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Scarce Evidence of Yogurt Lactic Acid Bacteria in Human Feces after Daily Yogurt Consumption by Healthy Volunteers

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In a double-blind prospective study including 114 healthy young volunteers, the presence in human feces of the yogurt organisms *Lactobacillus delbrueckii* and *Streptococcus thermophilus* after repeated yogurt consumption (15 days) was analyzed by culture, specific PCR, and DNA hybridization of total fecal DNA. Detection of yogurt lactic acid bacteria in total fecal DNA by bacterial culture and PCR assay was consistently negative. DNA compatible with yogurt bacteria was found by hybridization experiments in only 10 (10.52%) of 96 individuals after consumption of fresh yogurt and in 2 (2.10%) of 96 individuals after consumption of pasteurized yogurt ($P = 0.01$).

Yogurt has been traditionally considered a probiotic-carrier food with health-promoting effects. Very few studies have been done to scientifically ascertain these health-promoting effects, particularly in healthy people (1). It seems obvious that any presumed effect depends on the ability of probiotic organisms to survive and multiply in the gastrointestinal tract and persist at high levels in the intestine (8). *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have been classically used as starters for milk fermentation in yogurt production. The concentration of these organisms in the human or animal gastrointestinal tract has been poorly examined in comparison with that of other probiotic strains. The aim of this study was to assess the presence of yogurt microorganisms in human feces after repeated oral yogurt intake in healthy young volunteers, who represent one of the target populations in which presumptive intestinal health benefits are claimed by the dairy industry.

(Part of this work was presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy [abstr. C2-2169, p. 160].)

One hundred fourteen young volunteers (49 male, 65 female) with a mean age of 23.6 years were included. The healthy status of the individuals was confirmed by the Preventive Medicine Department with the gastrointestinal health test GIQLY (4), large-spectrum blood tests, and immunological tests (CD3, CD4, and CD8 lymphocytes, immunoglobulin A [IgA], IgG, and IgM). Except for the requirement of the daily consumption of a measured amount of a specific yogurt, no special dietary restrictions were imposed on the volunteers. In a first round of 2 consecutive weeks, 48 volunteers had a daily consumption of commercially produced fresh yogurt (375 g) containing 1.3×10^7 and 2×10^8 CFU of *L. delbrueckii* and *S. thermophilus* per g, respectively (total bacterial intake, about 10^{11} cells). The same schedule with pasteurized yogurt was applied to the other 48 volunteers. The same manufacturer provided both fresh and pasteurized preparations. This was a double-blind prospective study in which standard and pasteurized yogurts were labeled with code names so that neither the volunteers nor the microbiologists were aware of the type of preparation under study in each group. After a washing period of 2 weeks without any yogurt intake, the experimental groups were reversed in a second round. Each volunteer group then consumed the other type of yogurt used in the study (pasteurized or fresh). The control group of 18 volunteers did not ingest any yogurt. Three different fecal samples were recovered per individual from both study groups and the control group: a first baseline sample after a week without yogurt in the diet, a second sample after completion of the first round, and a third after the second round of yogurt intake. The Fleiss method was used to carry out statistical analysis of research results (5).

A sample of 0.5 g of fresh feces from each one of the volunteers was suspended in 5 ml of saline. Samples were centrifuged at low speed for 5 min, 1 ml of the upper phase was collected, and 100-μl portions of these samples were consecutively diluted in saline and seeded in appropriate plates for bacterial count determination and phenotypic colony detection. The morphology of all colonies growing in MRS and M17 plates inoculated with 10^{-3} and 10^{-4} dilutions of fecal samples was analyzed after 48 h. At least five different colonies with each morphology compatible with that of the *L. delbrueckii* or *S. thermophilus* control strains, grown from fresh yogurt, were subcultured for each fecal sample and used for PCR detection of these organisms with specific primers. For total fecal DNA extraction (200 μl of the centrifuge upper phase), the QIAamp DNA stool minikit (QiaGen, Hilden, Germany) was used as recommended for PCR experiments with fecal material (14). One milliliter of each sample was PCR tested with the specific *L. delbrueckii* or *S. thermophilus* primers. DNA extraction with a QIAamp minikit was applied to both fresh and pasteurized yogurts to assess positive amplifications for both bacterial types. To evaluate the limit of detection by amplification, con-

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trol amplification experiments were carried out with mixtures of both yogurt types with feces (1:10 to 1:1,000,000 proportions).

Cultures of *L. delbrueckii* and *S. thermophilus* (Christian Hansen industrial yogurt starter reference YF203) were, respectively, obtained with MRS agar (Difco, Detroit, Mich.) at 37°C and in M17 agar (Difco) at 42°C and 10% CO_2 after at least 48 h of incubation. Five different colonies with each different morphology were subcultured and preidentified by Gram staining. The identification of yogurt strains was confirmed by PCRs with the specific primers described by Lick et al. (12). Primers for *L. delbrueckii* based on the *add* gene sequence were DEL-F (5′ AATTCCGTCAACTCCTCATC 3′) and DEL-R (5′ TGATCCGCTGCTTCATTTCA 3′). Cycling conditions for these primers were 10 cycles of 20 s at 94°C, 75 s at 65°C, and 40 s at 72°C, followed by 35 cycles of 20 s at 94°C, 50 s at 55°C, and 30 s at 72°C. A final elongation of 3 min at 72°C was applied. The amplicon obtained with the positive controls had a size of 715 bp. The primers based on the *lacZ* gene used for *S. thermophilus* identification were THER-F (5′ CACTATGCTCAGAATACA 3′) and THER-R (5′ CGAACAGCATTGATGTTA 3′). The conditions of this PCR were 35 cycles of 20 s at 94°C, 60 s at 58°C, and 30 s at 72°C, followed by 3 min of elongation at 72°C. Positive controls produced an amplicon of 968 bp.

During the study, the bacterial content of fresh yogurt samples was in compliance with all legal regulatory requirements at the time of intake and the compliance status of all fresh yogurt samples held in storage was monitored weekly to ensure continued compliance. Measurements of the bacterial content of fresh yogurt samples were carried out in accordance with all applicable standards (International Dairy Federation international standard 1996, milk and milk products: preparation of samples and dilutions for microbiological examinations, no. 122C).

For hybridization experiments, probes were obtained from purified PCR-specific fragments. For dot blot hybridization, 5 μl of total fecal DNA obtained with the QIAamp minikit was transferred onto a nylon membrane (Hybond; Amersham) as previously described (13). All fecal samples were tested, and positive and negative controls were included. The probes were labeled by random primer labeling (Rediprime; Amersham) with [^{32}P]dCTP (Redivue; Amersham). Prehybridization and hybridization were carried out with Rapid Buffer (Amersham) at 60°C for 30 min and at 56°C for 18 h, respectively. The filters were washed twice at 56°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and then twice at room temperature with 1× SSC–0.1% SDS and 0.7× SSC–0.1% SDS, successively. Autoradiography was carried out by exposing the filters for 72 h at −80°C.

To recover yogurt lactic acid bacteria by culture, MRS or M17 plates were inoculated with fecal samples from all of the volunteers in both experimental groups and the control group. Only the colony morphotypes compatible with the target yogurt bacteria in the Gram stain were tested by PCR with the specific primers. A total of 427 PCR amplifications of the suspected colonies from the 114 volunteers were consistently negative for the target organisms, either in the baseline samples or in the phases of ingesting pasteurized or fresh yogurt, as well as in the control group. These results indicate the absence of viable *L. delbrueckii* and *S. thermophilus* at concentrations higher than 10^3 CFU/g (our limit of detection) in fecal samples.

The detection of yogurt lactic acid bacteria by direct DNA amplification in feces was also consistently negative. Not a single positive amplification for *L. delbrueckii* or *S. thermophilus* was obtained when the total DNA from the feces of the 114 human volunteers was studied. Negative detection occurred not only in the control group or the baseline fecal preparations but also in the samples obtained after repeated ingestion of either pasteurized or fresh yogurt. Clear positive amplification products with the expected size for *L. delbrueckii* and *S. thermophilus* were always obtained in PCR experiments with fresh or pasteurized yogurt or with mixtures (100,000:1) of feces with yogurt containing viable organisms, with an estimated limit of detection of 1 to 5 bacteria/ml, respectively. In summary, ingested yogurt bacteria were not detectable by conventional culture or by PCR in feces within the limits of the assays.

Detection of *L. delbrueckii* and *S. thermophilus* by specific DNA hybridization of fecal samples proved to be more sensitive than culture or amplification. Hybridization assays with specific probes yielded negative results for the control group and all baseline samples from the experimental group. Eight positive results were observed in the *L. delbrueckii* hybridization experiments, seven of them in DNA from fecal samples of different volunteers obtained at the end of fresh yogurt ingestion (8.4%) and in only one in DNA from feces recovered after the pasteurized yogurt round (1.05%). When the *S. thermophilus*-specific probe was used, four positive hybridizations were obtained for different volunteers, belonging in three cases to the fresh yogurt round (3.15%) and in one case to the pasteurized yogurt preparation (1.05%). Two patients (both in the fresh yogurt round) produced positive hybridization for both target lactic acid bacteria. DNA compatible with either of the yogurt starter bacteria was found by hybridization experiments in 10 (10.52%) of the 96 individuals in the fresh yogurt group and in 2 (2.10%) of the 96 in the pasteurized yogurt group ($P = 0.01$).

Despite the widely held assumptions about the probiotic properties of classic lactic acid bacteria in yogurt, *L. delbrueckii* and *S. thermophilus*, data about the function of these organisms in the human intestine remain scarce at best and unconvincing where they exist at all (16). The new molecular techniques provide a much greater opportunity to examine this important research question. In the carefully controlled experiments reported in this paper, we were consistently unable to detect viable yogurt lactic acid bacteria in fecal samples after repeated yogurt consumption by healthy volunteers. *L. delbrueckii* and/or *S. thermophilus* DNA remains were detected by hybridization assays in only 10% of volunteers who had ingested fresh yogurt.

In the experiment reported in this paper, we only tested the viability of yogurt bacteria in feces, and lack of survival of these organisms in the upper gastrointestinal tract cannot be inferred from these results. However, our negative results obtained by both culture and DNA detection in feces indicate that a substantial multiplication of yogurt bacteria in the small intestine is not expected to occur. In hybridization experiments, a significantly higher proportion of positive results was obtained in volunteers after intake of fresh yogurt than among

volunteers who ingested pasteurized yogurt, but even in the first group, 90% of the volunteers were negative for any remains of yogurt bacterial DNA. We cannot discard the possibility that the higher number of positive results among volunteers consuming fresh yogurt is not necessarily because of a higher colonization of the probiotic bacteria but is due to the more extensive degradation of the DNA of previously heat-killed bacteria in pasteurized yogurt.

Viable yogurt starter bacteria have been found in human duodenal samples (15), but the short-term viability of these organisms after contact with gastric juice or bile salts is very poor (2) and highly sensitive to the type and amount of food ingested (3, 10, 12). Consequently, the presumed probiotic effect of yogurt should depend on the frequency of ingestion (9, 11, 17). That is what is expected for organisms that, like *L. delbrueckii* and *S. thermophilus*, are not substantially represented in the natural human intestinal microbiota.

The definition recommended by the Food and Agriculture Organization and the World Health Organization for probiotics is "Live microorganisms which when administrated in adequate amounts confer a health benefit on the host" (6). Our results show that this may not be the case for yogurt bacteria. Obviously, we cannot discount on the basis of our experiments the possibility that the effect of yogurt is more related to prebiotics than to probiotics. With respect to the presumed health benefit (if health can be improved!), our experimental groups were analyzed for gastrointestinal comfort and again no difference was found between fresh and pasteurized yogurt (unpublished data). This is in contrast to the reports of other authors, who found that live bacteria are essential to a beneficial effect (7, 18). In summary, we have been unable to confirm, with either classic or molecular tools, the role of fresh yogurt preparations in supplying health-promoting bacterial organisms to the human intestinal flora.

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