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Early Intestinal Bacterial Colonization and Necrotizing Enterocolitis in Premature Infants: The Putative Role of *Clostridium*

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ABSTRACT

Necrotizing enterocolitis (NEC) is among the most severe conditions that can affect preterm infants. Although the etiology of NEC remains unknown, initial bacterial colonization could play a pivotal role in the development of NEC. To further explore the putative relationship between pathogen microorganisms and NEC, we conducted a prospective case-control study in 12 preterm infants with a new approach based on molecular techniques. Over an inclusion period of 24 mo, 12 neonates of <34 wk gestational age admitted to the neonatal unit were enrolled. The group included three cases of NEC, and nine control infants without evidence of NEC who were matched for gestational age and birth weight. Stool samples were collected at weekly intervals from all infants. PCR and temporal temperature gradient gel electrophoresis of 16S ribosomal DNA were used to detect the establishment of bacterial communities in the digestive tract. A salient feature of the bacteriological pattern was observed only in the three infants who later developed NEC: A band corresponding to the *Clostridium perfringens* subgroup could be detected in early samples, before diagnosis. There was no evidence for this specific band in any of the nine controls. To our knowledge, the current report is the first to demonstrate that the use of molecular techniques based on the study of bacterial 16S rRNA genes allowed the recognition of *C. perfringens* species in the first 2 wk of life of three infants who later displayed symptoms of NEC. A significant temporal relationship was thus established between early colonization by *Clostridium* and the later development of NEC. Compared with conventional bacteriological culturing methods, the use of this new molecular approach to analyze the gastrointestinal ecosystem should therefore allow a more complete and rapid assessment of intestinal flora. Although the current data do not constitute definitive proof that the identified bacterial species was a causative agent in the development of NEC, they outline the promise of this new technique based on molecular biology, and suggest that large-scale studies on a much wider population at high risk for NEC may be warranted. **(*Pediatr Res* 56: 366–370, 2004)**

Abbreviations

NEC, necrotizing enterocolitis
16S rDNA, 16S ribosomal DNA
TTGE, temporal temperature gradient gel electrophoresis
TAE, tris-acetate/EDTA
DDGE, denaturing gradient gel electrophoresis

NEC is a major cause of morbidity and mortality in preterm infants. Although the etiology of NEC remains unknown, primary risk factors include intestinal immaturity, intestinal ischemia, and bacterial colonization of the intestine (1). As the gastrointestinal tract of a normal fetus is sterile, bacterial colonization must begin in the birth process and soon thereafter.

Previous studies have explored the establishment of bacterial flora in term and preterm infants (2–4). Several studies noted that initial bacterial colonization could play a pivotal role in the development of NEC (5–7). Yet, classical methods using culture have not been able to consistently identify specific pathogens before the onset of NEC, presumably because of incomplete bacterial analysis (*e.g.* the search for anaerobic bacteria).

The first study using molecular methods to explore the establishment and succession of bacterial strains in the gastrointestinal tract of term infants was reported by Favier *et al.* (8) in two healthy babies by monitoring 16S rDNA diversity in fecal samples. They were able to show that band patterns were

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very simple during the first few days of life and became more complex over time. Yet little is known on the composition of the bacterial community of premature infants.

TTGE is a rapid and inexpensive molecular method to analyze diversity within bacterial communities. It is based on direct analysis of DNA in the environment and does not require cell cultivation. This approach involves extraction of bacterial DNA, amplification of genes (16S rDNA), and analysis of PCR products by genetic fingerprinting. TTGE successfully differentiates closely related target species, and is a powerful method for revealing sequence heterogeneities in the 16S rDNA. The early work of Godon *et al.* (9) on microbial communities expresses concern about the use of molecular biologic techniques, especially those that take advantage of the small-subunit of rDNA. The work of Vasquez *et al.* (10) gives a good example of the use of this new technique in the study of *Lactobacillus casei* complex.

To further explore the putative relationship between pathogen microorganisms and NEC, we conducted a case-control study with an approach based on molecular techniques. By those methods, it is possible to detect the presence of bacteria by identifying their 16S rDNA, irrespective of whether or not the bacteria can be cultured. The potential contribution of the detected bacteria in the pathogenesis of NEC is discussed.

METHODS

Subjects. Subjects were recruited from a single neonatal intensive care unit (NICU) at Nantes University Medical Center on the first day of life. As it was not an invasive study, requiring the collection of stools as the only samples, no written consent was required from the parents, according to policies defined by the local ethical committee (CCPPRB Pays-de-Loire France). Over a study period of 20 mo, 117 preterm infants of <30 wk of gestation were admitted to the NICU at Nantes University Hospital. Starting on the first day of life, stool samples were obtained at weekly intervals from infants at risk of NEC. Among this population, three infants happened to develop cataclysmic NEC with severe sepsis, and two of them died. They were sporadic cases at successive intervals of 3 and 6 mo. These three infants were matched, for gestational age and birth weight, with nine control infants without evidence of NEC.

DNA isolation and 16S rDNA amplification. Stool samples were collected in sterile tubes and immediately stored at −80°C until analysis. Total DNA was extracted as described previously (9). The concentration and integrity of the nucleic acids were determined visually after electrophoresis on a 1% agarose gel containing ethidium bromide. Primers U968-GC (5' CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC) were used to amplify the V6 to V8 regions of the bacterial 16S rDNA. PCR was performed using Hot Star *Taq* DNA polymerase (QIAGEN, Courtaboeuf, France). PCR mixtures of 50 μL contained 1× PCR buffer, 1.5 mM Mg Cl_2, 0.1 mM of each dNTP, 0.5 μM of primers U968-GC and L1401, 2.5 U of Hot Star *Taq* Polymerase, and approximately 1 ng of DNA. The samples were amplified in a Gene Amp PCR system 9700 (PerkinElmer, Nantes, France) using the following program: 95°C for 15 min; 30 cycles of 94°C for 1 min, 56°C for 1min, 72°C for 1.5 min, and finally 72°C for 15 min. Both negative (without DNA) and positive controls (samples with DNA extract from a *Clostridium perfringens* species) were used in every reaction. The rationale for choosing *C. perfringens* was because its 16S rDNA is always well amplified with primers U968-GC and L1401, so it is a good positive control for PCR. All isolates for positive controls were kindly supplied by the bacteriological laboratory of the hospital. Aliquots of 5 μL were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide to verify the size and concentration of the obtained amplicons.

TTGE analysis of PCR amplicons. The Dcode universal mutation detection system (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products. Electrophoresis was performed in a 1 mm polyacrylamide gel [8% (wt/vol) bisacrylamide, 7 M urea, 2.5% 50× TAE, 55 μL of Temed, and 550 μL of 10% ammonium persulfate], with 1.25× TAE as the electrophoresis buffer at a voltage of 20 V for 15 min, then 65 V for 999 min. Each sample had a DNA concentration of approximately 1 ng/μL. Standard ladders were created by individually PCR-amplifying DNA extracted from three intestinal bacterial strains using the same universal primers (in order of migration: *C. perfringens*, *Klebsiella oxytoca*, and *Bifidobacterium bifidum*). A temperature gradient from 65 to 70°C (ramp rate of 0.2°C/h) was applied during electrophoresis. After completion of electrophoresis, the gel was stained in a 30 μg/mL Sybr Green solution, destained in 1.25× TAE, and analyzed using Quantity One software of the Gel Doc 2000 system (Bio-Rad). Profiles were scanned and gray intensity recorded along a densitogram, each band giving rise to a peak.

Sequence analysis. To perform sequence identification, specific bands were cut out from polyacrylamide gel. Gel fragments were washed once in 200 μL PCR water and kept in 100 μL PCR water overnight at 4°C for diffusion. rDNA fragments were then amplified from the dialysate. The PCR reaction was the same as described above. The size and concentration of the amplicons were evaluated on agarose gel. The obtained PCR products were sequenced by Eurogentec (Seraing, Belgium). Newly determined sequences were aligned with closely related sequences of the Ribosomal Database Project II (Michigan State University, U.S.A.).

Statistical analysis was performed using Fisher's exact two-tailed test. A p value of <0.01 was considered significant.

RESULTS

Characteristics of the infants. Mean (SD) gestational age and birth weight were 28.5 (2.1) wk and 880 (170) g in the case group and 27.7 (2.0) wk and 940 (320) g in control group (Table 1).

Intestinal flora. The bacteriological data acquired over the timeframe of the stool collection period are depicted in Figure 1. A salient feature of the bacteriological pattern was the low diversity of the 16S rDNA sequences present in the feces

Table 1. *Clinical characteristics of premature infants*

Infant no.	Month of inclusion	Gestational age (wk)	Birth weight (g)	NEC onset	Total oral nutrition (d)	Initial antibiotics†	Type of surgery	Outcome (d)
1	2	29	680	Day 22	74	No		Survival
2	12	26	990	Day 24	22	Yes	Ileostomy	Death (26)
3	19	29	960	Day 37	14	No	Ileostomy	Death (49)
Mean	±SEM	28.5 ± 2.1	880 ± 170					
4	1	28	1110		30	Yes		Survival
5	1	28	1270		45	Yes		Survival
6	2	28	1150		26	Yes	*Patent ductus arteriosus*	Survival
7	2	28	1480		31	Yes		Survival
8	3	24	780		—	Yes		Death* (22)
9	3	24	690		46	Yes		Survival
10	12	29	650		21	Yes		Survival
11	19	29	820		24	No		Survival
12	20	28	540		40	Yes		Survival
Mean	±SEM	27.7 ± 2.0	940 ± 320					

† Antibiotics including amoxicillin, cefotaxim and netromycin.
* With severe neurological complications.

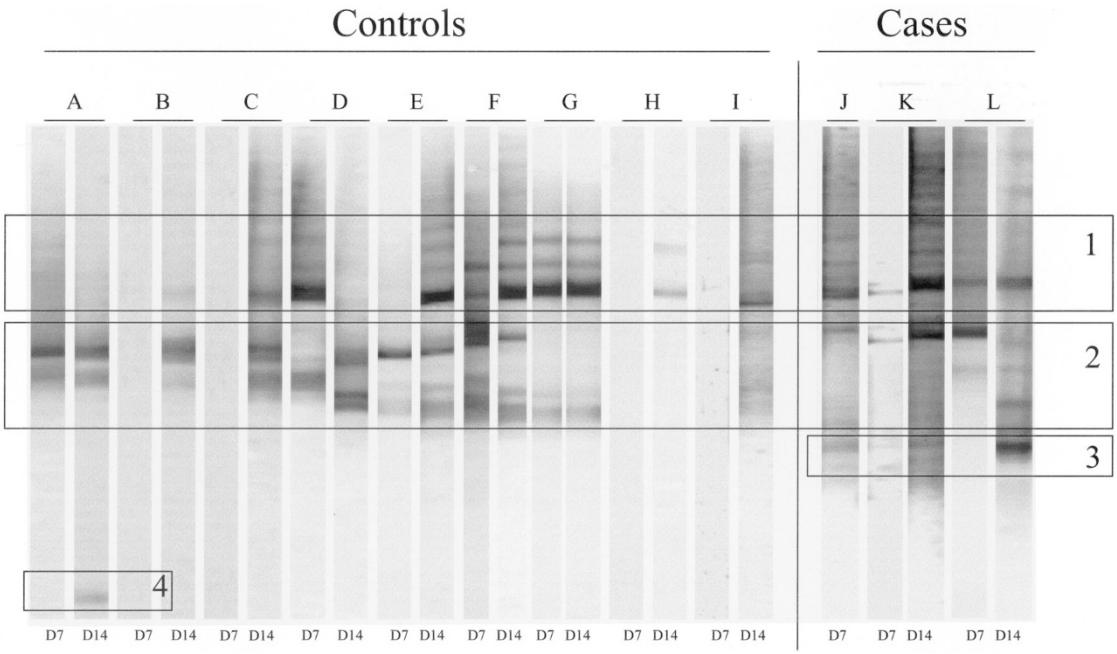

Figure 1. TTGE analysis of amplicons generated by PCR with established GC-containing universal primers from fecal samples taken on d 7 and d 14 of life in 12 neonates (*A–L*). Neonates *J* and *L* died from severe NEC. The bands identified from the 16S rDNA clone libraries are highlighted in *frames 1, 2, 3,* and *4.* In *frames 1* and *2*, the presence of heterogenous 16S rRNA genes results in several bands in TTGE profile. Closest relatives, as determined by comparative sequence analysis and level of identity with this relative are: frame 1: *Staphylococcus epidermidis*, 99%; frame 2: *Staphylococcus aureus*, 98%; frame 3: *C. perfringens*, 95%; frame 4: *Klebsiella oxytoca*, 98%. For clarity, the standard ladders are not shown on the figure.

during the first 2 wk of life. In addition, a peculiar profile was observed only in the three infants who later developed NEC: aside from the expected *Staphylococcus epidermidis* and *aureus* bands, a band could also be detected in early samples, and before diagnosis, whereas there was no evidence for this specific band in any of the nine controls. Our phylogenetic analysis revealed that the found strain was closely related to *C. perfringens*, as the level of similarity of the detected bands was 95%. In the infant (case #3) who developed NEC at a later date (d 37), the *Clostridium* colonization detected at d 14 persisted in the subsequent sample obtained at d 21 (data not shown).

Furthermore, the presence of a band with a sequence related to *Klebsiella oxytoca* was detected on d 14 in one control infant.

DISCUSSION

Although bacterial infection has long been suspected to play a key role in the pathophysiology of NEC, earlier studies using bacteriological culture have not produced consistent evidence for the initial colonization by a specific germ and the subsequent development of NEC. The present study was, therefore,

undertaken to determine whether more recently developed molecular methods would provide a better understanding of a suspected correlation between early intestinal colonization and the subsequent development of NEC. Before d 15, a species closely related to *C. perfringens* was detected in the three cases but not in any of the nine controls ($p = 0.005$). The NEC-suffering infants and the controls were hospitalized in the same ward but at successive intervals of 3 and 6 mo for the cases of NEC, and over a period of 20 mo for the controls. We did not notice any significant difference in clinical features between the two groups of infants. Among the cases of NEC, two children died and one survived. As for controls, the death of one child was due to neurologic complications without relation to NEC. The findings of the current study highlight the usefulness of a molecular method to assess differences in flora between control and NEC infants. They also highlight the fact that anaerobic flora has to be taken into account in bacterial analyses in cases of NEC. They furthermore raise the question of the putative role of a high initial level of *C. perfringens* in NEC.

To our knowledge, only two studies assessed the microbial colonic flora of premature infants by molecular methods. In the study by Schwiertz *et al.* (11), only one infant, however, was diagnosed with NEC, so they were unable to draw any realistic conclusions on the role of microbiota in premature infants with NEC. An already highly diverse flora was nevertheless noted in this preterm infant at birth. Although several infants developed NEC in the other relevant study performed by Millar *et al.* (12), stool samples collection did not, however, start until the day when clinical symptoms occurred, and no difference was detected between NEC patients and controls.

It is now recognized that 80% of the dominant fecal flora of adults is unculturable (13). The literature about the dominant fecal flora of infants is still poor. The aim of this work was the early detection of bacteria by identifying their 16S rDNA. In addition to molecular techniques, only the routine stool culture technique from our hospital laboratory was used. The presence of inhibitors in human fecal material is a major obstacle limiting the usefulness of PCR for detecting microorganisms in feces. These include bile salts, hemoglobin degradation products, and complex polysaccharides. Although a variety of strategies for removing PCR inhibitors from human stool have been devised (*e.g.* the use of polyvinylpyrrolidone to attenuate inhibitory effects of polyphenolic substances), competition for the PCR primers by the dominant DNA species is a limiting factor for TTGE sensitivity. PCR artifacts can be minimized (14). In the current study, we therefore used high-quality primers (purified by HPLC purification system), high-fidelity polymerases (the Hot Star Taq Polymerase has been developed to provide higher PCR specificity), and optimal PCR reaction conditions (data not shown).

Until now, conventional bacteriological culturing methods have not been able to establish a temporal relationship between initial bacterial colonization and NEC. The use of this new molecular approach to analyze the gastrointestinal ecosystem should therefore allow a more comprehensive and rapid assessment of intestinal flora and provide further insight into the dynamic evolution of the biodiversity in this ecosystem. In previous studies, Millar *et al.* (12) indeed attempted to determine to what extent bacteria not detected by culture contributed to the microbial flora of the bowel of preterm infants with and without NEC. Fecal samples were examined both by culture and PCR amplification of the 16S rDNA. The authors used DGGE to separate mixtures of 193-bp fragments amplified from the V3 region of the 16S rDNA. As expected, more bacterial strains were detected by direct PCR-DGGE analyses on fecal samples without any culture. In our study, we used TTGE instead of DGGE. DGGE is based on electrophoretic separation of DNA molecules that are the same length but have different nucleotide sequences. TTGE is a related but simpler method, in which a temperature gradient rather than a solvent gradient is used to denature the DNA (15). Both DGGE and TTGE are now methods of choice for environmental microbiologists. They have been used to determine the genetic diversities in natural microbial communities (16), human digestive tract (17), subgingival plaque (18), and, more recently, in association with quantitative dot blot hybridization, to follow the alteration of the dominant fecal bacterial groups in patients with Crohn's disease of the colon (19). Although a recent comparative analysis of DGGE and TTGE by Farnleitner *et al.* (20) indicated a significantly higher discrimination efficiency of the spatial chemical denaturing gradient as compared with the temporal temperature denaturing gradient, we considered it more appropriate to use TTGE, as the feasibility of using TTGE for bacterial identification has been confirmed in previous studies (15, 16). Millar *et al.* (12) separated mixtures of 193-bp fragments amplified from the V3 region of the 16S rDNA. Although the sequence information from a fragment of this size is insufficient to allow high precision in the construction of a phylogenetic tree, it did allow a presumptive identification. The primers used in our study amplified the V6 to V8 region of the 16S rDNA. This region was chosen as an appropriate variable region with high intraspecies diversity (21, 22). Thus, the 500-bp amplified fragments allow a more accurate identification of species.

Although Millar *et al.* (12) studied a population of preterm infants similar to that enrolled in the current study, all samples but one were collected either on the day of onset of clinical signs of NEC, or on subsequent days, and no differences were found between samples from infants with NEC and samples from infants without NEC. In contrast, in the current study stool samples were obtained weekly starting on the first day of life, before any clinical signs of NEC could be observed.

The present results are consistent with earlier studies. Indeed, in animal models of NEC, particularly in the quail, Butel *et al.* (23) showed that several species of *Clostridium* (*C. butyricum* or whole fecal flora containing *C. perfringens*) isolated from premature infants suffering from NEC were able to produce cecal lesions similar to those observed in human neonates affected with NEC, including gas cysts, hemorrhagic ulcerations, and necrotic areas. In one report, an especially severe form of NEC was found to be associated with the presence of *C. perfringens* in blood culture and peritoneal fluid. Mortality in this group was 78% (24).

As the limit of detection of TTGE is not well known, the early detection of a *C. perfringens* band in the three NEC-

suffering infants and not in the controls may simply reveal the presence of this strain above a critical threshold level in the group who later developed NEC. So the current findings do not prove the existence of a causal relationship between the *Clostridium* strain identified and the occurrence of NEC. The early presence of *Clostridium* DNA could indeed be either a cause or, alternatively, a consequence of early subtle, infraclinical alterations in the gut of the infants who later developed clinical symptoms of NEC. The presence of *Clostridium* may simply be an early index of intestinal frailty, or a consequence of early alterations in gut barrier function and/or microflora that precede the true occurrence of NEC. The proof for a causative role of *Clostridium* in the pathogenesis of NEC could only be provided by mechanistic studies such as the production of cecal lesions similar to those observed in humans upon inoculation of the isolated germ strains in the quail, which may be a suitable animal model for the human disease (23). This approach was not feasible in the current study since this *C. perfringens* could not be isolated from the patients' stools using routine techniques in the hospital bacteriology laboratory. A global analysis of the fecal flora comparing the two methods would therefore be of great interest. The isolation of the *C. perfringens*–related species and the assessment of its putative pathogenicity in an animal model would definitely validate the molecular methods.

In conclusion, the current report provides evidence for a temporal relationship between an initial colonization with *Clostridium* and the later onset of NEC. To our knowledge, this study therefore is the first to demonstrate that the use of molecular techniques based on the study of bacterial 16S rRNA genes allowed the recognition of a *C. perfringens* closely related species in the first 2 wk of life in three infants who later displayed symptoms of NEC, from which two of them eventually died. The current data do not, however, constitute definite proof that the identified bacterial species was a causative agent in the development of NEC. It is obvious that a combination of the current method with adequate bacteriological cultures would be needed for validation. The current finding, nevertheless, highlights the promise of this new technique based on molecular biology, and suggests that large scale studies on a much wider population at high risk for NEC may be warranted. The combined uses of the described technique, along with the use of a fast diagnostic technique should allow an early detection of the infants at risk, and would help in the choice of preventive treatment.

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REFERENCES

1. Claud EC, Walker WA 2001 Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 15:1398–1403
2. Sakata H, Yoshioka H, Fujita K 1985 Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. Eur J Pediatr 144:186–190
3. Westra-Meijer CM, Degener JE, Dzoljic-Danolovic G, Michel MF, Mettau JW 1983 Quantitative study of the aerobic and anaerobic faecal flora in neonatal necrotising enterocolitis. Arch Dis Child 58:523–528
4. Hall MA, Cole CB, Smith SL, Fuller R, Rolles CJ 1990 Factors influencing the presence of faecal lactobacilli in early infancy. Arch Dis Child 65:185–188
5. Foglia RP 1995 Necrotizing enterocolitis. Curr Probl Surg 32:757–823
6. Hoy C, Millar MR, MacKay P, Godwin PG, Langdale V, Levene MI 1990 Quantitative changes in faecal microflora preceding necrotising enterocolitis in premature neonates. Arch Dis Child 65:1057–1059
7. Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL 1985 Enteric colonization in sporadic neonatal necrotizing enterocolitis. J Pediat Gastroenterol Nutr 4:591–595
8. Favier CF, Vaughan EE, De Vos WM, Akkermans AD 2002 Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68:219–226
9. Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R 1997 Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl Environ Microbiol 63:2802–2813
10. Vasquez A, Ahrné S, Pettersson B, Molin G 2001 Temporal temperature gradient gel electrophoresis (TTGE) as a tool for identification of *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus zeae* and *Lactobacillus rhamnosus.* Lett Appl Microbiol 32:215–219
11. Schwiertz A, Gruhl B, Löbnitz M, Michel P, Radke M, Blaut M 2003 Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. Pediatr Res 54:393–399
12. Millar MR, Linton CJ, Cade A, Glancy D, Hall M, Jalal H 1996 Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J Clin Microbiol 34:2506–2510
13. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J 1999 Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807
14. Mackie RI, Sghir A, Gaskins HR 1999 Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 69:1035S–1045S
15. Muyzer G 1999 DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2:317–322
16. Ogier JC, Son O, Gruss A, Tailliez P, Delacroix-Buchet A 2002 Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl Environ Microbiol 68:3691–3701
17. Zoetendal EG, Akkermans AD, De Vos WM 1998 Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 64:3854–3859
18. Fujimoto C, Maeda H, Kokeguchi S, Takashiba S, Nishimura F, Arai H, Fukui K, Murayama Y 2003 Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of microbial communities of subgingival plaque. J Periodontal Res 38:440–445
19. Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P, Jian R, Dore J 2003 Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut 52:237–242
20. Farnleitner AH, Kreuzinger N, Kavka GG, Grillenberger S, Rath J, Mach RL 2000 Comparative analysis of denaturing gradient gel electrophoresis and temporal temperature gradient gel electrophoresis in separating *Escherichia coli* uidA amplicons differing in single base substitutions. Lett Appl Microbiol 30:427–431
21. Schmalenberger A, Schwieger F, Tebbe CC 2001 Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetics profiling. Appl Environ Microbiol 67:3557–3563
22. Zhu XY, Zhong T, Pandya Y, Joerger RD 2002 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl Environ Microbiol 68:124–137
23. Butel MJ, Roland N, Hibert A, Popot F, Favre A, Tessedre AC, Bensaada M, Rimbault A, Szylit O 1998 Clostridial pathogenicity in experimental necrotising enterocolitis in gnotobiotic quails and protective role of bifidobacteria. J Med Microbiol 47:391–399
24. Kosloske AM, Ball Jr WS, Umland E, Skipper B 1985 Clostridial necrotizing enterocolitis. J Pediatr Surg 20:155–159