Vol. 72, No. 7

Survival of Yogurt Bacteria in the Human Gut

Marina Elli,[1]* Maria Luisa Callegari,[2] Susanna Ferrari,[2] Elena Bessi,[1] Daniela Cattivelli,[1]
Sara Soldi,[1] Lorenzo Morelli,[1] Nathalie Goupil Feuillerat,[3] and Jean-Michel Antoine[3]

*AAT-Advanced Analytical Technologies S.r.l., 29100 Piacenza, Italy[1]; Centro Ricerche Biotecnologiche,
26100 Cremona, Italy[2]; and Danone Vitapole, Nutrivaleur, 91767 Palaiseau Cedex, France[3]*

Whether *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* can be recovered after passage through the human gut was tested by feeding 20 healthy volunteers commercial yogurt. Yogurt bacteria were found in human feces, suggesting that they can survive transit in the gastrointestinal tract.

Yogurt, defined as the product of milk fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, has a long history of beneficial impact on the well-being of humans. In a few articles workers have focused on the scientifically documented impact of yogurt cultures alone on gut metabolism (1, 23). Yogurt bacteria have been shown to improve lactose digestion in lactose-intolerant individuals (16, 17, 20, 21, 27), to affect the intestinal transit time (11), and to stimulate the gut immune system (2, 14, 31), although some authors failed to confirm these conclusions (5, 6, 35). The immunological and genetic basis of the immunostimulatory properties of yogurt starters has also been investigated (8, 15).

Nevertheless, the scientific background of these studies and the application of the term "probiotic" to the starter bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* are still strongly debated (13, 33). As indicated by Guarner et al. (13), recent scientific developments have challenged the validity and usefulness of the in vitro selection criteria traditionally proposed for probiotics.

Traditional yogurt starters have nonhuman origins, and they (especially streptococci) are known to suffer from exposure to gastric acidic conditions (7) and to have a moderate ability to adhere to intestinal epithelial cells (12).

Survival during passage through the gastrointestinal tract is generally considered a key feature for probiotics to preserve their expected health-promoting effects (3).

There have been conflicting studies concerning the recovery of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* from fecal samples after daily yogurt ingestion. Some authors reported that *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were not recovered from feces of young (9) and elderly (26) subjects. On the other hand, Brigidi et al. (4) reported that for 6 days after the end of treatment, they recovered *S. thermophilus* from fecal samples from 10 healthy subjects who had ingested a pharmaceutical preparation orally for 3 days. The persistence of a yogurt culture in the human gut was also recently confirmed by Mater et al. (22), who studied 13 healthy volunteers fed yogurt containing rifampin- and streptomycin-resistant strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

The aim of this study was to investigate the recovery of viable *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* from fecal samples from 20 healthy volunteers fed commercial yogurt for 1 week.

Our study included 10 male and 10 female healthy subjects whose mean age was 32.3 years. These subjects ate 125 g of commercial yogurt (Bianco Naturale; Danone) twice a day for 1 week. The volunteers, who were divided into two groups of 10 subjects, were investigated once to determine the persistence of yogurt cultures in fecal samples.

Two subsequent trials (trials 1 and 2) were perfromed with 10 subjects each. The volunteers were required to abstain from consumption of yogurt and fresh dairy products for 2 weeks before the beginning of the trials. The daily intake of bacteria from yogurt, containing 2.4×10^7 CFU/g of *L. delbrueckii* subsp. *bulgaricus* and 2.0×10^8 CFU/g of *S. thermophilus*, was about 6×10^9 CFU of *L. delbrueckii* subsp. *bulgaricus* and 5×10^{10} CFU of *S. thermophilus*.

Fecal samples, which were obtained at the beginning of each trial (zero time) and after 2 and 7 days, were stored for a maximum of 12 h at 4 to 8°C. A 1-g sample of fecal material was decimally diluted in sterile saline and plated onto skim milk medium (RSMA) (Difco, BD, Sparks, Md.) in trial 1. In trial 2, RSMA was supplemented with 0.05% ruthenium red dye (Sigma, St. Louis, Mo.) to obtain modified RSMA (m-RSMA). In both cases the plates were incubated at 44°C for 48 h.

In this study great emphasis was placed on selection of a medium suitable for reliable and reproducible detection of lactobacilli and streptococci in feces. The ability of RSMA to support the growth of clearly distinguishable colonies was determined, and the performance of this medium was refined by addition of ruthenium red dye (24) (m-RSMA), which allowed us to clearly distinguish the white halos of small *Enterococcus* colonies from the pink halos of *Lactobacillus* and *Streptococcus* colonies. After prolonged incubation at 44°C, colonies on m-RSMA plates changed from pink to fuchsia for streptococci and from old rose to yellow for lactobacilli, which allowed us to prescreen the fecal microflora of treated volunteers and to

* Corresponding author. Mailing address: AAT-Advanced Analytical Technologies S.r.l., Spin-off of the Catholic University of Piacenza, Via Emilia Parmense, 84, 29100 Piacenza, Italy. Phone: 39-335-7988122. Fax: 39-0523-599246. E-mail: marina.elli@unicatt.it.

TABLE 1. Recovery of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* from fecal samples after yogurt ingestion

No. of detections per subject[a]	No. of subjects whose feces contained viable yogurt strains	
	L. delbrueckii subsp. *bulgaricus*	*S. thermophilus*
Trial 1		
Two	1	0
One	5	0
None	4	10
Trial 2		
Two	6	0
One	1	1
None	3	9
Total	20	20

[a] Two indicates detection of the microorganism on days 2 and 7, while one indicates that the strain was detected only on day 7.

TABLE 2. Concentrations of viable *L. delbrueckii* subsp. *bulgaricus* in feces of subjects who consumed yogurt[a]

Trial	Subject	Concn ($\log_{10}$ CFU/g [wet wt] feces)	
		Day 2	Day 7
1	1	ND[c]	3.7
	2	4.0	3.9
	3	ND	3.6
	4	ND	3.3
	5	ND	3.0
	6	ND	4.0
2	7	5.5	5.1
	8	4.0	4.0
	9	5.6	3.8
	10	5.2	4.7
	11	ND	5.7
	12[b]	4.0	5.0
	13	4.5	5.0

[a] Subjects whose feces did not contain viable yogurt strains were not included (see Table 1).
[b] The feces of this subject also contained 5.6 $\log_{10}$ CFU of *S. thermophilus* per g (wet weight) on day 7.
[c] ND, viable *L. delbrueckii* subsp. *bulgaricus* not detected.

reduce the number of isolates that were identified by molecular tools.

Colonies having *Lactobacillus*- and *Streptococcus*-like morphologies on days 2 and 7 were replica plated, using 10% of the colonies recovered from the first readable plate, onto MRS medium (Difco, Detroit, Mich.) and LM17 medium (Oxoid, Basingstoke, Hampshire, United Kingdom), respectively, incubated at 44°C for 48 h, and directly lysed with MicroLYSIS (Microzone Ltd., Haywards Heath, West Sussex, United Kingdom). Lysed suspensions were used as templates in species- and strain-specific PCRs.

L. delbrueckii subsp. *bulgaricus* and *S. thermophilus* strains that were used as controls in amplification experiments were isolated from commercial yogurt by plating on RSMA, and their taxonomic identities were confirmed by using molecular tools.

A three-step identification protocol was used for *L. delbrueckii* subsp. *bulgaricus*-like colonies grown on selective medium, using primers SS1 and DB1 (10) for *L. delbrueckii* species and primers 34/2 and 37/1 (18) for *L. delbrueckii* subsp. *bulgaricus* and the cycling conditions described previously. Colonies from days 2 and 7 that gave the expected amplicon size with the PCR primers of Lick et al. (18) were then analyzed by the repetitive extragenic palindromic PCR (Rep-PCR) technique using primer GTG5, as described by Versalovic et al. (32), to confirm the identities of fecal isolates from subjects who had consumed yogurt and orally administered *L. delbrueckii* subsp. *bulgaricus* isolates.

A two-step identification protocol was used for *S. thermophilus*-like colonies, which involved primers upstream and downstream of the *lacZ* gene (19). Isolates that were confirmed to be *S. thermophilus* isolates were then analyzed by pulsed-field gel electrophoresis (PFGE) because of the lack of reliable PCR primers that could distinguish between the closely related species *S. thermophilus* and *Streptococcus salivarius* present in human oral cavity (28, 34). Profiles of SmaI-digested chromosomes, obtained as described by O'Sullivan and Fitzgerald (25), were compared in order to determine strain identities.

Molecular biology tools were used for strain-specific identification of orally administered yogurt strains isolated from fecal samples previously plated onto RSMA (trial 1) and m-

RSMA (trial 2). The total counts obtained on these media resulted in a maximum concentration of 10^7 viable cells per g (fresh weight) of feces in both trials before consumption of yogurt (zero time). Moreover, a significant increase in the counts, especially in trial 2, was observed after 1 week of yogurt consumption (day 7) for 4 of 10 volunteers whose initial counts in this medium were very low (around 10^3 CFU per g [fresh weight] of stool).

The absence of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains from fecal samples obtained at zero time was investigated and confirmed (data not shown).

Recovery of viable yogurt strains in trial 1. After 1 week of treatment, 41 fecal isolates were subjected to PCR analysis with *L. delbrueckii* species-specific primers, which led to identification of *L. delbrueckii* subsp. *bulgaricus* in 35 cases involving 6 of 10 volunteers, as shown in Table 1. The numbers of viable *L. delbrueckii* subsp. *bulgaricus* CFU in fecal samples are shown in Table 2.

No *Streptococcus*-like colonies were retrieved from plates on day 2 or on day 7.

Recovery of viable yogurt strains in trial 2. Fecal colonies from day 2 samples (110 *Streptococcus*-like colonies and 110 *Lactobacillus*-like colonies) and from day 7 samples (23 *Streptococcus*-like colonies and 19 *Lactobacillus*-like colonies) were replica plated onto MRS and LM17. Higher numbers of colonies were investigated for day 2 in order to confirm the reliability of m-RSMA for distinguishing between enterococci, lactobacilli, and streptococci.

The presence of *L. delbrueckii* subsp. *bulgaricus* in feces was confirmed for 6 of 10 human subjects on day 2, and the number increased by day 7, when orally administered *Lactobacillus* was present in 7 volunteers (Table 1). The fecal counts for *L. delbrueckii* subsp. *bulgaricus*, as confirmed by using strain-specific molecular tools, appeared to increase in trial 1 (Table 2), which confirmed the improved performance of m-RSMA compared to the performance of RSMA.
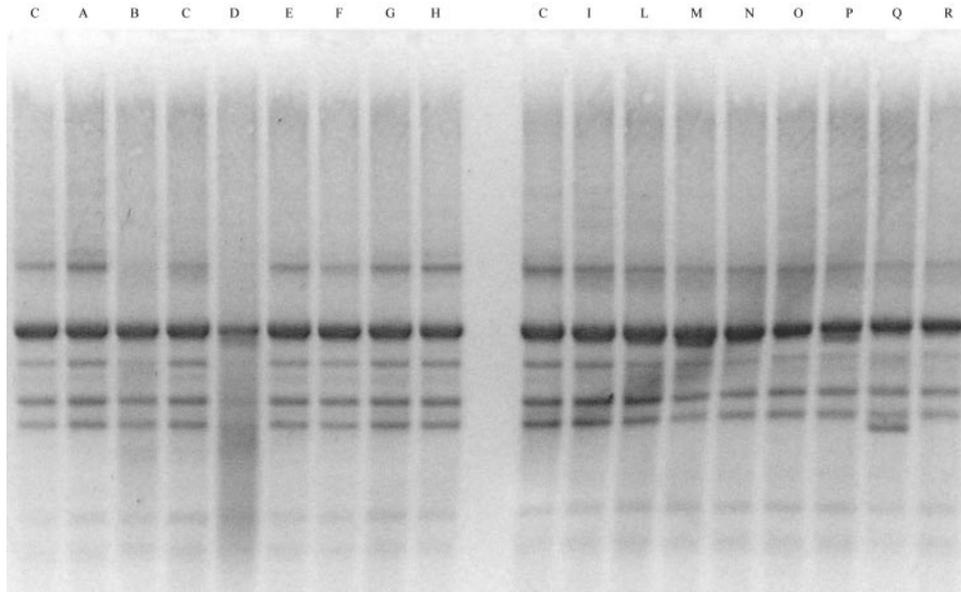
FIG. 1. Strain-specific Rep-PCR detection of orally administered *L. delbrueckii* subsp. *bulgaricus* in fecal samples from subjects who consumed yogurt. Isolates were previously confirmed to belong to the *L. delbrueckii* group by species-specific PCR. Lanes C, profile of orally administered *L. delbrueckii* subsp. *bulgaricus*; lanes A to H, profiles of isolates obtained on day 2 in trial 2; lanes I to R, profiles of isolates obtained on day 7 in trial 2. Lanes D and Q were not considered comparable to controls.

Rep-PCR strain-specific profiles of yogurt cultures and fecal isolates are shown in Fig. 1.

Molecular identification led to accurate detection of an orally administered *Streptococcus* strain in only one subject on day 7, despite the fact that another colony was recognized as an *S. thermophilus* colony by the PCR approach. Figure 2 shows that the orally administered *S. thermophilus* strain and the strain isolated from one volunteer were identical, while *S. thermophilus* isolated from another volunteer in trial 2 had a clearly different PFGE profile.

After 2 days of treatment, the recovery of orally administered *L. delbrueckii* subsp. *bulgaricus* in trial 2 was significantly greater than the recovery of orally administered *L. delbrueckii* subsp. *bulgaricus* in trial 1 (Table 2), which confirmed the contribution of m-RSMA to refinement of the microbiological approach used to assess the survival of the yogurt culture. The viability of orally administered *L. delbrueckii* subsp. *bulgaricus* during passage through the gastrointestinal tract of humans was therefore confirmed, as described by Mater et al. (22), who recovered viable *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* from feces of healthy volunteers fed 375 g of yogurt per day. These conclusions were in contrast with the conclusions of Del Campo et al. (9), who reported consistently negative results for detection of plate colonies of yogurt bacteria from fecal samples of 114 volunteers, even if there were many positive results following hybridization of fecal DNA with species-specific probes. In this case, fecal specimens were plated onto media with poor selective properties, like MRS and M17, and plates were incubated in nonstringent conditions (37°C for thermophilic lactic acid bacteria), which resulted in growth of a background intestinal microflora that masked *Streptococcus*- and *Lactobacillus*-like colonies, particularly
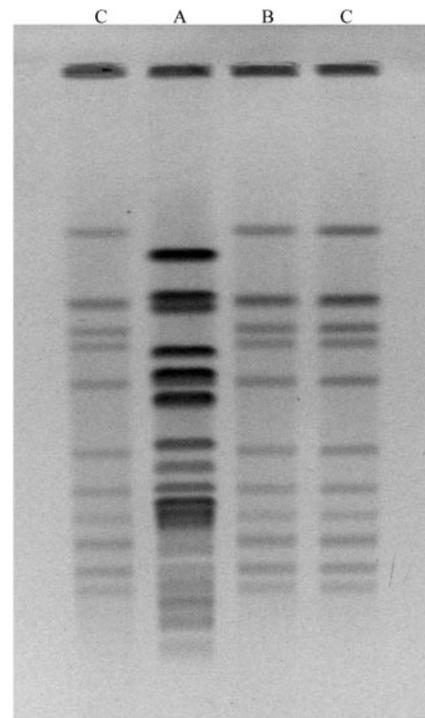

FIG. 2. Strain-specific identification of orally administered *S. thermophilus* by PFGE analysis of two colonies from day 7 samples (trial 2) (lanes A and B) previously identified as belonging to *S. thermophilus* by species-specific PCR. The PFGE profile in lane B is identical to the profile for the orally administered strain in lanes C.

at low dilutions. Therefore, the presence of orally administered strains could have been underestimated.

In our study, *S. thermophilus* was retrieved from only one volunteer on day 7, but we cannot exclude the possibility that a prolonged ingestion period or a larger amount of ingested yogurt, as described by Mater et al. (22), could positively affect the rate of *S. thermophilus* recovery from fecal samples. However, several authors have shown that *S. thermophilus* suffers from the environmentally adverse gastric conditions (9, 26).

A recent report (4) described detection and enumeration of *S. thermophilus* from stool samples of 10 healthy subjects and 10 patients with irritable bowel syndrome, half of whom were treated with 250 g of yogurt per day and half of whom were treated with a probiotic pharmaceutical preparation. The thermophilic streptococci in feces were quantified by the authors using primers ThI and ThII (30), which we also tested in order to monitor the reliability of this species-specific primer pair. Our results confirmed that primers ThI and ThII could not discriminate between *S. thermophilus* and *S. salivarius*, as previously described by Tilsala-Timisjarvi and Alatossava (30). Species-specific PCR amplification performed with primers ThI and ThII could lead to incorrect results and, together with the lack of subsequent strain-specific recognition, could easily explain the retrieval of thermophilic streptococci in fecal samples described by Brigidi et al. (4). These observations confirm that assessment of yogurt culture survival in the human gut requires reliable molecular tools, such as PCR primers for species- and strain-specific identification of orally administered strains.

Since recently there have been conflicting reports, several authors are still debating the probiotic properties of traditional yogurt starters. Probiotic traits are currently considered to be strictly strain specific (29); therefore, the use of strain-specific molecular approaches is very relevant to the study of the survival and effects of yogurt starters on host health.

Moreover, the ability to survive transit through the gastrointestinal tract, the ability to reach the distal tract in a viable form, and the ability to be recovered from feces by culture methods are unequivocally considered key features for a probiotic. These considerations led us to focus on accurate strain-specific retrieval of viable yogurt cultures in fecal samples, while other authors failed to consider this aspect (9).

In conclusion, we confirmed that yogurt bacteria, especially *L. delbrueckii* subsp. *bulgaricus*, can be retrieved from feces of healthy individuals after a few days of ingestion of commercial yogurt. Moreover, our results indicate that very careful setup of the analytic procedures can dramatically improve the reliability of studies of the survival of yogurt starters.

REFERENCES

1. **Adolfsson, O., S. N. Meydani, and R. M. Russell.** 2004. Yogurt and gut function. Am. J. Clin. Nutr. **80:**245–256.
2. **Aldinucci, C., L. Bellussi, G. Monciatti, G. C. Passàli, L. Salerni, D. Passàli, and V. Bocci.** 2002. Effects of dietary yoghurt on immunological and clinical parameters of rhinopathic patients. Eur. J. Clin. Nutr. **56:**1155–1161.
3. **Bezkorovainy, A.** 2001. Probiotics: determinants of survival and growth in the gut. Am. J. Clin. Nutr. **73**(Suppl. 2):399S–405S.
4. **Brigidi, P., E. Swennen, B. Vitali, M. Rossi, and D. Matteuzzi.** 2003. PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces of human subjects after oral bacteriotherapy and yogurt consumption. Int. J. Food Microbiol. **81:**203–209.
5. **Campbell, C. G., B. P. Chew, L. O. Luedecke, and T. D. Shultz.** 2000. Yogurt consumption does not enhance immune function in healthy premenopausal women. Nutr. Cancer **37:**27–35.
6. **Carlsson, P., and D. Bratthall.** 1985. Secretory and serum antibodies against *Streptococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* in relation to ingestion of fermented milk products. Acta Odontol. Scand. **43:**147–153.
7. **Conway, P. L., S. L. Gorbach, and B. R. Goldin.** 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. **70:**1–12.
8. **Cross, M. L., L. M. Stevenson, and H. S. Gill.** 2001. Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? Int. Immunopharmacol. **1:**891–901.
9. **Del Campo, R., D. Bravo, R. Canton, P. Ruiz-Garbajosa, R. Garcia-Albiach, A. Montesi-Libois, F.-J. Yuste, V. Abraira, and F. Baquero.** 2005. Scarce evidence of yogurt lactic acid bacteria in human feces after daily yogurt consumption by healthy volunteers. Appl. Environ. Microbiol. **71:**547–549.
10. **Drake, M., C. L. Small, S. D. Kemet, and B. G. Swanson.** 1996. Rapid detection and identification of *Lactobacillus* spp. in dairy products by using the polymerase chain reaction. J. Food Prot. **59:**1031–1036.
11. **Fioramonti, J., V. Theodorou, and L. Bueno.** 2003. Probiotics: what are they? What are their effects on gut physiology? Best Pract. Res. Clin. Gastroenterol. **17:**711–724.
12. **Greene, J. D., and T. R. Klaenhammer.** 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl. Environ. Microbiol. **60:**4487–4494.
13. **Guarner, F., G. Perdigon, G. Corthier, S. Salminen, B. Koletzko, and L. Morelli.** 2005. Should yoghurt cultures be considered probiotic? Br. J. Nutr. **93:**783–786.
14. **Halpern, G. M., K. G. Vruwink, J. Van de Water, C. L. Keen, and M. E. Gershwin.** 1991. Influence of long-term yoghurt consumption in young adults. Int. J. Immunother. **7:**205–210.
15. **Kitazawa, H., H. Watanabe, T. Shimosato, Y. Kawai, T. Itoh, and T. Saito.** 2003. Immunostimulatory oligonucleotide, CpG-like motif exists in *Lactobacillus delbrueckii* ssp. *bulgaricus* NIAI B6. Int. J. Food Microbiol. **85:**11–21.
16. **Labayen, I., L. Forga, A. Gonzalez, I. Lenoir-Wijnkoop, R. Nutr, and J. A. Martinez.** 2001. Relationship between lactose digestion, gastrointestinal transit time and symptoms in lactose malabsorbers after dairy consumption. Aliment. Pharmacol. Ther. **15:**543–549.
17. **Lerebours, E., N. C. N'Djitoyap, A. Lavoine, M. F. Hellot, J. M. Antoine, and R. Colin.** 1989. Yogurt and fermented-then-pasteurized milk: effects of short-term and long-term ingestion on lactose absorption and mucosal lactase activity in lactase-deficient subjects. Am. J. Clin. Nutr. **49:**823–827.
18. **Lick, S., E. Brockmann, and K. J. Heller.** 2000. Identification of *Lactobacillus delbrueckii* and subspecies by hybridization probes and PCR. Syst. Appl. Microbiol. **23:**251–259.
19. **Lick, S., M. Keller, W. Bockelmann, and K. J. Heller.** 1996. Rapid identification of *Streptococcus thermophilus* by primer-specific PCR amplification based on its *lacZ* gene. Syst. Appl. Microbiol. **19:**74–77.
20. **Marteau, P., B. Flourie, P. Pochart, C. Chastang, J. F. Desjeux, and J. C. Rambaud.** 1990. Effect of the microbial lactase (EC 3.2.2.23) activity in yoghurt on the intestinal absorption of lactose: an *in vivo* study in lactase-deficient humans. Br. J. Nutr. **64:**71–79.
21. **Martini, M. C., D. Kukielka, and D. A. Savaiano.** 1991. Lactose digestion from yogurt: influence of a meal and additional lactose. Am. J. Clin. Nutr. **53:**1253–1258.
22. **Mater, D. D. G., L. Bretigny, O. Firmesse, M.-J. Flores, A. Mogenet, J.-L. Bresson, and G. Corthier.** 2005. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yoghurt. FEMS Microbiol. Lett. **250:**185–187.
23. **Meydani, S. N., and W.-K. Ha.** 2000. Immunologic effects of yogurt. Am. J. Clin. Nutr. **71:**861–872.
24. **Mora, D., M. G. Fortina, C. Parini, G. Ricci, M. Gatti, G. Giraffa, and P. L. Manachini.** 2002. Genetic diversity and technological properties of *Streptococcus thermophilus* strains isolated from dairy products. J. Appl. Microbiol. **93:**278–287.
25. **O'Sullivan, T. F., and G. F. Fitzgerald.** 1998. Comparison of *Streptococcus thermophilus* strains by pulsed field gel electrophoresis of genomic DNA. FEMS Microbiol. Lett. **168:**213–219.
26. **Pedrosa, M. C., B. B. Golner, B. R. Goldin, S. Barakat, G. E. Dallal, and R. M. Russell.** 1995. Survival of yogurt-containing organisms and *Lactobacillus gasseri* (ADH) and their effect on bacterial enzyme activity in the gastrointestinal tract of healthy and hypochlorhydric elderly subjects. Am. J. Clin. Nutr. **61:**353–359.
27. **Pochart, P., O. Dewit, J. F. Desieux, and P. Bourlioux.** 1989. Viable starter cultures, beta-galactosidase activity, and lactose in duodenum after yoghurt ingestion in lactase-deficient humans. Am. J. Clin. Nutr. **49:**828–831.
28. **Sachs, G., D. L. Weeks, K. Melchers, and D. R. Scott.** 2003. The gastric biology of *Helicobacter pylori*. Annu. Rev. Physiol. **65:**349–369.
29. **Senok, A. C., A. Y. Ismaeel, and G. A. Botta.** 2005. Probiotics: facts and myths. Clin. Microbiol. Infect. **11:**958–966.
30. **Tilsala-Timisjarvi, A. T., and T. Alatossava.** 1997. Development of oligonu-

cleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int. Food Microbiol. **35:**49–56.

31. **Van der Water, J., C. L. Keen, and M. E. Gershwin.** 1999. The influence of chronic yogurt consumption on immunity. J. Nutr. **129**(Suppl. 7)**:**1492S–1495S.

32. **Versalovic, J., M. Schneider, F. J. de Brujin, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. **5:**25–40.

33. **Von der Weid, T., A. Donnet-Hughes, S. Blum, E. J. Schiffrin, J. R. Neeser, and A. Pfeifer.** 2001. Scientific thoroughness of human studies showing immune-stimulating properties of yogurt. Am. J. Clin. Nutr. **73:**133–134.

34. **Watanabe, T., H. Shimohashi, Y. Kawai, and M. Mutai.** 1981. Studies on streptococci. I. Distribution of fecal streptococci in man. Microbiol. Immunol. **25:**257–269.

35. **Wheeler, J. G., M. L. Bogle, S. J. Shema, M. A. Shirrell, K. C. Stine, A. J. Pittler, A. W. Burks, and R. M. Helm.** 1997. Impact of dietary yogurt on immune function. Am. J. Med. Sci. **313:**120–123.