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Development of a *Lactobacillus* Specific T-RFLP Method to Determine Lactobacilli Diversity in Complex Samples

Long Chen¹, Matt T. Teasdale¹, Melissa M. Kaczmarczyk², Gregory G. Freund^{2,3}, and Michael J. Miller^{1,2}

¹Department of Food Science & Human Nutrition, University of Illinois, Urbana, IL 61801

²Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801

³Department of Pathology, Program in Integrative Immunology and Behavior, University of Illinois, Urbana, IL, 61801.

Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis has been widely used for studying microbial communities. However, most T-RFLP assays use 16S rDNA as the target and are unable to accurately characterize a microbial subpopulation. In this study, we developed a novel T-RFLP protocol based on *Lactobacillus hsp60* to rapidly characterize and compare lactobacilli composition. The theoretical terminal restriction fragment (TRF) profiles were calculated from 769 *Lactobacillus hsp60* sequences from online databases. *In silico* digestion with restriction endonucleases *AluI* and *TacI* on *hsp60* amplicons generated 83 distinct TRF patterns, of which, 70 were species specific. To validate the assay, five previously sequenced lactobacilli were cultured independently, mixed at known concentrations and subjected to analysis by T-RFLP. All five strains generated the predicted TRFs and a qualitative consistent relationship was revealed. We performed the T-RFLP protocol on fecal samples from mice fed 6 different diets (n=4). Principal component analysis and agglomerative hierarchical clustering revealed that the lactobacilli community was strongly connected to dietary supplementation. Our study demonstrates the potential for using *Lactobacillus* specific T-RFLP to characterize lactobacilli communities in complex samples.

Keywords

terminal restriction fragment length polymorphism; *Lactobacillus hsp60*; community research

1. Introduction

Lactobacillus is one of the most important bacterial species in gut microbiota research. It is well established that lactobacilli play an important role in the functioning of the intestinal ecosystem (Fuller, 1989). The balance of intestinal lactobacilli closely relates to the health of the individual (Sanders *et al.*, 2003). The composition of *Lactobacillus spp.* is greatly

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Correspondence: Michael J Miller, 905 S.Goodwin Ave. Rm 439, Urbana, IL 61801. Tel.: 217 244 1973; mille216@illinois.edu.

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dependent on the diet, but currently this is poorly understood due to limitations in measuring the vast diversity of microorganisms and monitoring their changes.

To study the structure of gastrointestinal bacterial community, standard plating of media agar with selective agents such as bile, esculin or antibiotics can be used for selective enrichment of certain species (Engelkirk *et al.*, 1992). Phenotypic tests such as serotyping and carbohydrate utilization can only be applied after cultivation. Traditionally, it was thought that approximately 20-40% of the microorganisms in the gastrointestinal tract could be cultivated by using enrichment media (Dicksved *et al.*, 2007). Recently, Goodman *et al.* found that 56±4% of species-level taxa, which were detected by 16S rDNA pyrosequencing, could be also identified by sequencing cultivable isolates (Goodman *et al.*, 2011). However, this method is extremely laborious and time consuming. Therefore, novel molecular methods have been developed over the last 15 years. Polymerase chain reaction (PCR) amplification of the specific 16S rDNA-based oligonucleotide probes has been widely used to detect different groups of bacteria directly in fecal or intestinal content samples (Singh *et al.*, 2009).

Terminal restriction fragment length polymorphism (T-RFLP) is an increasingly popular molecular approach which can determine taxa dominance and richness within samples (Engelbrektson *et al.*, 2006; Kitts *et al.*, 2001). T-RFLP allows the assessment of complex bacterial communities and rapid comparison of the community diversity between different ecosystems (Christensen *et al.*, 2004). The typical T-RFLP analysis is based on bacterial 16S rDNA specificity. Briefly, T-RFLP uses a set of fluorescently labeled primers in a PCR reaction to amplify partial 16S rDNA sequences from bacterial community DNA. The resulting mixed amplicons are then digested with restriction enzymes to create a mixture of fluorescence tagged terminal restriction fragments (TRFs), which have specific sizes corresponding to specific microbes. The exact size of each TRF is determined by standard capillary electrophoresis and can be used in community level analysis methods such as agglomerative hierarchical clustering (AHC) and principle component analysis (PCA).

In the past 20 years, various protocols using partial 16S rDNA have been developed which were able to identify *Lactobacillus spp.* isolated from different environments (Nieminen *et al.*, 2011; Christensen *et al.*, 2004; Davis *et al.*, 2010; Collen *et al.*, 2005). However, 16S rDNA based molecular analysis is not efficient enough to reveal significant differences between closely related species (Blaiotta *et al.*, 2008; Walter *et al.*, 2000). Additionally, the rDNA copy number in lactobacilli varies from species to species (Fogel *et al.*, 1999), which makes it difficult to quantify specific amplicons in digested products without a prior calibration. Hsp60 is a highly conserved heat shock protein found in all prokaryotic and eukaryotic cells (Kwok *et al.*, 1999). Polymorphisms within *hsp60* among different *Lactobacillus spp.* can be used to discriminate recently diverged species (Blaiotta *et al.*, 2008; Dellaglio *et al.*, 2005). Additionally, the higher variability of the *hsp60* nucleotide sequences compared to 16S rDNA offers greater opportunities to generate distinguishable TRFs which can be used to detect closely related *Lactobacillus spp.* at the strain level. Moreover, *hsp60* presents a single gene copy in each individual bacterial cell (Goh *et al.*, 1996; Kwok *et al.*, 1999), which makes it possible to develop quantitative or semi-quantitative assays.

The primary aim of this project was to develop a T-RFLP protocol using the *Lactobacillus hsp60* gene as a template. We optimized conditions for PCR and restriction enzyme digestion in order to minimize sampling, extraction and template biases. Furthermore, we used this method to evaluate the potential of dietary factors in modulating the gut lactobacilli community.

2. Methods

2.1 TRF pattern prediction in silico analysis

A set of 769 *Lactobacillus hsp60* sequences were obtained from NCBI gene and genome project databases for analysis. All sequences were imported and analyzed using the sequence analysis software CodonCode Aligner (CodonCode Corporation, Dedham, MA). Alignment was performed using the “sequence assembly” tool. The theoretical PCR amplicons were extracted by deleting all the sequences flanking the binding sites of the two primers. The *hsp60* restriction profiles by enzymes *AluI* and *TacI* of each strain were obtained by using the “restriction mapping” tool. Cleavage site positions were recorded and imported into Microsoft Excel (Microsoft, Seattle, WA) for TRF size calculation.

2.2 Development of T-RFLP protocol based on *Lactobacillus hsp60*

Nested PCR was used to generate *Lactobacillus hsp60* segments for T-RFLP. In the first PCR reaction, universal bacterial *hsp60* primers H279 and H280 were used to amplify a 650 bp fragment internal to the *hsp60* gene (Goh *et al.*, 1996).

PCR amplification was performed with a 50 μ L total volume. The reaction system includes 5 μ L of target DNA used directly from the bacterial community or pure culture DNA extraction, 5 μ L of Taq DNA polymerase 10X Buffer (Lucigen Corporation, Middleton, WI), 2.5 μ L of 50 mM $MgCl_2$, 0.5 μ L of a dNTP mix (25 mM each), 0.125 μ L of each primer (0.1 mM), and 0.5 μ L of Taq DNA polymerase. The PCR consisted of 40 cycles (30s at 94°C, 30s at 37°C, and 1 min at 72°C) and one final cycle at 72°C for 5 min. Two replicate PCRs in 50 μ L reaction mixtures were performed for each sample and the PCR products of the replicates were pooled. PCR products were purified using Zymoclean DNA Clean & Concentrator Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions. The purified DNA was diluted 10-fold and used as the templates for a second round of PCR.

In the second round of PCR, lactobacilli specific *hsp60* primers, LB308F (forward) and LB806RM (reverse), previously described by Blaiotta *et al.* (Blaiotta *et al.*, 2008), were used to amplify a 499 bp fragment internal to the first round PCR product. In order to make the TRFs visually detectable in the later steps, two fluorescent dyes, 6-FAM (6-carboxyfluorescein) and HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein) were separately attached to the forward and reverse primers (LB308F-D and LB806RM-D respectively) before the PCR reaction (IDT, Coralville, IA). The reaction condition of PCR remained the same as described above.

The PCR products were subjected to electrophoresis at 100 V in a 1% (w/v) agarose gel containing 1 mg mL⁻¹ ethidium bromide prior to enzyme digestion. Bands of approximately 500 bp were visualized by ultraviolet (UV) transillumination, cut from the gel, and then recovered using a Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA).

Restriction enzyme digests were performed by digesting 200 ng of a gel-purified PCR product with 20 U of either restriction enzyme *AluI* (New England Biolabs, Ipswich, MA) at 37°C for 2 h or *TacI* (Promega Corporation, Madison, WI) at 65°C for 1.5 h. Following the restriction digestion, the DNA was analyzed with an ABI 3730xl capillary systems (Applied Biosystems Inc, Foster City, CA) according to the manufacturer's instructions.

2.3 Application of T-RFLP with mixed culture

To validate the T-RFLP protocol and determine detection limits, a mixed *Lactobacillus* spp. culture was made with 5 *Lactobacillus* spp.: *L. acidophilus* (NCFM), *L. jensenii* (ATCC 25258), *L. sakei* (ATCC 15521), *L. rhamnosus* (ATCC 53103), *L. plantarum* (ATCC 11146). These 5 strains have distinct TRF patterns (Table S1) and were selected as reference strains for this study. Working cultures were prepared in 10 mL of MRS broth (BD, Franklin Lakes, NJ) and incubated for 24 h at 37°C. 1 mL of the cultures was serially diluted and then plated on LBS agar (BD, Franklin Lakes, NJ) plates. The plates were cultivated in an anaerobic chamber at 37°C for 24 h and then enumerated. After plating, the remaining culture was stored in a -80°C freezer immediately to end bacterial growth.

After colony forming units (CFU) numbers were calculated, the frozen media was thawed on ice, and then homogenized using a vortex. The lactobacilli cells were mixed at a ratio of 10⁴: 10³: 10²: 10¹: 1, respectively. The final concentrations of cells in the mixed culture depicted in Fig. 1 were: *L. rhamnosus*: 72,000 cells mL⁻¹, *L. plantarum*: 7,200 cells mL⁻¹, *L. acidophilus*: 720 cells mL⁻¹, *L. sakei*: 72 cells mL⁻¹, *L. jensenii*: 7 cells mL⁻¹. Additional trials were performed so that each species was represented at each concentration.

The total DNA of the mixed bacterial culture was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA), following the manufacturer's protocol. The DNA was finally eluted in 200 µL of Qiagen Buffer AE, and T-RFLP was applied using the protocol described above.

2.4 Application of T-RFLP on lactobacilli community research

C57BL/6J male mice (3 weeks old) were purchased from Jackson Laboratories (Bar Harbour, ME). Mice were housed (8 per cage) in large standard shoebox cages (length 28 cm; width 17 cm; height 12.5 cm) and allowed free access to food and water. Mice were fed open source uniform-base diets purchased from Research Diets (New Brunswick, NJ). These diets varied based on amount and type of fiber as well as fat content (Table 2). Housing temperature (22°C) and humidity (45-55%) were controlled as was a 12/12 h reversed dark-light cycle (2200-1000 h). Animal use was conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) approved protocols at the University of Illinois Urbana-Champaign.

Fecal samples were collected from each mouse at the end of the treatment phase (either 3 month or 8 month). Approximately 0.5 g of fresh fecal pellets from every mouse were collected into a 1.5 mL centrifuge tube using sterile forceps, and the fecal DNA was extracted and purified using the commercial QIAamp DNA Stool Mini Kit as described above. T-RFLP was then applied to the extracted DNA.

To specifically analyze the dominant cultivable *Lactobacillus* population in subjects from group 7 and 8, an extra 0.1 g of fresh fecal sample from each mouse in these 2 groups was collected. The fecal pellets were vortex-homogenized in 10 mL saline-peptone buffer for 1 hour after collection; serially diluted and then plated on LBS agar (BD, Franklin Lakes, NJ) plates. The plates were cultivated in an anaerobic chamber at 37°C for 24 h. CFUs were calculated and twelve colonies were randomly selected from each sample and subcultured in MRS broth for 24 h. Ten colonies are believed to provide adequate representation of the major bacterial strains which were cultivated with a selective medium (Hartley *et al.*, 1977).

Genomic DNA was extracted from the *Lactobacillus* isolates using the method described by Korhonen *et al.* with slight modifications (Korhonen *et al.*, 2007). Briefly, bacterial cultures grown overnight in 3 mL MRS broth were centrifuged (10,000 xg, 2 min) and the cells were re-suspended and washed twice in 1.5 mL molecular biology grade water (MO BIO

Laboratories, Inc., Carlsbad, CA). The supernatant was discarded and the pellet was re-suspended in 750 μL of TE buffer (10mM Tris-HCl-1 mM EDTA [pH 8.0]). The bacterial cells were lysed by shaking for 1 min on a mini-bead beater (Bio-Rad Laboratories, Hercules, CA) at a speed of 6 m s^{-1} in 2 mL screw-cap tubes, to which 350 to 400 mg of 100- μm -diameter Silica beads (Research Products International Corp., Mt. Prospect, IL) had been added. After centrifugation at 12,000 $\times g$ for 2 min, 500 μL of supernatant was transferred to a 1.5 mL centrifuge tube and stored at -20°C .

In order to identify the *Lactobacillus* isolates, primers LB308F and LB806RM lacking fluorescent tags were used to generate 0.5 kb unlabeled amplicons, which were cleaned using the Zymoclean DNA Clean & Concentrator Kit (Zymo Research Corporation, Irvine, CA) and sequenced on ABI 3730xl capillary systems by either primer LB308F or LB806RM. A FASTA file containing all of the sequences was constructed and uploaded into the BLAST search engine to compare records in the NCBI nucleotide collection database. Based on previous studies with *Bifidobacterium* (Zhu *et al.*, 2003), *Vibrio* (Kwok *et al.*, 2002), and *Staphylococci* (Goh *et al.*, 1996), records with maximal similarity values greater than 97% were used to identify species in this study. The sequences of recovered in this study were deposited with GenBank under accession numbers JN998111- JN998113.

2.5 T-RFLP dataset analysis

All digitized TRF datasets were retrieved and analyzed by the Applied Biosystems Peak Scanner (Applied Biosystems, Foster City, CA) and Microsoft Excel. The word “dataset” is used to define a particular microbial community. Each dataset consisted of 4 data matrices which were derived from the same template DNA and reflected the same community, named *AluI*-forward, *AluI* -reverse, *TacI*-forward, *TacI*-reverse.

In Peak Scanner, the analysis method for the T-RFLP matrices was set to “PP” (factory default sizing algorithm for samples containing primer peaks) and size standard GS500 was used. A baseline threshold of 25 fluorescence units was used to eliminate background noise with all datasets.

Those TRFs which differed by less than 0.5 bp in different profiles were considered identical and were clustered (Korhonen *et al.*, 2007). The relative peak area was calculated by dividing each raw peak area by the cumulative peak area of the sample and was shown as a percentage value. The reproducibility of the procedure was confirmed by repeating the entire PCR, digestion and data manipulation procedure three times. While absolute peak areas varied between trials, the relative peak area varied less than 5% for each TRF from each sample across the three replicates. Incomplete digests for TRFLP profiles were also resolved by comparisons between replicate digests. (Mills *et al.*, 2003).

The relative T-RFLP matrices were entered into an Excel spreadsheet that consisted of the TRFs as variables and individuals as objects. A binary data table (presence or absence of individual peaks) was generated, in which peaks with percentage of total area $>1\%$ were assigned as 1 (presence) and $<1\%$ were assigned as 0 (absence) at every possible TRF size in all 4 matrices. The binary table was imported into the statistics software XLStat (Addinsoft Inc., Brooklyn, NY) to generate principal component analysis (PCA) plots and an agglomerative hierarchical clustering (AHC) dendrogram.

The identification of the *Lactobacillus spp.* was done by matching the TRFs in the generated database. As described by other authors, we noted some minor difference between the predicted and observed TRF lengths (Kaplan *et al.*, 2003). So, a variation tolerance of -1 to 10 bp was applied in this study. In addition, an online tool using Google Spreadsheet (Google Inc., Mountain View, CA) was developed to rapidly conduct the pattern

comparison; with all of the *Lactobacillus spp.* matching the known TRF patterns were highlighted.

3. Results

3.1 In silico evaluation of the *Lactobacillus hsp60* T-RFLP method

A total of 769 *Lactobacillus hsp60* partial sequences were obtained from NCBI databases for *in silico* analysis. Computer simulated *Lactobacillus hsp60* T-RFLP using restriction endonucleases *AluI* and *TacI* on fluorescently labeled PCR products was able to generate 83 distinct TRF patterns, of which, 70 were specific for one species (Table S1). In addition, 23 *Lactobacillus spp.* had more than one corresponding pattern.

3.2 Validation of Method

In order to verify the T-RFLP protocol, 5 lactobacilli with distinct TRF patterns were selected and mixed as reference strains. The five strains were mixed so that their concentration was 7, 72, 720, 7,200 and 72,000 CFU mL⁻¹. All species generated clear and distinct peaks at the location predicted *in silico* (Fig. 1). Only the peak generated by *L. jensenii* was close to background noise due to the low starting cell number, indicating the high sensitivity of this assay. There were -5 to 5 bp variations between the predicted and observed TRF lengths, this variation was previously observed and described by Kaplan *et al.* (Kaplan *et al.*, 2003). When the experiment was repeated with different ratios of these five lactobacilli, we found that the minimum concentration of cells in pure culture needed to readily generate distinguishable peaks was 1×10³ CFU mL⁻¹. T-RFLP demonstrated a qualitative relationship between the size of peaks and bacterial cell concentration. The species' with a larger relative abundance generated the larger peak while the species' with a lesser relative abundance generated smaller peaks.

3.3 Use of the T-RFLP method for analysis of fecal lactobacilli community

T-RFLP was used to obtain fingerprints of the fecal lactobacilli communities from 4 mice in each of 8 treatment groups. Each fecal lactobacilli community was evaluated by a dataset consisting of 4 data matrices: *AluI*-forward, *AluI* -reverse, *TacI* -forward and *TacI* -reverse. A total of 53 TRF patterns were generated. Each mouse had a unique lactobacilli community profile, consisting of different mixtures of TRFs. Within each treatment group, the most dominant TRFs were observed in all subjects, and the intensity for each TRF was similar. However, between groups, there were differences in both intensity and sizes of the dominant TRFs present. Agglomerative hierarchical clustering (AHC) analysis was applied to visualize the *Lactobacillus* communities (Fig. 2). Samples from different treatment groups showed much greater differences than samples within the same group, which demonstrated that both dietary fiber supplements and the duration of time on the diets significantly impact the fecal lactobacilli community.

3.4 Species identification by culture dependent method and T-RFLP

To compare the traditional culture dependent method and T-RFLP in analyzing the *Lactobacillus* population, we compared the T-RFLP results from the fecal extraction (Figure 3) with a culture based approach. Twelve randomly selected isolates from the same feces were isolated from LBS plates for each of the four mice in group 7 and group 8. These isolates were speciated based on partial *hsp60* sequence (Table 3). All 48 isolates from group 7 were identified as *L. salivarius* with a predicted TRF for *AluI*-forward of 84 bp. In group 8, 18 isolates were identified as *L. johnsonii* (37%; predicted TRF for *AluI*-forward of 75 bp) and 30 as *L. reuteri* (63%; predicted TRF for *AluI*-forward of 150 bp). When

compared to the T-RFLP data set for these two groups (Fig. 3), the dominance of *L. salivarius* in group 7 and *L. johnsonii* and *L. reuteri* in group 8 is confirmed.

4. Discussion

Selection of suitable primers and enzymes is a key step in designing T-RFLP protocols. Many investigators have used the 16S rDNA primers and enzymes *HhaI*, *MspI*, *RsaI* in T-RFLP analysis (Culman *et al.*, 2008) because extensive databases of sequences, primer sets and tools for analysis of 16S rDNA TRFs are well established. However, 16S rDNA gene sequence analysis is not able to reveal significant differences between recently diverged species, such as *L. plantarum*, *L. paraplantarum*, and *L. pentosus*; or *L. casei*, *L. rhamnosus*, and *L. zeae*; or *L. johnsonii* and *L. gasseri* (Blaiotta *et al.*, 2008; Walter *et al.*, 2000). In some cases the poor specificity of 16S rDNA based TRF approaches make it difficult to identify bacteria at the species or genera level (Culman *et al.*, 2008). The 16S-23S rRNA internal transcribed spacer (ITS) has greater variation, which allows for discrimination of a larger number of *Lactobacillus* spp. (Moreira *et al.*, 2005). In addition, the 16S-23S rRNA ITS has been used as a template for T-RFLP in detecting 19 other species (Spasenovski *et al.*, 2009). However, Culman *et al.* suggested that the most optimal PCR primers for T-RFLP should have product length between 400 and 700 bp since this allows for the best possible estimation of diversity while avoiding the loss of data associated with long amplicons (Culman *et al.*, 2008). Song *et al.* indicated that most of the *Lactobacillus* spp. have an ITS between 200 to 300 bp (Song *et al.*, 2000), so the amplicons of ITS are usually too short to generate informative patterns in bacterial fingerprint analysis. The *hsp60* based T-RFLP developed in this research overcomes limitations of the existing methods using either 16S rDNA or 16S-23S ITS. Seventy-nine distinct overall TRF patterns were generated *in silico* by a set of fluorescently labeled *Lactobacillus* specific primers and endonuclease *AluI* and *TacI*, of which, 70 were specific for one species. The high variability and comparably long lengths of the *hsp60* nucleotide sequences allowed for the discrimination of very closely related species and even strains within these species, including *L. casei* and *L. rhamnosus*; *L. acidophilus* and *L. crispatus*; *L. acidophilus*, *L. helveticus*, and *L. amylovorus*; and *L. plantarum* and *L. pentosus* (Blaiotta *et al.*, 2008). These data confirm the usefulness of using *hsp60* as a target in *Lactobacillus* phylogenetic analyses.

Previous studies have shown that the number of rRNA genes varies significantly between different species (Culman *et al.*, 2008) and others have postulated that an average bacterial community has 3.8 16S rDNA copies per genome (Fogel *et al.*, 1999) while *L. acidophilus* has four copies in the genome (Roussel *et al.*, 1993). The TRF abundance may not describe the relative abundance of specific amplicons in a mixture, and in turn not be able to estimate relative bacterial abundance. Since the *hsp60* is present in only a single copy on the chromosome (Goh *et al.*, 1996; Kwok *et al.*, 1999), amplicon abundance after PCR is more proportional to cell abundance in the original sample without any prior calibration, which makes it possible to relate T-RFLP analysis to relative cell abundance in the original sample. Additionally, the single copy of *hsp60* avoids the potential problem associated with polymorphisms found in different copies of the same gene as has been seen with 16S rRNA (Pillidge *et al.*, 2009). This feature of *hsp60* makes it ideal for either generating a similarity index or being used in principle components analysis (PCA) (Culman *et al.*, 2008). We demonstrated that the *hsp60* based T-RFLP could correctly reflect the quantitative relationship of the tested species in the mixed culture (Fig. 1). This result demonstrates the potential utility of this tool for comparing the abundance of *Lactobacillus* spp. in the community.

In order to test the capability of *hsp60* based T-RFLP in profiling the fecal *Lactobacillus* communities, fecal samples from 8 treatment groups of mice were analyzed. The graphs

generated using raw data in the software Peak Scanner demonstrate that the TRF patterns from different animals within the same treatment group are very similar, while comparison between groups are significantly different (Fig. 3). PCA plot and AHC dendrogram were generated using a binary table. Individuals within the same dietary groups could be easily clustered together with either PCA plot or the AHC dendrogram. We also constructed PCA and AHC tests that incorporate peak area which generated very similar results with only trivial variation. However, using binary values was preferred, due to a greater resistance to errors from incomplete digestion. This test has also indicated that diet can have a great influence on the fecal *Lactobacillus* microbiota composition.

We compared the T-RFLP results with groups 7 and 8 to results from culture dependent analysis. The major peaks found in T-RFLP can be clearly identified by *in silico* analysis of the *hsp60* sequences generated from the lactobacilli isolates. The TRF for *L. reuteri* and *L. salivarius* did not previously exist in the database and required *hsp60* sequencing of lactobacilli isolates to identify. However, *hsp60* sequencing of a few isolates will provide the necessary information to identify the major peaks in the TRFLP data set if species identification is necessary. At a community level, our *Lactobacillus* T-RFLP could readily compare the lactobacilli community from one treatment to another (Fig. 2).

In conclusion, we have shown that the *hsp60* based T-RFLP method is a valid monitoring tool for *Lactobacillus* community dynamics, the high variability, suitable amplicon size and stable copy number in the chrome offers a higher usefulness than other PCR based molecular approaches. The application of this method could considerably enhance and extend our current understanding of intestinal microbiota.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We developed a novel T-RFLP protocol based on *Lactobacillus hsp60*
- We determined the terminal restriction fragment (TRF) profiles of 96 *Lactobacillus spp.* or strains.
- This new protocol is semi-quantitative
- We performed this protocol on mice fecal samples for community and species level analysis.

Fig. 1.

TRF profile generated by mixed bacterial culture with *AluI* and forward labeled *hsp60* primers. The X axis indicates the size of the TRFs, and the Y axis indicates the percentage of peaks height compared to the tallest peak. The original concentrations of the bacterial cells in 1 mL culture were: *L. rhamnosus*: 72,000 cells mL⁻¹, *L. plantarum*: 7,200 cells mL⁻¹, *L. acidophilus*: 720 cells mL⁻¹, *L. sakei*: 72 cells mL⁻¹, *L. jensenii*: 7 cells mL⁻¹. The TRF sizes (indicated in the brackets) identified corresponded with the prediction. In addition, TRF signal strength demonstrated a semi-quantitative relationship.

Fig. 2.

AHC dendrogram of the T-RFLP data for the fecal lactobacilli community of 32 mice from 8 treatment groups (4 mice per group). These figures were constructed by the software XLStat (Addinsoft Inc.) based on a binary data table, which indicates presence or absence of individual peaks in all 4 matrices from the dataset of each individual samples. Samples from different groups showed much greater differences than samples within the same group.

Group 7

Group 8

Fig. 3.

T-RFLP raw data from the software Applied Biosystems Peak Scanner. Each panel represents a fecal extraction from a different mouse and only *A/uI* with labeled forward primer for Group 7 and 8 is depicted here. The X axis indicated the size of the TRFs, and the Y axis indicated the percentage of peaks height compared to the tallest peak. The TRF patterns within the sample dietary groups are very similar to each other, while between these two dietary groups are very different.

Table 1

Quantitative real-time PCR primers used in this study

Primer	Sequence (5'-3')	Reference
H279	GAATTCGAIIGCIGGIGA(TC)GGIACIACIAC	Goh <i>et al.</i> , 1996
H280	CGCGGGATCC(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCGGIGC(TC)TT	Goh <i>et al.</i> , 1996
LB308F	TGAAGAAAGTNRNGCYGG	Blaiotta <i>et al.</i> , 2008
LB806RM	AANGTNCCVCGVATCTTGTT	Blaiotta <i>et al.</i> , 2008
LB308F-D	/56-FAM/TGAAGAAAGTNRNGCYGG	This study
LB806RM-D	/5HEX/AANGTNCCVCGVATCTTGTT	This study

Table 2

Animal grouping and diet information

Group	Fiber (%)[*]	Fat (%)^{**}	Age (months)^{***}	Diet NO.^{****}
1	cellulose (10)	60	8	D07102501
2	pectin (10)	10	8	D06082202
3	pectin (10)	60	8	D08111803
4	cellulose (5)	60	8	D12492
5	cellulose (5)	10	8	D12450B
6	cellulose (10)	10	8	D06082201
7	cellulose (10)	10	3	D06082201
8	pectin (10)	10	3	D06082202

^{*} Percent fiber on a weight basis

^{**} Percent fat on caloric basis

^{***} The animals were fed the indicated diet from weaning (21 days) to the age indicated.

^{****} Product number for each diet as assigned by Research Diets