2004; Satokari et al., 2003; Temmerman, Masco, Vanhoutte, Huys, & Swings, 2003; Ventura, Van Sinderen, Fitzgerald, & Zink, 2004; Ventura & Zink, 2002).

For this study, we have selected *Bifidobacterium animalis* subsp. *lactis* strain DN-173010 contained in the most popular and consumed commercial dairy product in Spain. There are some studies about this strain (Duez et al., 2000; Lepercq, Relano, Cayuela, & Juste, 2004; Marteau et al., 2002; Tavan et al., 2002) but in this study we test different techniques to detect bifidobacteria (*B. animalis* subsp. *lactis* DN-173010) in human feces and describe the results of an in vivo study in which healthy human subjects consumed this product with viable bifidobacteria. This paper describes the results of an in vivo study in which healthy human subjects consumed a probiotic product with a viable bifidobacteria DN-173010 strain. The aim of this study was to demonstrate that *B. animalis* subsp. *lactis* DN-173010 can survive through the gastrointestinal tract and to obtain accurate data on the fate of ingested bifidobacteria in humans as a first step towards assessing the physiological importance of ingested bifidobacteria. We have monitored total bifidobacteria in fecal samples of healthy subjects throughout the course of a controlled DN-173010 administration using different techniques including selective culture techniques, molecular typing based on amplified 16S rRNA gene and digestion with different restriction enzymes of *Bifidobacterium* isolates.

2. Materials and methods

2.1. Subjects

Healthy volunteers ($n = 12$) aged 25–40 years of age (mean 32.5 years), who did not include dairy products in their regular diet, participated in the study. Throughout the study period, the subjects were asked to avoid other fermented milks, oligosaccharides and any food or antibiotic that may influence the fecal microbiota other than used in the study. Otherwise there were no specific dietary restrictions. Also, the subjects did not have any history of milk allergy or intolerance nor other symptoms during the study. A total of 10 subjects ingested commercial fermented milk and two out of these 10 subjects were monitored as controls; negative control (individual without product ingestion) and positive control (subject with continuous product ingestion for three months prior the study).

The volunteers provided written informed consent prior to the start, and the study was conducted based on the principles of the World Medical Association Declaration of Helsinki (1964) (Ethical Principles for Medical Research Involving Human Subjects).

2.2. Fermented milk administration

Commercial fermented milk with *B. animalis* subsp. *lactis* DN-173010 produced by Danone España S.A. was administered to healthy subjects. Also, the product contains *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains. Fermented milk was administered at weekly intervals up to 2 weeks before their expiry dates. The number of viable bifidobacteria in 250 ml of the fermented product was 1.4×10^9 CFU/ml. These results were obtained by traditional plate counting using specific bifidobacteria media (BFM agar; Nebra & Blanch, 1999) following incubation at 37 °C for 48 h under anaerobic conditions. The study lasted 12 weeks, with the following schedule: control period (4 weeks with no ingestion of any fermented milk with bifidobacteria), 250 ml product administration period (4 week period with administration of fermented milk) and post-administration period (4 week period with no administration of bifidobacteria). During the bifidobacteria administration period, subjects consumed 250 ml of the DN-173010 fermented milk. Throughout the entire experimental period the volunteers did not consume any liquid milk products or fermented dairy products prepared using lactic acid bacteria (LAB).

2.3. Examination of fecal samples

Fecal analysis was performed at the end of control period (1st sample) and every week during the administration (2nd, 3rd, 4th and 5th sample) and post-administration (6th, 7th, 8th and 9th sample) periods. Samples were collected fresh in sterile plastic recipients, refrigerated and processed without further delay. After thorough mixing, the fecal sample (2 g wet weight) was weighted and serial 10-fold dilutions from 10^{-10} to 10^{-8} were prepared in PBS buffer (130 mM sodium chloride, 10 mM sodium phosphate, [pH 7.2]) and plated on the appropriate agar media. Bifidobacteria were enumerated on BFMAgar (Nebra & Blanch, 1999) and the plates were incubated at 37 °C for 72 h in an anaerobic jar filled with an atmosphere of oxygen free CO_2 using anaeroGen sachets (Oxoid, Hampshire, England). Total enterobacteria were enumerated on McConckey (Merck, Darmstadt, Germany) agar and plates were incubated aerobically at 37 °C for 24 h. All colonies grown on McConckey were assumed to be enterobacteria. Duplicate plate values were averaged and bacterial densities were expressed as the log of the number of CFU/g wet weight of feces. After incubation at 37 °C for 3–4 days in anaerobic conditions, 10–12 colonies from BFM media agar were selected and re-plated on the same media. Colonies were examined and those showing

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