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Determining the Genetic Diversity of Lactobacilli from the Oral Cavity

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Summary

Several methods for determining the diversity of *Lactobacillus spp* were evaluated with the purpose of developing a realistic approach for further studies. The patient population was comprised of young children with an oral disease called severe early childhood caries. The ultimate goal of these studies was to ascertain the role of lactobacilli in the caries process. To accomplish that goal, we evaluated several methods and approaches for determining diversity including AP-PCR, chromosomal DNA fingerprinting, denaturing gradient gel electrophoresis, and 16S rRNA gene sequencing. Central to these methods was the gathering and screening of isolates from cultivation medium. Using various estimates of diversity, we addressed the question as to how many isolates represent the overall diversity and how cultivation compares to non-cultivation techniques. Finally, we proposed a working approach for achieving the goals outlined framed by both practical constraints in terms of time, effort and efficacy while yielding a reliable outcome.

Keywords

Oral lactobacilli; genotyping; 16S rRNA gene; DGGE; AP-PCR; CDF; dental caries

1. Introduction

The genus *Lactobacillus* comprises over 100 species exhibiting a wide range of diversity in terms of genomic size, G+C content, ecological or host preferences and expression or absence of specific genetic loci (Canchaya et al., 2006; Claesson et al., 2007; Makarova and Koonin, 2007; Tannock, 2002). Besides their enormous economic value in the fermentation and preservation of various food products, lactobacilli (LB) colonize the gastrointestinal tract (GI) of humans and urogenital tract of women, and play an ever increasing role as a probiotic for promoting overall health (Bernardeau et al., 2006).

In contrast, the presence of LB in the oral cavity plays a substantial role in dental caries progression which has been well established from both epidemiological surveys (Beighton, 2005; Burt et al., 1985; Leverett et al., 1993) and the genetic-based determination of the bacterial composition associated with caries lesions (Byun et al., 2004; Caufield et al., 2007;

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Chhour et al., 2005; Marchant et al., 2001; Munson et al., 2004). Indirect support for the role of LB in dental caries comes from its ability to generate low pH from fermentation of carbohydrates, including sucrose, and its tolerance to low pH environment (Cotter and Hill, 2003). The same physiological properties of LB underpin both their use in food fermentation and their role in dental caries: LB prefer carbohydrate-rich environments in a stagnation state where they can lower the pH to the extent of inhibiting the growth of other less acid tolerant organisms. LB performs well its degradative role, both of food products and teeth.

Methods that are capable of identifying unique strains or genotypes of LB, often referred to as genetic fingerprinting, include arbitrarily-primed PCR (AP-PCR or RAPD) (Dal Bello and Hertel, 2006; Matsumiya et al., 2002; Sanchez et al., 2005; Tynkkynen et al., 1999) and chromosomal DNA restriction digest profiles (CDF) (Caufield et al., 2007). Less genotype-specific yet powerful methods for estimating overall diversity of LB at the genus or species level include denaturing gradient gel electrophoresis (DGGE) (Dal Bello and Hertel, 2006; Ercolini, 2004; Heilig et al., 2002; Walter et al., 2000; Zoetendal et al., 2002), 16S rRNA gene sequencing (phylotyping) (Byun et al., 2004; Caufield et al., 2007; Chhour et al., 2005; Marchant et al., 2001; Munson et al., 2004), multiple locus sequence typing (MLST) (Cai et al., 2007), ribotyping (Rodtong and Tannock, 1993; Zhong et al., 1998), pulse-field gel electrophoresis (PFGE) (Tynkkynen et al., 1999), and repetitive elements profiling (Antonio and Hillier, 2003). The latter methods can sometimes give resolution to the genotypic level within a sample of limited diversity, but are generally not useful for identifying unique, host-specific genotypes.

Much remains to be determined about the ecology of LB in the oral cavity, their origins, diversity and stability. For example, do LB constitute stable members of the indigenous oral biota of humans or are they transient and opportunistic colonizers originating from food products or from other internal sources such as the GI tract? What determines the extent of genetic and species diversity of the LB in the oral cavity? Are LB transmitted vertically, along familial lines? To answer these and other queries about LB diversity, methods are required to identify LB at both the species and strain (genotypic) level. Once a specific genotype is defined, its distribution, genetic composition and likely origin can be determined.

This report described and evaluated the usefulness of various approaches for delineating diversity and genotypes of LB in the oral cavity. The aim of our study was to propose a single and unified approach that can yield the most informative, reproducible, and time-effort efficient method of characterizing the diversity of LB in the oral cavity.

2. Materials and Methods

2.1. Study population

The study population was comprised of seven children (six males, one female) of Hispanic origin with severe early childhood caries (S-ECC) (defined here as a child under 5 years of age with greater than or equal to six active caries lesions in primary teeth that include the maxillary anterior teeth (http://www.aapd.org/media/Policies_Guidelines/D_ECC.pdf). The children were evaluated by pediatric dental specialists and scheduled for extensive caries restorative treatment under general anesthesia in the operating room at the Bellevue Hospital, New York, NY. The study protocol was approved by the Institutional Review Board of New York University School of Medicine and the Bellevue Hospital for human subjects.

2.2. Bacterial sample collection and preparation

One unstimulated saliva and one pooled plaque samples were obtained from the children using the methods previously described (Caufield et al., 2007; Li et al., 2005). In addition, four individual plaque sample were obtained from caries lesions of four posterior quadrants of the dentition with a sterile round bur (Kidd et al., 1996). A total of six bacterial samples were collected from every child, hence 42 bacterial samples were processed for the assessment of genetic diversity of LB (Fig. 1). Samples were placed in 1 ml reduced transport medium (Syed and Loesche, 1972) and stored on ice for no more than 4 h.

2.2.a. Cultivation for LB—All of the bacterial samples were first dispersed by sonication and plated onto various select and non-select media, including Rogosa agar (Difco BD, Sparks, MD, USA) using a Autoplate 4000 spiral plater (Spiral Biotech, Inc., Bethesda, MD, USA) (Fig. 1). Colonies were obtained after incubation of the plates for 48 h at 37 °C in an anaerobic workstation (MACS, dw Scientific Ltd, West Yorkshire, England). To capture as many representative genotypes of LB as practically possible, 50 colony forming units (cfus) per sample (a total of 300 cfus per child) were picked from each Rogosa plate by the method described by described by Munson and co-workers (Munson et al., 2004) to minimize bias and maximize randomness in selection. We also made an effort to including different colony morphologies when present. All pure-streaked colonies were transferred to MRS broth (Difco BD, Sparks, MD, USA), grown overnight at 37 °C in the anaerobic workstation, and gram-stained to ascertain that they were gram positive bacilli. The isolates were stored at –80°C in MRS broth (Difco) with 15% glycerin until further testing.

2.2.b. Total cultivated LB—After picking 50 cfus per plate, the remaining colonies on the Rogosa plate were gathered with a sterile cotton swab and washed thoroughly in TE solution (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) for DNA extraction (Fig. 1). The DNA extraction protocol can be found in our previous reports (Caufield et al., 2007; Li et al., 2005).

2.2.c. Total culture independent bacterial samples—To access the LB genotypic diversity, a portion (0.5 ml) of the pre-plating bacterial sample of each type was saved frozen at –20 C° (Fig. 1); total genomic DNA was isolated for the survey by DGGE using LB-specific primers (Walter et al., 2001). The results were used to compare with the results from Rogosa-cultivated samples.

2.2.d. LB reference strains—Seven *Lactobacillus* reference strains were obtained from the American Type Culture Collection (ATCC). These included *L. gasseri* (ATCC33323), *L. acidophilus* (ATCC4356), *L. fermentum* (ATCC4356), *L. salivarius* (ATCC11741), *L. casei* (ATCC393), *L. oris* (ATCC49062) and *L. vaginalis* (ATCC49540). Strains were selected based on different G+C content and their frequent appearance in the oral cavity of humans (Byun et al., 2004; Caufield et al., 2007; Chhour et al., 2005; Marchant et al., 2001; Munson et al., 2004).

2.3. Arbitrarily primed PCR (AP-PCR)

Genotypes of LB were initially determined by AP-PCR using primer OPA3 (5'-AGTCAGCCAC-3'), a method described for *S. mutans* (Li and Caufield, 1998; Li et al., 2001). OPA3 was selected for LB genotyping based on a previous study in which 40 primers from commercially available kits, OPA and OPB (Operon Technologies Inc, CA) were screened for suitability (Lu et al., 1999). Template DNA was prepared by growing overnight cultures of LB in MRS broth, lysing cells with lysozyme and mutanolysin and a final treatment with proteinase K and phenol extraction (Li et al., 2001; Li and Caufield, 1998; Zhong et al., 1998). The AP-PCR amplification was performed in a GeneAmp PCR 9700

thermocycler (PE Applied Biosystems, Foster City, CA, USA) in a total volume of 50 μ l containing PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4); 200 μ M of each dNTP (Invitrogen, Carlsbad, CA, USA); 7 mM MgCl₂; 2.5 U of Taq DNA polymerase (Invitrogen), 100 pmol of primer OPA3 and 50 ng DNA template. The PCR reaction was run 45 cycles of 94°C for 30 s, 36°C for 30 s and 72°C for 1 min, followed by an extension of 5 min at 72°C. The AP-PCR products were separated by electrophoresis on a 1.5% agarose gel in Tris-borate-EDTA buffer and results were captured with a digital imaging system (Alpha IS-1000, Alpha Innotech Corp., San Leandro, CA, USA).

2.5. Chromosomal DNA fingerprinting

Isolates displaying unique AP-PCR fingerprints were subjected to chromosomal DNA fingerprinting (CDF). Large-scale isolation of chromosomal DNA for CDF was accomplished using previously published methods (Caufield and Walker, 1989; Li and Caufield, 1995) with the modification that following cell lysis and centrifugation, DNA was isolated using QIAGEN Genomic-tip 100 following manufacturer instructions. DNA quantity and purity were assessed by spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA, USA). A battery of restriction enzymes was screened for suitability based on the literature (Caufield et al., 2007; Stahl et al., 1990; Zhong et al., 1998) and on *in silico* digestion profiles of reference genomes *L. gasseri*, *L. casei*, *L. salivarius*, *L. fermentum* and *L. acidophilus*. *In silico* profiles were generated for approximately 30 restriction enzymes with the Restriction Digest Tool at the JCVI Comprehensive Microbial Resource website (http://cmr.jcvi.org/cgi-bin/CMR/shared/MakeFrontPages.cgi?page=restriction_digest&crumbs=genomes). We confined our selection of restriction digests to those that yielded discrete fragments below 23 kb and larger than 4 kb. The selected set of restriction enzymes for delineating LB genotypes included *Hind*III, *Hae*III, *Bcl*I, *Hpa*I, *Eco*RI, *Spe*I, and *Pst*I. Restriction digestions were performed as previously described (Li and Caufield, 1995). Briefly, 1 μ g of chromosomal DNA was digested in a final volume of 30 μ l with 20–100 U of the appropriate restriction enzyme. The resulting fragments were separated on 0.55% agarose gels for 17 h at 40 V.

2.6. 16S rRNA gene amplification and sequencing analysis

Isolates displaying unique AP-PCR fingerprints and confirmed by CDF were subjected to 16S rRNA gene sequencing analysis for species identification. DNA template was prepared as above for AP-PCR. A 232-bp fragment of the V2-V3 region of 16S rRNA was amplified with primers LactoF and LactoR (Byun et al., 2004; Caufield et al., 2007), and sequenced from each unique genotype determined based on the AP-PCR screening results. Briefly, 50 μ l reaction mixtures consisted of PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 2.5 mM MgCl₂, 200 μ M of each dNTP, 10 pmol of each primer, 2.5 U of Taq DNA polymerase and 50 ng DNA template. PCR conditions consisted of an initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 15 sec, 61°C for 1 min and 72°C for 30 sec, and an additional cycle of 7 min at 72°C for chain elongation. PCR products were evaluated by electrophoresis on 1.0 % agarose gels and purified with the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The sequences obtained (Genewiz, South Plainfield, NJ) were compared to other 16S rRNA genes in the Ribosomal Database Project II (Caufield et al., 2007; Cole et al., 2009). Taxonomic identity to the species level was assigned based on the highest similarity score equal or greater than 99%.

2.7. DGGE

A second of pair of LB-specific 16S rRNA-targeted primers (Lac1 and Lac2_GC; (Walter et al., 2001)) generated an approximately 340-bp amplicon. Each PCR reaction mixture contained PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 200 μ M of each dNTP, 20

pmol of each primer, 1 mM MgCl₂, 2.5 U of *Taq* DNA polymerase and 50 ng DNA template. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of 30 sec at 94°C, 1 min at 61°C, 1 min at 68°C, and an additional cycle of 7 min at 68°C for chain elongation. The PCR products were evaluated by electrophoresis on 1.0 % agarose gels.

DGGE was carried out with Bio-Rad DCode System (Hercules, CA, USA). The PCR-amplified products were separated on 30–50% linear denaturing gradient (Walter et al., 2001) formed in 8 % (w/v) polyacrylamide gels. The electrophoresis was conducted with a constant voltage of 70 V at 60 °C for about 7 h in 1×TAE buffer. Gels were stained and recorded as previous described (Li et al., 2005).

2.8. Data analyses

AP-PCR, CDF and DGGE profiles were first normalized and analyzed using Bionumerics version 6.0 (Applied Maths, Belgium). Bands were predicted from standardized density curves. Minimum profiling and gray zone settings were both adjusted to 5% for AP-PCR and CDF experiments, and used uniformly across samples and gels. For DGGE experiments, the minimum profiling and gray zone settings were adjusted to 4% and 1%, respectively. Similarity matrices of pair wise comparisons for AP-PCR and CDF were evaluated using the Dice coefficient algorithm (optimization and tolerance both set to 1%), and differences were tested using Student t-test. Clustering was based upon the unweighed pair group method with Arithmetic mean (UPGMA) for the hierarchical clustering of pair wise distances.

Rarefaction curves were generated for each sample type from each subject (7 subjects × 6 sites = 42) using the AP-PCR genotype of each colony isolate as the taxonomical unit as they accumulated for 50 isolates from each site (collector's curve). Rarefaction estimates were based on 1000 re-samplings without replacement from each of 50 isolate identities. A median and 95% confidence intervals were calculated for each sample site.

3. Results

The aim of the present study was to compare several methods and approaches for assessing the diversity of lactobacilli in clinical isolates from the oral cavity of children with severe early childhood caries. AP-PCR with primer OPA3 produced unique and well-discerned patterns for the seven *Lactobacillus* type strains (Fig 2A). The number of amplicons present was sufficient for differentiating strains with individual amplicon size ranging from 300 to 3000 bp. For the clinical samples, we analyzed 50 isolates each from the saliva, pooled plaque and four caries lesions from seven children for a total of 2,100 isolates. Among those isolates, we identified between one and six unique genotypes for each subject, and a total of 29 LB genotypes (Table 1) for all the children.

The genetic uniqueness of these genotypes was confirmed by CDF. First, we selected restriction enzymes used previously in the published literature. We also examined digest profiles of five complete and assembled *Lactobacillus* genomes *in silico* with approximately 30 different restriction enzymes, using the Restriction Digest Tool available at the JCVI website. Seven restriction enzymes were selected for further application (*EcoRI*, *HpaI*, *SpeI*, *PstI*, *HaeIII*, *HindIII* and *BclI*) on the basis of their *in silico* digestion pattern. We found that among all the enzymes examined, restriction enzymes *BclI* and *HindIII* yielded the most discriminative patterns based on visual examination and digital reconstruction using the fragment normalization program implemented in Bionumerics ver 6.0. As shown in Fig 2B, enzyme *BclI* generated unique patterns for the genomes of the seven type *Lactobacillus* strains, except for *L. vaginalis*, which appeared largely undigested by *BclI* (Fig 2B, lane 7). On the other hand, *HindIII* could differentiate all seven type strains (Fig. 2B) and was,

therefore our enzyme of choice to assess the diversity of LB in clinical isolates by CDF. CDF yielded a greater number of bands than AP-PCR (Fig 2).

To compare the discriminating power between the AP-PCR and CDF methods, we analyzed the average magnitude of band dissimilarity via a pair wise distance matrix generated from the Dice algorithm across 20 of 29 genotypes (180 pairwise comparisons), averaging the distance values for each genotyping method. The mean dissimilarity value calculated for AP-PCR was 51% compared to only 12% for CDF. Both methods produced clear and well-discerned profiles for the clinical isolates similar to those shown in Fig. 3 for the type strains. The AP-PCR genotypes were clustered according to their similarity as seen in the dendrogram (Fig 3). The similarity values $\leq 90\%$ were in agreement with the genotypes being unique, except for isolates 87-A and 88-A, isolated from two different subjects, which appeared to have similar CDF and AP-PCR profiles. Because they come from different unrelated subjects, however, we propose that they are separate genotypes, understanding that this may be proven not to be the case after additional genetic profiling.

To obtain an overall estimation of diversity of LB populations within the clinical samples, DGGE was performed with LB-specific primers to the variable portion of the 16S rRNA gene. Primers and conditions were first tested with the reference strains; all displaying both discrete amplicons and migration distances consistent with their G+C content (data not shown). Fig 4 depicts the DGGE profiles for each clinical sample. Following gel normalization to standardize reading across gels, the number of bands present in each lane was estimated with Bionumerics. The detected number of amplicons (phylotypes) present ranged from 4 to 12 per sample (Table 1).

For subjects 80, 86, 87 and 88 the number of bands in the Rogosa-cultivated sample was smaller than for the culture independent sample (Table 1). This suggests that the culture of the clinical samples on Rogosa medium results in the loss of some phylotypes that are resilient to cultivation. Conversely, for patients 81, 84 and 85 the Rogosa-cultured profiles showed more bands than the culture-independent profiles. This suggests that the passage through Rogosa medium might enrich the samples in phylotypes that are otherwise not abundant and thus not detected in the culture-independent sample. However, when the average number of bands is compared between culture-independent (8.7 ± 1.6) and Rogosa-cultured (8.4 ± 2.9) samples, the difference is negligible. This suggests that overall, cultivation does not significantly impact the number of phylotypes detected by DGGE.

The average number of phylotypes detected per subject by DGGE on Rogosa-cultured samples was approximately twice the average number of unique genotypes detected per subject by AP-PCR (8.4 ± 2.9 vs. 4.1 ± 1.7 , Table 1). Subject 87 constituted an extreme example of this: only one genotype was identified by AP-PCR, but the corresponding DGGE profile indicated that as many as seven phylotypes were present on the Rogosa plate. This suggests that the diversity of the samples might be under-represented as a result of restricting colony-picking from the Rogosa plates to 50 cfus.

The species affiliation of the 29 genotypes was determined by sequencing of a 232-bp DNA fragment encompassing the V2-V3 region of the 16S rRNA gene. All sequences had identity scores of 1.0 with the Ribosomal Database curated collection. The 29 genotypes corresponded to seven different *Lactobacillus* species: *L. rhamnosus*, *L. gasseri*, *L. casei*, *L. fermentum*, *L. oris*, *L. salivarius* and *L. vaginalis*. The number of species found for each subject ranged between 1 and 4, with an average of 3.14 species per subject (Table 1).

4. Discussion

The purpose of this study was to test various methods for estimating the diversity of lactobacilli in the oral cavity that, in turn, would lead to the development of an efficient as well as effective overall approach for subsequence studies. Assessing overall diversity using culture-independent methods such as 16S rRNA sequencing or DGGE, while efficiently and amply utilized by others (Byun et al., 2004; Chhour et al., 2005; Marchant et al., 2001), was not an option because viable isolates of LB were necessary for more detailed genetic characterization. Methods evaluated here included cultivation of samples on Rogosa medium, picking and screening isolates by AP-PCR, confirming with CDF, and DGGE profiles of both cultivated and culture independent samples followed by sequencing of the 16S rRNA gene for species assignment.

Perhaps the most labor-intensive aspect of this study was picking colonies from Rogosa medium and screening for unique genotypes. We modeled our colony picking based on the random selection process described by Munson and co-workers (Munson et al., 2004) so as to minimize bias in colony selection. Our method was not entirely random, however, because we first tried to select representatives with different colony morphologies, if present. Although we recovered 29 genotypes from seven species of LB, we found that the difference in colony morphology present on Rogosa was not helpful in differentiating strains of LB. Rogosa medium has enjoyed widespread acceptance for selective cultivation of LB (Caufield et al., 2007; Dal Bello and Hertel, 2006; Marchant et al., 2001). However, Rogosa also permits the growth of oral streptococci including *S. mutans* (streptococcal colonies are readily observed based on their small and transparent morphologies). Moreover, not all *Lactobacillus* genotypes/species grow on Rogosa anaerobically at 37°C as some food-associated LB prefer different growing conditions, e.g., lower temperatures, likely more closely associated with their natural habitat as opposed to the oral cavity (Dal Bello et al., 2003; Munson et al., 2004; Walter et al., 2001). DGGE profiles comparing the number of putative LB phylotypes found on the entire Rogosa plate (not cfus picked) versus culture independent profiles, however, were essentially the same, indicating that Rogosa medium supported the growth of the majority of LB found in the oral cavity (Table 1 and Fig. 4).

The biggest discrepancy became evident comparing culture-independent DGGE and actual genotypes recovered (Table 1), showing a difference of almost two-fold. The most likely explanation is that we did not screen enough colonies to capture all species present. Some of the bands in the DGGE profiles could be due to artifacts (Ercolini, 2004; Meroth et al., 2003; Walter et al., 2001) or species present in low abundance. As mentioned above, Rogosa medium incubated at 37°C may not support food-associated LB that would appear on DGGE profiles (Dal Bello and Hertel, 2006; Dal Bello et al., 2003; Walter et al., 2001). The number of colonies picked from plates (300 per subject) was dictated in part by our previous work (Caufield et al., 2007) but also upon time-effort constraints. Moreover, the number of colonies needed to be screened is a direct function of the number of genotypes (species) present in each sample. In our cohort, the number of genotypes ranged from one to five per individual (average of 4.1). The rarefaction curves constructed based on random re-sampling estimated that for a subject harboring five genotypes of LB, between 20 and 40 isolates would be required to recover over 80% of the total genotypes present based on cultivation (data not shown). Obviously the number of colonies required to recover the majority of genotypes is a function of the number of genotypes present in each subject; the more genotypes present, the greater depth of sampling required. Although we did not follow this sequence of screening in our experimental design, results presented here indicates that a better design would be to obtain a rough estimate of overall diversity of species present using a 16S rRNA survey such as DGGE or 16S rRNA sequencing, then picking the number of colonies for genotyping based on a rarefaction curve generated from the 16S rRNA data.

Although not part of this study, we observed less diversity of genotypes within samples from caries lesions (average of 2 genotypes/site) compared to saliva or plaque with average of 4 genotypes (data not shown). This suggests that fewer cfus need be picked from caries lesion samples.

The application of AP-PCR as a screening tool, while tedious in repetition, identified genotypes as effectively as the more time and labor-intensive CDF. Our initial plan was to use AP-PCR for screening purposes then confirm using CDF. Our analysis of pair wise distance scores convinced us that AP-PCR had more resolving power than CDF, making CDF redundant and unnecessary. Interestingly, when we compared CDF profiles with AP-PCR genotypes that differed by a few bands as seen in Fig. 3, we found that CDF profiles also varied by a few bands (data not shown). The major advantage of AP-PCR over CDF and other typing methods such as ribotyping (Rodtong and Tannock, 1993; Tynkkynen et al., 1999; Zhong et al., 1998) and pulse field gel electrophoresis (Tynkkynen et al., 1999) is that it is considerably less demanding and requires a less stringent preparation of template DNA. From the over 2100 isolates of LB screened by AP-PCR, we found the method capable of identifying unique genotypes both within the same species as well as different species. All AP-PCR profiles were repeated with different DNA preparations on different days and found reproducible in the vast majority of cases although lab-to-lab differences are not uncommon (personal observation).

Assigning genotypes via differences in AP-PCR or CDF profiles results in an overestimation of diversity compared to using species designation only. For example, the genotype profiles of subject 86 within the *L. casei* species (Fig 3) show small differences in banding patterns suggesting they are derivatives or sub-types of the same strain. The overall difference between the number of genotypes versus the number of species per subject is small, 3.1 versus 4.1, respectively (Table 1). However, we will not know whether these small differences reflect real differences in the genetic composition or origination of the strains.

The choice of primers for AP-PCR differed across laboratories but the final profiles appeared similar in terms of resolving power. We tested other primers including ERIC (Marchant et al., 2001) and M13V (Dal Bello and Hertel, 2006; Meroth et al., 2003) and obtained comparable results (data not shown). A study by Tynkkynen et al. (Tynkkynen et al., 1999) supported the utility of primer OPA3 used in this study, as first reported by Lu and coworkers (Lu et al., 1999).

We found seven LB species in the oral cavity of the subjects: *L. vaginalis*, *L. oris*, *L. gasseri*, *L. salivarius*, *L. fermentum*, *L. rhamnosus* and *L. casei*. Our results generally agree with previous reports both in the species identified and in the number of species found, usually between four and eight (Byun et al., 2004; Caufield et al., 2007; Chhour et al., 2005; Dal Bello and Hertel, 2006; Munson et al., 2004). Larger discrepancies appear comparing 16S rRNA surveys of diversity to our post-cultivation screen. For example, Byun and coworkers (Byun et al., 2004) reported 12 LB species present in pooled bacterial samples from 58 extracted teeth. The DGGE profiles shown in Fig. 4 support the idea that species identification by 16S rRNA sequencing post-cultivation results in a lower number of species identified, likely because of under sampling of cultivable colonies. Further studies will likely benefit from an initial survey of diversity using 16S rRNA-based methods, followed by colony screening based on rarefaction curves from richness and abundance estimates of diversity from 16S rRNA profiling. However, the addition of genotyping individual isolates showing 29 genotypes suggest an even greater diversity than predicted by 16S rRNA gene surveys. Intra-species variation is thought to be important among other bacterial species, such as *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Helicobacter pylori*, to name a few, where niche specific attributes and virulence determinants are strain specific. Strains

of LB within the same species also exhibit genetic variation that might impact variations in fermentation end products or organoleptic qualities of foods such as cheese. We believe studying diversity at the strain or genotypic level will ultimately enhance our understanding as to questions about variation in strain distribution and virulence as well as transmission between individuals. In addition, strains of LB selected for their probiotic benefits might be screened to exclude those genotypes or species associated with the promotion of dental caries.

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Fig. 1. Flowchart of the study design for bacterial sample collection and preparation, and the assessment of genetic diversity of lactobacilli.

Fig. 2.

Comparison of AP-PCR (A) and CDF (B) on seven *Lactobacillus* standard species. 1: *L. gasseri* (ATCC33323), 2: *L. acidophilus* (ATCC4356), 3: *L. fermentum* (ATCC14931), 4: *L. salivarius* (ATCC11741), 5: *L. casei* (ATCC393), 6: *L. oris* (ATCC49062), 7: *L. vaginalis* (ATCC49540).

Fig. 3. Genotyping of *Lactobacillus* clinical isolates by AP-PCR. Clustering according to similarity was done from the AP-PCR profiles using the Dice assignment of similarities and UPGMA clustering metric. The scale on top indicates similarity (%) and the dotted line indicates the similarity threshold of 90%. The species and strain names are indicated on the right.

Fig. 4.

DGGE profiles of PCR-amplified bacterial 16S rRNA gene fragments from seven S-ECC clinical samples (patients B080-B088). DNA aliquots from culture-independent (CI) saliva, plaque and four lesions, or from Rogosa-cultured (R) saliva, plaque and four lesions, were pooled together in equal amounts before DGGE PCR was performed. M: reference marker composed of seven type *Lactobacillus* strains (from top to the bottom) *L. gasseri* (ATCC33323), *L. acidophilus* (ATCC4356), *L. fermentum* (ATCC14931), *L. salivarius* (ATCC11741), *L. oris* (ATCC49062), *L. casei* (393) and *L. vaginalis* (ATCC49540).

Table 1

Lactobacilli genotypes detected by using different survey methods

Subject	AP-PCR Genotypes	No. Species (16S rRNA)	DGGE bands	
			Culture-independent	Rogosa
B080	5	3	8	4
B081	5	4	7	9
B084	5	4	8	12
B085	4	4	9	10
B086	6	3	12	11
B087	1	1	9	7
B088	3	3	8	6
MEAN±SD	4.14±1.68	3.14±1.07	8.71±1.60	8.43±2.88