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FEMS Microbiology Ecology 47 (2004) 337–343

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Gnotobiotic rats harboring human intestinal microbiota as a model for studying cholesterol-to-coprostanol conversion

Philippe Gérard, Fabienne Béguet, Pascale Lepercq, Lionel Rigottier-Gois, Violaine Rochet, Claude Andrieux, Catherine Juste *

Unité d'Écologie et Physiologie du Système Digestif, Institut National de la Recherche Agronomique, Bâtiment 405, Domaine de Vilvert, 78352 Jouy-en-Josas, France

Received 26 September 2003; received in revised form 20 November 2003; accepted 21 November 2003

First published online 14 January 2004

Abstract

The efficiency of microbial reduction of cholesterol to coprostanol in human gut is highly variable among population and mechanisms remain unexplored. In the present study, we investigated whether microbial communities and their cholesterol metabolism characteristics can be transferred to germ-free rats. Two groups of six, initially germ-free rats were associated with two different human microbiota, exhibiting high and low cholesterol-reducing activities. Four months after inoculation, enumeration of coprostanoligenic bacteria, fecal coprostanol levels and composition of the fecal microbial communities were studied in gnotobiotic rats and compared with those of the human donors. Combination of culture (most probable number enumeration of active bacteria) and biochemical approaches (extraction followed by gas chromatography of sterols) showed that gnotobiotic rats harbored a coprostanoligenic bacterial population level and exhibited coprostanoligenic activities similar to those of the corresponding human donor. On the other hand, molecular approaches (whole-cell hybridization with fluorescently labeled 16S rRNA-targeted oligonucleotide probes, and temporal temperature gradient gel electrophoresis of bacterial 16S rRNA gene amplicons) demonstrated that gnotobiotic rats reproduced a stable microbial community, close to the human donor microbiota at the group or genus levels but different at the dominant species level. These results suggest that the gnotobiotic rat model can be used to explore the still unknown human intestinal microbiota involved in luminal cholesterol metabolism, including regulation of expression of its activity and impact on health.

Keywords: Cholesterol-to-coprostanol conversion; Human intestinal microbiota; Animal model; Temporal temperature gradient gel electrophoresis; Fluorescent in situ hybridization

1. Introduction

In mammals, it has long been known that luminal cholesterol (5-cholesten-3β-ol) from both exogenous and endogenous origins (diet, bile, and desquamated intestinal cells) is metabolized by the intestinal microbial community. The end-product is mainly the fully saturated and poorly absorbable coprostanol (5β-cholestan-3β-ol) whose formation would facilitate the elimination of cholesterol from the body [1,2], and would therefore decrease the risk of cardiovascular diseases [2]. The ambiguity of coprostanol in a clinical perspective must however be pointed out, since this metabolite could be associated with colorectal carcinogenesis [3–5]. Interestingly, the efficiency of microbial conversion of cholesterol to coprostanol among human populations has been found to be bimodal, with a vast majority of high transformers and a minority of low-to-inefficient transformers [6,7]. Yet, differences in the intestinal microbial community of both populations remain unexplored, and no colony-forming strain capable of reducing cholesterol to coprostanol has hitherto been isolated from the human intestinal microbiota. Dietary habits do influence the rate of cholesterol to coprostanol conversion, as inferred from epidemiological studies [8,9] and nutritional trials in man [10,11] and animals [12,13], but the main luminal factors that regulate the expression of the cholesterol-reducing microbial activity have yet to be elucidated.

Various models have been developed for studying the

* Corresponding author. Tel.: +33 (1) 34 65 24 87; Fax: +33 (1) 34 65 24 92.
E-mail address: juste@jouy.inra.fr (C. Juste).

0168-6496/04/$22.00
doi:10.1016/S0168-6496(03)00285-X

activities of the intestinal microbial communities in vivo including human volunteers, conventional laboratory animals and gnotobiotic animals [14]. Since gnotobiotic animals maintain the microbial community of the human gut in an in vivo environment similar in many respects to that of the human intestine, the associated microbiota was thought to retain its original composition and metabolic activities. However, it was recently shown that bacterial metabolism in the intestine of gnotobiotic mice reflected that of human feces with respect to some metabolic activities but not others [15,16], suggesting the need for care when using gnotobiotic animals as a model. Ex-germ-free animals harboring human intestinal microbiota would certainly aid in exploring microbial cholesterol metabolism, the effect of diet on populations involved and on expression of their activity, as well as its potential impact on health, provided that the model reproduced both microbial composition and coprostanoligenic activity of human donors. This model has been evaluated in the present study using: (1) anaerobic culture and biochemical techniques to estimate the most probable number (MPN) of coprostanol-forming bacteria and the cholesterol-to-coprostanol transformation rate in human and animal feces and (2) two molecular approaches (whole-cell hybridization with fluorescently labeled 16S rRNA-targeted oligonucleotide probes (FISH) and temporal temperature gradient gel electrophoresis (TTGE) of bacterial 16S rDNA amplicons) to compare the composition of the fecal microbiota of human donors and gnotobiotic rats.

2. Materials and methods

2.1. Human fecal samples and inoculation of germ-free rats

Two fresh stools were provided in anaerobic boxes (Anaerocult; Merck, Darmstadt, Germany) by two healthy Caucasian women (42 and 45 years) on an unrestricted Western diet, without laxative or antibiotic use for the last three months, and previously identified as a high and a low cholesterol-to-coprostanol transformer, respectively. A 10^{-2} dilution of each stool was anaerobically prepared in (in g l^{-1}) NaCl (5.0)-glucose (2.0)-cysteine-HCl (0.3) and immediately administered to 6 male adult germ-free Fisher 344 albino rats through a single oral gavage (1 ml/rat). Both groups of 6 rats were housed in 2 distinct sterile Trexler plastic isolators (La Calhène, Vélizy, France), with 2 cages per isolator (3 rats/cage). All rats were fed ad libitum on the same semi-purified diet, containing in % (wt wt^{-1}) casein (5), soy isolate (12), maize starch (28), cooked potato starch (28), sucrose (4), maize oil (3), tallow (5), cellulose (7), mineral mix (7), and vitamin mix (1) for 1 week before inoculation, and for the following 4 months of gnotobiotic life, at the end of which fresh feces were individually collected from the rectum of each rat.

2.2. MPN enumeration of coprostanoligenic bacteria and measurement of the coprostanol level in feces

MPN enumerations were performed using the strictly anaerobic technique of Hungate [17], with all dilution and culture tubes pre-reduced under O_2-free N_2. An aliquot of each stool (0.5 g for rats and 1 g for humans) was rapidly diluted 10-fold under a stream of O_2-free N_2. The dilution solution (4.5 ml or 9.0 ml) contained, per liter, casitone 2.0 g, yeast extract 2.0 g, NaCl 5.0 g, and KH_2PO_4 1.0 g. After aseptic sealing, these first dilutions were homogenized by vigorous vortexing, and then serially diluted 10-fold to 10^{-12} in the same dilution solution. One-milliliter aliquots of each dilution were then transferred in triplicate to the MPN culture tubes containing 9 ml of growth medium enriched with cholesterol solubilized in soy lecithin. Briefly, 0.2 g of cholesterol (Sigma-Aldrich Chimie, St Quentin Fallavier, France) was thoroughly mixed with 1 g of L-α-phosphatidylcholine (Type IV-S, Sigma-Aldrich Chimie) in a minimal volume of water under heating at less than 50°C. All other ingredients, i.e. brain heart infusion (10 g), yeast extract (10 g), L-cysteine (0.5 g), 0.1% hemin solution in water (10 ml), were mixed together in water. The lecithin-cholesterol preparation was added, the pH was adjusted to 7.4 and the volume was adjusted to 1 liter. After the MPN cultures had been incubated at 37°C for 7 days, sterols (cholesterol and its bacterial metabolites) were extracted from 1 ml of each culture with 2 ml of *n*-hexane by magnetic stirring for 3 h [18]. The samples were centrifuged and the sterols in the hexane supernatant were analyzed by gas chromatography (GC) as their silyl derivatives [19]. MPN results were calculated using a micro-computer program [20] and were expressed as cell number g^{-1} (fresh stools). The coprostanol level in feces (0.5–1.0 g aliquots) was measured by GC after the neutral sterols had been extracted, saponified and derivatized [19]. Results were expressed as the percentage of coprostanol within the total neutral sterols present.

2.3. FISH analysis

FISH analysis was performed in duplicate from a single 0.5 g aliquot of fresh stools. Fixation and permeabilization of samples were carried out as described previously [21,22]. Permeabilized cells were hybridized for 16 h at 35°C in 50 μl of hybridization buffer (900 mM NaCl, 20 mM Tris–HCl, pH 7.2; 0.01% SDS; 15% formamide; and 4 ng μl^{-1} of fluorescent probe) in a 96-well microtiter plate. The eleven 16S rRNA-targeted oligonucleotide probes used in this study are listed in Table 1. They were covalently linked with fluorescein isothiocyanate (FITC) or indodicarbocyanin (Cy5) at the 5′-end and were purchased from Qbiogene (Evry, France). Eub338 is specific for the domain Bacteria [23]. NonEub338 is the complement of Eub338 and is used as a negative con-

trol [24] to account for non-specific binding and background fluorescence. Other probes are group specific without any cross hybridization, so that the summation of the percentages of target groups could be calculated. Following hybridization, a volume of 150 μl of hybridization solution was added to each well and cells were pelleted at 4000×*g* for 15 min. Non-specific binding of the probe was removed by incubating the bacterial cell suspension at 37°C for 20 min in a washing solution (64 mM NaCl, 20 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.01% SDS). Cells were pelleted and resuspended in phosphate-buffered saline (PBS, pH 7.2). Aliquots of 100 μl were added to 0.5 ml of FACS FLOW (Becton Dickinson) for data acquisition by flow cytometry. Data acquisition was performed as described previously [21]. Cell enumeration was performed by combining one group Cy5-probe with the Eub338 FITC-probe in one hybridization tube. Results were expressed as cells hybridizing with group-Cy5 probe as a proportion of the total bacteria hybridizing with the general Eub338 FITC-probe.

2.4. DNA isolation, PCR and TTGE analysis

Total DNA was extracted for PCR-TTGE as previously described [32] from 0.2 g fecal samples frozen at −70°C. The concentration and integrity of the nucleic acids were determined visually by electrophoresis on a 1% agarose gel containing ethidium bromide. Primers GCclamp-U968 (5′ GCclamp-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 bacterial 16S rRNA genes [33]. PCR was performed using *HotStar Taq* DNA polymerase (Qiagen, Courtaboeuf, France). The PCR mix (50 μl) contained 15 PCR buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP, 20 pmol of primers U968-GC and L1401, 2.5 U of *HotStar Taq* DNA polymerase and approximately 2 ng of DNA. The samples were amplified in a PCT 100 thermocycler (MJ Research, USA) using the following program: 95°C for 15 min; 30 cycles of 97°C for 1 min, 58°C for 1 min, 72°C for 1 min 30 s and finally 72°C for 15 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide to verify their size (500 bp) and estimate their concentrations. We used the DCode Universal Mutation Detection System (Bio-Rad, Paris, France) for sequence-specific separation of PCR products. Electrophoresis was performed through a 1 mm-thick, 16×16 cm polyacrylamide gel (8% w/v acrylamide/Bis, 7 M urea, 1.25× Tris-(hydroxymethyl)aminomethane-acetate-ethylenediamine tetraacetic acid [pH 8.0] (TAE) and 55 μl and 550 μl of TEMED and 10% ammonium persulfate, respectively) using 7 L of 1.25× TAE as the electrophoresis buffer. Electrophoresis was run at a fixed voltage of 76 V for 16 h with an initial temperature of 66°C and a ramp rate of 0.2°C h^{-1}. For better resolution, the voltage was fixed at 20 V for 15 min at the beginning of electrophoresis. Each well was loaded with 100–200 ng of amplified DNA plus an equal volume of 25 gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol and 70% glycerol). A marker consisting of a PCR amplification mix of cloned rDNA from *Clostridium coccoides* (no. 157, 93 and 40), *Clostridium leptum* (no. 365 and 296), and *Bacteroides* (no. 303 and 73) [34] was used as a normalization standard. Gels were stained in the dark by immersion for 30 min in a solution of SYBR Green I Nucleic Acid Gel Stain (Roche Diagnostics, GmbH, Mannheim, Germany) and read on a Storm system (Molecular Dynamics). Analyses (including Principal Component Analysis) of the TTGE profiles were performed using GelCompar software (version 2.0 Applied Maths, Kortrijk, Belgium). Analysis included calculation of the number, position and intensity of bands for each lane, and between-pattern comparisons using the Pearson coefficient calculated as a measure of the degree of similarity. A value of zero indicates that the samples are completely different, whereas a value of 100 indicates that they are identical. Principal Component Analysis (PCA) ordinations were calculated using the Pearson product-moment correlation coefficient. This coefficient subtracts each character set by its average and divides the characters by the variance of the character set.

3. Results and discussion

Using MPN enumeration, the cholesterol-to-coprostanol reducing bacterial community of the human volunteers was estimated at 9.2×10^8 and 9.2×10^3 cells g^{-1} of fresh stools in the high and low cholesterol transformer, respectively (Table 2). The coprostanol levels measured by GC in the corresponding stools were 85.8% and 1.3% of total neutral sterols, respectively (Table 2). This work provides the first MPN estimates of viable coprostanoligenic bacterial communities in an ecosystem. It also provides the first indication that the abundance of this population in human feces coincides with the rate of conversion of cholesterol to coprostanol in the large intestine, as reflected by the fecal coprostanol level. When the same measurements were carried out in rats colonized for 4 months with these human microbiota, the coprostanol-forming microbial community was estimated at 2.1×10^9 cells g^{-1} of fresh stools in the group harboring the highly coprostanoligenic human microbiota, whereas it was not detectable in the other group. The corresponding coprostanol levels were 58.9 ± 9.1 and 3.0 ± 1.9% of total neutral sterols, respectively (Table 2). These results showed that the coprostanoligenic microbial community from the high cholesterol converter colonized the intestine of rats at a level close to that observed in the donor, and persisted for at least four months, the duration of the experiment. Moreover, the cholesterol reducing activity of this human donor was retained in rats, although at a slightly reduced level. To our knowledge, only one study has compared the cholesterol-to-coprostanol trans-

Table 1
Designations, sequences and targets of probes

Probe	Sequence (5′→3′)	Target	Fluorochrome	Reference
Eub338	GCT GCC TCC CGT AGG AGT	domain Bacteria	5′FITC	[23]
Eub338	GCT GCC TCC CGT AGG AGT	domain Bacteria	5′CY5	[23]
NonEub338	ACA TCC TAC GGG AGG C	none	5′FITC	[24]
NonEub338	ACA TCC TAC GGG AGG C	none	5′CY5	[24]
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium* cluster	5′CY5	[25]
Bac303	CCA ATG TGG GGG ACC TT	*Bacteroides-Prevotella* group	5′CY5	[26]
Bif164	CAT CCG GCA TTA CCA CCC	*Bifidobacterium* genus	5′CY5	[27]
Enter1432	CTT TTG CAA CCC ACT	Enteric group	5′CY5	[28]
Erec482	GCT TCT TAG TCA GGT ACC G	*Clostridium coccoides* group	5′CY5	[29]
Fprau645	CCT CTG CAC TAC TCA AGA AAA AC	*Faecalibacterium prausnitzii* group	5′CY5	[30]
Lab158	GGT ATT AGC AYC TGT TTC CA	*Lactobacillus-Enterococcus* group	5′CY5	[31]

formation rate in a human donor and 12 corresponding gnotobiotic rats [35]. After one month of gnotobiotic life, the bacterial conversion of cholesterol in rats was 5 times less than in the donor. The discrepancy between these data and our own might come from the diet, whose composition was far from that of a human diet in the previous study. In our study, the cholesterol-to-coprostanol reducing bacteria were below the minimum detectable level of 10 microorganisms g^{-1} of feces in gnotobiotic rats inoculated with the low coprostanoligenic microbiota which originally contained 9.2×10^3 of these bacteria g^{-1}. However, a residual level of coprostanol was detected in these gnotobiotic rat feces indicating that bacteria unable to grow on the media used may be involved in the cholesterol reducing activity.

Recent advances in rRNA-based molecular techniques have made it possible to identify different bacterial communities in environmental samples without prior cultivation [36]. In particular, fluorescent *in situ* hybridization (FISH) with specific 16S rRNA-based oligonucleotide probes has been used for analysis of human gut and fecal microbial communities [21,22,25,29,31,37]. In this study, we used probes specific to major genera and groups present in the human gut to compare the composition of fecal microbiota of human donors and recipient gnotobiotic rats four months after inoculation. The probe set consisting of seven probes made it possible to detect about 50% of the total bacterial cells detected with the Eub338 probe in both human and gnotobiotic rat fecal samples (Fig. 1A,B). The three major bacterial groups in the donor feces, in decreasing order, *Clostridium coccoides* (targeted by Erec482), *Bacteroides–Prevotella* (targeted by Bac303), and *Faecalibacterium prausnitzii* (targeted by Fprau645), were recovered in approximately the same proportions in rats, whereas the less represented enteric (targeted by Enter1432) and *Bifidobacterium* groups (targeted by Bif164) in donors, remained low in rats. This profile approaches that previously reported in studies with several healthy volunteers, using the same whole-cell hybridization technique [21,22]. The only significant difference between profiles of human and gnotobiotic rat fecal microbiota concerned the *Lactobacillus/Enterococcus* group (targeted by Lab158), which was higher than expected in rats associated with the non-coprostanoligenic microbiota (Fig. 1A). This observation is consistent with previous reports showing that lactobacilli have a strong host specificity [38,39]. Regarding the profiles of both human fecal microbiota, the most striking difference between the two donors concerned the *Atopobium* cluster (targeted by Ato291), the proportion of which was about 10 times greater in the high cholesterol converter (Fig. 1A,B). This was reproduced in the corresponding rats, so that the difference between both animal groups was highly significant. Whether the *Atopobium* cluster supported a cholesterol-to-coprostanol reducing activity and accounted for, or at least participated in the differences in cholesterol metabolism, was not investigated in the present study. However, results from a current study involving 21 human stool samples showed no correlation between the proportion of *Atopobium* and cholesterol conversion (data not shown) suggesting that this bacterial group is not responsible for

Table 2
MPN estimates of cholesterol-to-coprostanol-converting bacteria, and measurement of the coprostanol levels in fresh stools from two human donors and the six corresponding gnotobiotic rats

	MPN results		Mol% coprostanol[a] in GC
	No. of cells g^{-1} (fresh stools)	Confidence interval (95%)	
High transformer woman	9.2×10^8	2.2×10^8–3.78×10^9	85.8
Corresponding 6 gnotobiotic rats	2.1×10^9	1.1×10^9–4.03×10^9	58.9 ± 9.1
Low transformer woman	9.2×10^3	2.2×10^3–3.77×10^4	1.3
Corresponding six gnotobiotic rats	<10		3.0 ± 1.9

[a]Coprostanol×100/(cholesterol+coprostanol+others minor sterols).

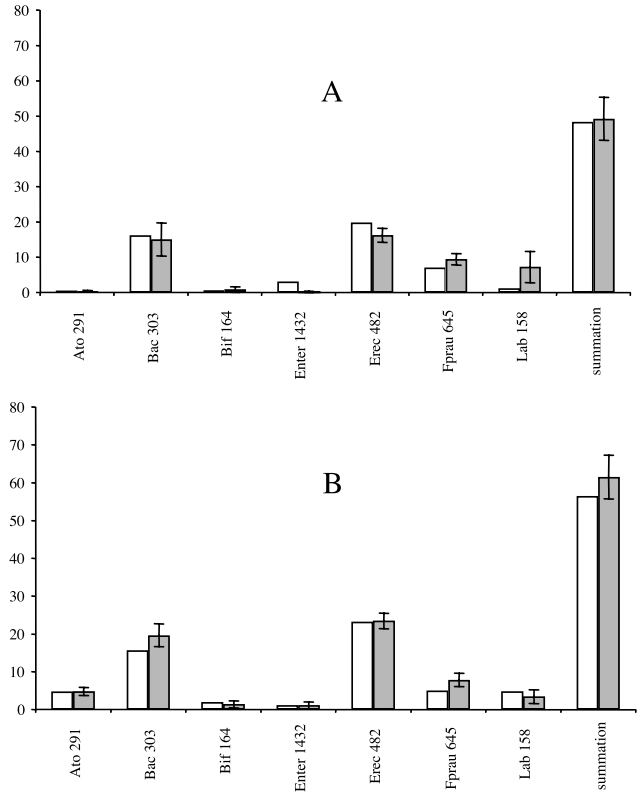

Fig. 1. Percent composition of fecal microbiota from two healthy women (white bars) (a low [A] and a high [B] cholesterol-to-coprostanol transformer) and three corresponding gnotobiotic rats (gray bars) (means ± standard errors of the means), as studied by fluorescent whole-cell hybridization with seven group-specific 16S rRNA-targeted oligonucleotide probes. Results are expressed as percent of the total cells detected by the bacterial domain probe Eub338.

the difference in coprostanoligenic activity between our two groups.

Altogether, the present FISH, MPN and biochemical studies converged to suggest that gnotobiotic rats reproduce a microbial community close to that of the human donor and may therefore constitute a reliable model to study the human intestinal microbiota, its metabolic activities or its responses to environmental changes. However, in other studies gnotobiotic mice were also shown to reproduce the overall composition of their inoculated human microbiota, as inferred from culture on specific media, whereas only part of the metabolic activities was recovered [15,16]. This has been suggested to be related to a different species distribution among each phylogenetic group between human and gnotobiotic animal microbiota. We therefore further explored and compared the composition of microbial communities from human donors and their corresponding gnotobiotic rats at the predominant species level, through TTGE analysis. Recently, temperature and denaturing gradient gel electrophoresis of the PCR-amplified sequences of fecal 16S rRNA and 16S rRNA genes have been shown to be a powerful tool to determine and monitor the bacterial community in feces [33,40]. These methods allow differentiation at the species level and sometimes at the subspecies level and reveal that the dominant microbiota in human feces is stable and host specific. The TTGE profiles obtained with fecal samples of human donors and corresponding gnotobiotic rats are presented in Fig. 2. Comparison of gel patterns first showed that rats inoculated with the same human microbiota presented a very close banding profile four months after the inoculation. Thus the Pearson coefficients for comparisons between patterns ranged between 72 and 95% for the non-coprostanoligenic group (lanes R1 to R6) and between 87 and 97% for the coprostanoligenic group (lanes R7 to R12). Moreover gel patterns from rats inoculated with the two different human fecal samples are remarkably different as the inter-group Pearson coefficients ranged between 21 and 43%. These results indicate that gnotobiotic rats which have been inoculated with a same human microbiota were essentially colonized with the same predominant species, different from those which received a different microbiota, demonstrating that microbial community composition of gnotobiotic animals is highly dependent on the human microbiota used for inoculation and is therefore not only dependent on host factors. This was confirmed by PCA, which clearly separated the two groups of rats (Fig. 3). PCA allows ordering of samples along axes (principal components) on the basis of the banding patterns [41]. PCA is applicable to single treatments, thus allowing statistical analysis of unreplicated ecosystems. Whereas similarities between complex communities are not always apparent by manual inspection of TTGE profiles or similar data, PCA provides insight into overall community composition. Here PCA ordination further demonstrated that TTGE profiles from the human donors

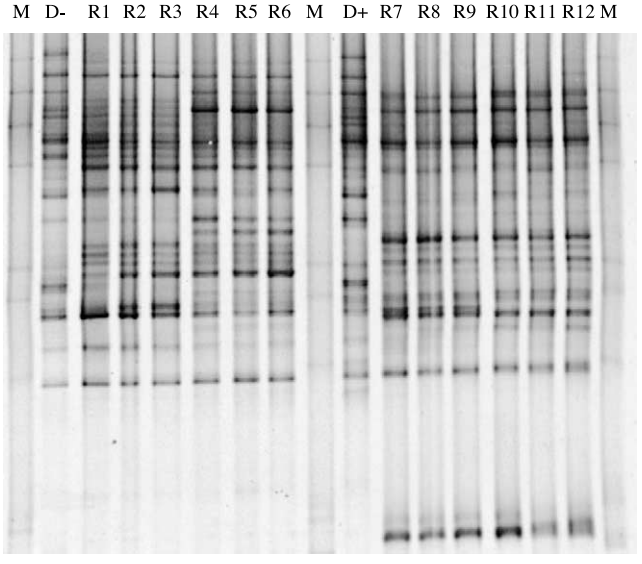

Fig. 2. Temporal temperature gradient gel electrophoresis of 16S rRNA gene amplicons (obtained using primers for the V6–V8 region of the gene) of fecal samples taken from human donors (lanes D− and D+, low and high cholesterol converter, respectively) and from corresponding gnotobiotic rats (lanes R1 to R6 and R7 to R12, respectively). Bacterial standard marker lanes are denoted as M.

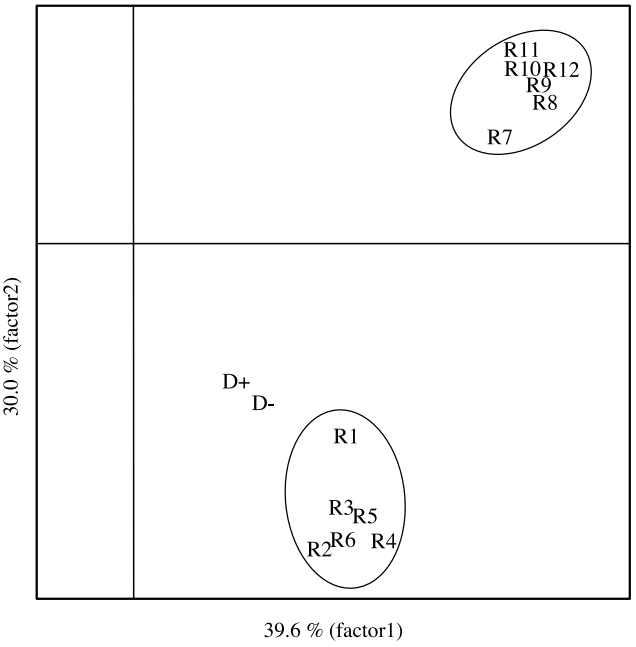

Fig. 3. Ordination produced from PCA of the TTGE banding patterns of fecal samples taken from human donors (D− and D+, low and high cholesterol converter, respectively) and from corresponding gnotobiotic rats (R1 to R6 and R7 to R12, respectively). The amount of variability accounted for by each factor is indicated on the axes.

differed from those of their corresponding gnotobiotic rats. However, non-coprostanoligenic rats (R1 to R6) were clustered closer to their human donor (D−) than were coprostanoligenic rats (R7 to R12 vs D+) (Fig. 3). This is otherwise illustrated by the degrees of similarity between profiles from the human donors (lanes D− and D+, Fig. 2) and their corresponding gnotobiotic rats (lanes R1 to R6 and R7 to R12, respectively) which ranged between 49 and 75% and between 19 and 30% for the low and high coprostanoligenic samples, respectively. These observations are in agreement with the suggestion that host factors greatly influence the composition of the dominant microbial community when focusing at the species level [33,40] and further demonstrate that conservation of the predominant human bacterial species in gnotobiotic rats is also dependent on the human microbiota used for inoculation. As-of-yet-unexplored host–microbe interactions may be responsible for these findings. Interestingly, this shift in predominant bacterial species between donors and recipient rats did not modify the expression of the coprostanoligenic activity, whereas other functions could have been modified.

To our knowledge, this is the first time that culture-independent approaches based on the sequence variability of 16S rRNA genes have been used to study gnotobiotic animal microbial communities and compare them with human donor microbiota. These approaches showed that, four months after the inoculation, dominant microbial communities from gnotobiotic rats and human donors are similar with regard to major phylogenetic groups but are distinct when focusing at the level of bacterial species. This suggests that the dominant species of each bacterial group in gnotobiotic rats may change from those present in inoculated human feces. Using culture-based methods, similar results were previously obtained when comparing the compositions of microbial communities from pig and gnotobiotic mice associated with pig microbiota. While bacterial group distributions were similar in both feces, colony and cell morphologies of bacteria grown on specific media differed between pig and gnotobiotic mice [42]. Thus, using gnotobiotic animals as a model for studying defined bacterial populations or metabolic activities requires verification that the studied bacteria are present in the gnotobiotic animal gut at population and metabolic activity levels approaching those of the inoculum. In this work, culture and biochemical studies showed that the coprostanoligenic human microbiota retained its level of population and cholesterol-reducing activity in gnotobiotic rats indicating that the Fisher 344 albino rat harboring an intestinal human microflora can be used as a model to explore the still unknown human intestinal microbiota involved in luminal cholesterol metabolism, including regulation of expression of its genetic potency and impact on health.

Acknowledgements

We thank M. Sutren for supplying the bacterial standard marker and expertise in PCR–TTGE.

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