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Lactobacillus species and genotypes associated with dental caries in Thai preschool children

S. Piwat[1], R. Teanpaisan[1], S. Thitasomakul[2], A. Thearmontree[2] and G. Dahlén[3]

1 Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand
2 Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand
3 Department of Oral Microbiology, Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

Correspondence: Rawee Teanpaisan, Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Songkhla, 90112, Thailand Tel.: + 66 74 429878; fax: + 66 74 212922; E-mail: rawee.t@psu.ac.th

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SUMMARY

Lactobacilli have been associated with the presence and progression of dental caries. Nevertheless, the relation between certain species or genotypes of *Lactobacillus* and caries is unclear and there are no data available for the Thai population. This study aimed to examine the distribution of species and genotypes of oral *Lactobacillus* among children with rather high caries prevalence, and to investigate whether certain species or genotypes were more related to caries activity than others. One hundred and sixty-five children were examined for caries status. Saliva samples were collected and the numbers of lactobacilli were counted. A total of 357 *Lactobacillus* isolates from 59 children were identified to species level by 16S ribosomal RNA genes polymerase chain reaction (PCR) –restriction fragment length polymorphism and 16S ribosomal RNA gene sequencing. Furthermore, 304 isolates from 56 children were genotyped using arbitrarily primed PCR. Significant correlation was found between levels of lactobacilli and dental caries ($P < 0.001$). Among the 10 identified species of *Lactobacillus*, *L. salivarius* was more prevalent in children with moderate to high caries prevalence compared with children with low caries prevalence, while *L. fermentum* was the most predominant species in all study groups. Moreover, a genetic heterogeneity of *Lactobacillus* species was found among the children and those with high caries prevalence tended to be colonized with more than one clonal type. In summary, *L. salivarius* may be a putative caries pathogen among preschool Thai children.

INTRODUCTION

Lactobacillus is part of the normal oral microflora, and it has been recognized for decades as a major contributor in the caries process (van Houte, 1994). Our previous reports have shown that *Lactobacillus* species are strongly associated with the presence and progression of dental caries in Thai children and adults (Teanpaisan *et al.*, 2007, 2009). Nevertheless, some *Lactobacillus* species have been introduced as probiotics in caries prevention, mainly because of their inhibitory activities against cariogenic *Streptococcus* spp. (Nase *et al.*, 2001; Chung *et al.*, 2004; Simark-Mattsson *et al.*, 2007). Although there is a strong association between lactobacilli and caries, less is known of the relationship at species level because of difficulties in identifying *Lactobacillus* species. It is important to understand the role of various lactobacilli, whether they are harmful, beneficial or neutral for the development of dental caries.

Genotypic studies of bacterial species are of interest in the search for more pathogenic clones. Recent

findings indicate that specific clones of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* from cases of severe periodontitis can be associated with higher virulence (Enersen *et al.*, 2008). It has also been demonstrated that caries-associated bacteria such as *Streptococcus mutans* and *Streptococcus sobrinus*, are usually presented as one single or a very limited number of genotypes in the predominant oral flora at a given time (Kilian *et al.*, 2006). However, genetic studies that relate the severity of caries with *Lactobacillus* genotypes are diverse and controversial.

As a consequence, it is important to define the roles of various species or genotypes of *Lactobacillus* in the caries process, because this will lead to better understanding of the natural habitat of various *Lactobacillus* species. The aims of the present study were to investigate the distribution of species and genotypes of oral *Lactobacillus* among Thai children with rather high caries prevalence (Teanpaisan *et al.*, 2007), and to determine whether certain species or genotypes were more related to caries activity than others.

METHODS

Subjects and clinical examination

One hundred and sixty-five Thai children aged 2–5 years old were recruited from children who attended a well-baby clinic at the hospital and health centers in Thepa district, Songkhla province, Thailand. The study protocol was approved by the National Ethical Committee, at the Ministry of Public Health, Thailand, and parental informed consent was obtained. The individual's caries status was recorded as dmft/dmfs indices (decayed, missing, filled teeth/decayed, missing, filled tooth surfaces) according to the criteria adapted from the World Health Organization's 1997 criteria (World Health Organization, 1997).

Bacterial sampling

A modified spatula method (Kohler & Bratthall, 1979) was used to obtain bacterial samples. A spatula was inserted into the mouth to moisten it with saliva. Each side of spatula was then placed directly on the surfaces of Rogosa SL agar (Difco, Sparks, MD) for recovery of lactobacilli and incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37°C for 72 h. The numbers of lactobacilli colonies on two predetermined areas, approximately 1.5 cm^2 of each spatula-pressed area, were counted as colony-forming units (CFU). For further analysis, colonies were collected from plates that contained average numbers of lactobacilli of more than 5 CFU per 1.5 cm^2.

A random sampling method was used for all culture plates. At least three colonies of either the same or different colonial appearance were collected from the culture plates. The colonies were tentatively identified as *Lactobacillus* based on their growth on Rogosa SL agar, colonial morphology, Gram staining and by being catalase negative (Felis & Dellaglio, 2007). After pure culture, all isolates were kept at −80°C until use.

Lactobacillus species identification

DNA samples were prepared using a Genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan), following the manufacturer's protocol for gram-positive bacteria. The *Lactobacillus* isolates were identified to species level by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) -amplified 16S ribosomal RNA (rRNA) genes by the method of Teanpaisan & Dahlen (2006). Briefly, the 16S rRNA genes were amplified by PCR using the universal primers 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGGTACCTTGTTACGACTT-3′) (Sato *et al.*, 2003). The PCR 50-μl reaction mixture contained 100 ng of DNA template, 1.0 μM of each primer, 5 μl 10 × buffer with 2.0 mM $MgCl_2$, 1.0 U of *Taq* DNA polymerase, and 0.2 mM of each dNTP. Amplification proceeded using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA) programmed as follows: initial heat activation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 1.5 min and a final extension step at 72°C for 10 min. The PCR products were individually digested with *Hpa*II or *Hae*III (New England Biolab, Ipswich, MA) according to the manufacturer's instructions. Digestion products were separated through 7.5% polyacrylamide and stained with silver nitrate. Discriminations between *L. casei* and *L. rhamnosus*, and between *L. acidophilus* and *L. crispatus*, which were not possible from

the PCR-RFLP pattern, were performed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Teanpaisan & Dahlen, 2006). Initially 14 type strains of *Lactobacillus* were included in the panel: *L. acidophilus* ATCC 4356^T, *L. brevis* ATCC 14869^T, *L. casei* ATCC 393^T, *L. crispatus* ATCC 33820^T, *L. curvatus* ATCC 25601^T, *L. delbrueckii* ATCC 9649^T, *L. fermentum* ATCC 14931^T, *L. gasseri* ATCC 33323^T, *L. paracasei* CCUG 32212^T, *L. plantarum* ATCC 14917^T, *L. reuteri* CCUG 33624^T, *L. rhamnosus* ATCC 7469^T, *L. salivarius* ATCC 11741^T, *Olsenella* (formerly *Lactobacillus*) *uli* CCUG 31166^T. Three other clinical isolates, *L. mucosae* CCUG 43179^T, *L. oris* CCUG 37396^T, and *L. vaginalis* CCUG 31452^T, were identified by 16s rDNA sequencing and were included in the panel. The isolates that did not fit to the panel above were identified by 16S rRNA gene sequencing. Also, several strains of the same species, identified by PCR-RFLP of 16S rRNA genes, were chosen for sequencing of 16S rRNA genes to confirm the results.

Sequencing was performed using an ABI PRISM Big Dye Terminator Kit and ABI PRISM 377 genetic analyzer (Applied Biosystems). In a 50-μl volume, the PCR mixture consisted of 500 ng template, 0.8 μl Terminator Ready Reaction Mix (Applied Biosystems), and 3.2 pmol each universal primer (8UA and 1492R primers). PCR was performed at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for a total of 25 cycles using the Gene Amp® PCR System 2400 (Applied Biosystems). Analysis of the alignment of % homology for the sequences was performed using the blast programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Lactobacillus paracasei CCUG 32212^T and all clinical strains identified as *L. paracasei* showed minor bands of PCR-RFLP and SDS–PAGE patterns different from *L. casei* ATCC 393^T. Therefore, these isolates were presented as *L. casei/paracasei* group.

Genotyping

After identification, three or more colonies of the same *Lactobacillus* species from the same child were collected for genotyping using arbitrarily primed PCR (AP-PCR) with the primers; ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Matsumiya *et al.*, 2002). The reaction mixture in a 50-μl reaction mixture contained 100 ng of DNA template, 1.0 μm of each primer, 5 μl 10 × buffer with 2.0 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase, and 0.2 mM of each dNTP. The mixture was subjected to 35 cycles of denaturation at 95°C for 1 min; ramping to 35°C in 3 min; annealing at 35°C for 1 min; extension at 74°C for 2 min, and a final extension at 74°C for 5 min. PCR products were separated through 7.5% polyacrylamide and silver-stained.

Analysis of data

Because there were no missing teeth and only one filling among the children investigated, the numbers of decayed teeth/surfaces (dt/ds) were depicted. The children were divided into three groups according to the first and third quartile cut-off points of dt; low caries was dt range 0–4, moderate caries was dt range 5–10, and high caries was dt more than 10. The average numbers of lactobacilli were categorized as: 0 CFU/1.5 cm^2, 1–10 CFU/1.5 cm^2, and >10 CFU/1.5 cm^2. The Kruskal–Wallis and chi square tests were used to evaluate the relationships between caries status and levels of salivary lactobacilli. The distribution of *Lactobacillus* species and genotypes was calculated as a percentage. The associations between frequency of isolation of each *Lactobacillus* species and caries group were compared using chi square test and Fisher's exact test. The analyses were performed with the SPSS statistical program (SSPS Inc., Chicago, IL). The differences were considered significant when $P < 0.05$.

RESULTS

A total of 165 children aged 2.2 ± 0.8 years had mean dt and ds of 5.7 ± 4.8 and 12.3 ± 13.3, respectively. In this study, the prevalence of caries-free children was quite low, with 22/165 of children (13.3%) being caries-free and 15/22 (68.2%) of children being negative for lactobacilli detection. Ninety-two out of 165 children (55.8%) carried salivary lactobacilli. Among these, 59 children produced more than five colonies of lactobacilli per 1.5 cm^2 and these were retained for species identification (total 357 isolates). The species frequency analysis of lactobacilli was therefore performed only for those 59 children with a predominant presence of lactobacilli.

Salivary lactobacilli levels in children with low caries prevalence were significantly different from those in children with moderate to high caries prevalence ($P < 0.001$) (Table 1). There were significantly higher mean dt and ds as numbers of lactobacilli increased ($P < 0.001$) (Table 1). The distribution of *Lactobacillus* species between different frequencies of caries lesions in children is shown in Table 2. *L. fermentum* (83% of the children) and *L. salivarius* (25% of the children) were the predominant species found. *L. salivarius* was the only species found in significantly higher numbers in the moderate to high caries group (35.9%) compared with the group with low caries (5%) ($P = 0.01$). The presence of other species was not related to the caries status. *L. fermentum* was the most frequently found (more than 80% of children) species in all groups. *L. plantarum* and *L. mucosae* were found only in the moderate to high caries group, while *L. gasseri*, *L. vaginalis*, and *L. oris* were identified more frequently in the low-caries group. The significance of these differences could not be further evaluated because too few subjects harbored these species. Most children (79.6%) harbored only one or two species of *Lactobacillus* with the maximum of five species detected in one individual. However, the diversity of *Lactobacillus* species was not statistically different between the groups ($P = 0.17$) All 10 *Lactobacillus* species from 56 children were further investigated by AP-PCR. The numbers of subjects and isolates of each species are shown in Table 3. Generally, isolates from each individual showed a distinct genotypic pattern, and between one and five different genotypes could be detected in a single child (Fig. 1). It was noted that children who had high caries prevalence tended to be colonized by more than one genotype. This distribution was statistically significant for *L. fermentum* ($P < 0.01$), but was not significant for *L. salivarius* because too few isolates were recovered (Table 3).

Table 1 Mean of decayed teeth/surfaces and prevalence of oral lactobacilli in low-caries group (dt ≤ 4, $n = 84$) and in moderate to high-caries group (dt > 4, $n = 81$)

	Mean ± SD of decayed		Number of children (%)	
Lactobacilli (CFU/1.5 cm^2)	Teeth	Surfaces	Low-caries group	Moderate to high- caries group
0	3.9 ± 3.5	7.7 ± 8.7	49 (58.3)	24 (29.6)
1–10	6.2 ± 4.9	12.7 ± 13.7	20 (23.8)	25 (30.9)
> 10	8.2 ± 5.4	19.0 ± 15.8	15 (17.9)	32 (39.5)
P-values	< 0.001[1]	< 0.001[1]	< 0.001[2]	

[1]Kruskal–Wallis test.

[2]Chi-square test.

CFU, colony-forming units; dt, decayed teeth score.

Table 2 Distribution of *Lactobacillus* isolated from children in low-caries group (dt ≤ 4) and children in moderate to high-caries group (dt > 4)

	All subjects		Low-caries group		Moderate to high-caries group	
Species	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)
L. fermentum	49 (83.1)	195 (54.6)	17 (85)	74 (59.7)	32 (82.1)	121 (51.9)
L. salivarius	15 (25.4)	53 (14.8)	1 (5)	2 (1.6)	14[1] (35.9)	51 (21.9)
L. casei/paracasei	11 (18.5)	32 (8.9)	5 (25)	14 (11.3)	6 (15.4)	18 (7.7)
L. mucosae	6 (10.2)	12 (3.4)	0	0	6 (15.4)	12 (5.2)
L. rhamnosus	5 (8.5)	14 (3.9)	2 (10)	4 (3.2)	3 (7.7)	10 (4.3)
L. oris	5 (8.5)	12 (3.4)	3 (15)	6 (4.8)	2 (5.1)	6 (2.6)
L. gasseri	4 (6.8)	18 (5)	3 (15)	14 (11.3)	1 (2.6)	4 (1.7)
L. plantarum	4 (6.8)	11 (3.1)	0	0	4 (10.3)	11 (4.7)
L. vaginalis	2 (3.4)	10 (2.8)	2 (10)	10 (8.1)	0	0
Total	59 (100)	357 (100)	20 (100)	124 (100)	39 (100)	233 (100)

[1]Fisher's exact test: $P = 0.01$.

Table 3 Genotypes of 304 *Lactobacillus* strains (with genotype = 1 or >1) of 56 children

Species (no. of children/isolates)	No. of genotypes	No. (%) of children/isolates (%)		
		Low-caries group	Moderate-caries group	High-caries group
L. fermentum (38/180)[1]	1	12 (85.7)/60 (88.2)	13 (92.9)/52 (92.9)	4 (40)/17 (30.4)
	>1	2 (14.3)/8 (11.7)	1 (7.1)/4 (7.1)	6 (60)/39 (69.6)
L. salivarius (10/45)	1	0	5 (100)/19 (100)	1 (20)/3 (11.5)
	>1	0	0	4 (80)/23 (88.5)
L. casei/paracasei and *L. rhamnosus* (9/34)	1	4 (100)/13 (100)	1 (25)/4 (22.2)	1 (100)/3 (100)
	>1	0	3 (75)/14 (77.8)	0
Others[2] (10/45)	1	2 (75)/11 (57.9)	4 (100)/15 (100)	3 (100)/11 (100)
	>1	1 (25)/8 (42.1)	0	0

[1]Chi-square test only for *L. fermentum*: $P = 0.02$.

[2]Others species included *L. mucosae* (1/5), *L. oris* (3/10), *L. gasseri* (2/12), *L. plantarum* (3/10), *L. vaginalis* (1/8).

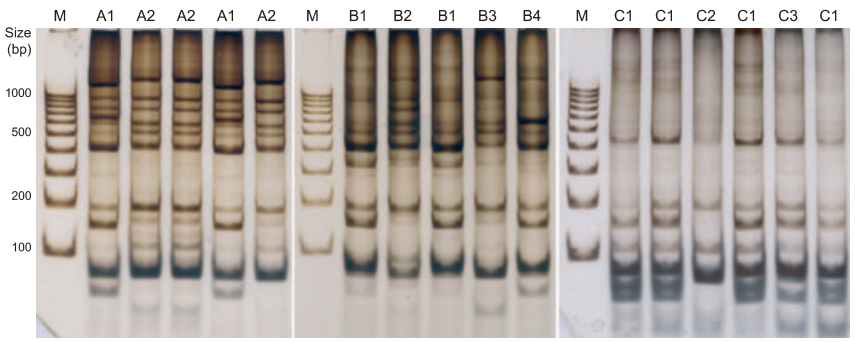

Figure 1 Arbitrarily primed polymerase chain reaction profiles of 16 *Lactobacillus fermentum* isolates from saliva of three children (A–C) with high caries, which showed A1–A2, B1–B4, and C1–C3 genotypes, respectively. M, molecular size markers – 100 base pairs (bp) DNA Ladder; Bio-Rad, Hercules, CA.

DISCUSSION

There are few studies on species and genotypes of *Lactobacillus* in relation to dental caries in young children. This study included a greater number of subjects and strains than previous reports (Milnes & Bowden, 1985; Marchant *et al.*, 2001), and also included children with low caries levels. A limitation of this study is related to the unexpected high caries prevalence (86.7%) in our children aged 2.2 ± 0.8 years. There were only five caries-free children who carried sufficient lactobacilli for species identification. As the patterns of *Lactobacillus* species distribution between caries-free children ($n = 5$) and other children in the low-caries group ($n = 15$) were similar (data not shown), those subjects were included in the low-caries group to gain sufficient numbers of children for statistical analyses. The prevalence of salivary lactobacilli in our subjects was found to be 55.8%, which was similar to culture-based studies in other populations (Nancy & Dorignac, 1992). Moreover, the children in the moderate to high-caries groups were frequently and heavily colonized by lactobacilli compared with the low-caries children.

This is the first attempt to speciate and genotype a substantial number of oral lactobacilli from Thai children. The application of PCR-RFLP and SDS–PAGE for comparison of isolates with type strains representative of known *Lactobacillus* species is suitable for discrimination of oral lactobacilli, simple to perform, and reproducible (Teanpaisan & Dahlen, 2006). Ten different *Lactobacillus* species were found in saliva samples from the children. Byun *et al.* (2004), using 16S rDNA sequence analysis and real-time PCR, found 18 different phylotypes of lactobacilli in carious dentine. About 50% of these were novel and it was concluded that diversity among lactobacilli was much greater than previously thought. *L. fermentum* was present in only 22% of the samples. The present study might have underestimated the diversity of lactobacilli by relying on culture on Rogosa agar plates, but the predominant species in carious dentine could be different from those in saliva. Ultimately, *Lactobacillus* taxonomy is still complex and conclusions about species relationships with clinical conditions should be made with caution.

When the distributions of *Lactobacillus* species of the group with low caries prevalence and the group

with moderate to high caries were compared, it was found that most children were colonized by several species, as observed by others (Marchant *et al.*, 2001; Caufield *et al.*, 2007). Interestingly, *L. salivarius* was found to be more highly associated with caries than the other lactobacilli in the present study. Caufield *et al.* (2007) showed that *L. salivarius* was one of nine taxa commonly found in subjects with active caries. *L. salivarius* may play a role in the caries process because it is acidogenic and can produce lactate, acetate and hydrogen peroxide (Martin *et al.*, 2006). *L. salivarius* is also aciduric and is reported to survive 4 h incubation at pH 2.5 (Strahinic *et al.*, 2007). Generally, lactobacilli have low affinity for the sound tooth surface and are recovered in low numbers in plaque samples, although they can be presented in high levels in saliva (van Houte *et al.*, 1972; van Houte, 1980). It has also been shown that *L. salivarius* adheres to saliva-coated hydroxyapatite *in vitro* (Matsumoto *et al.*, 2005). It is possible that *L. salivarius* is more likely to be incorporated into dental plaque than other lactobacilli. Fitzgerald *et al.* (1981) reported that *L. salivarius* isolates from human dental plaque could induce severe caries in the fissures of molars in germ-free rats receiving either the glucose-containing or sucrose-containing diet. This cariogenic capacity was further supported by the findings that *L. salivarius* strains were more cariogenic than *S. mutans* Ingbritt in gnotobiotic rats (Seppa *et al.*, 1989). In the presence of sucrose and low pH, *L. salivarius* further lowered the pH and this resulted in changes in the bacterial community within oral biofilms (Pham *et al.*, 2009). Therefore, the closer association of *L. salivarius* with caries prevalence found in the present study, together with other evidence above, indicates strongly that *L. salivarius* may be cariogenic.

The relationship of *L. fermentum* to the caries process is not clear. This was the most predominant species found in saliva of caries-free subjects in some studies (Colloca *et al.*, 2000; Ahumada Mdel *et al.*, 2003), while others have reported a high prevalence of *L. fermentum* in subjects with caries (Milnes & Bowden, 1985; Marchant *et al.*, 2001; Caufield *et al.*, 2007). In the present study, *L. fermentum* was the most common species presented in saliva of both the low-caries and the moderate to high-caries groups. Strains of this species have been previously isolated from Thai traditional foods (Klayraung *et al.*, 2008). Possibly food-associated lactobacilli survive within the normal resident microflora of the human mouth, and so may not associate with caries.

Other species that have been associated with caries such as *L. casei*, *L. paracasei* and *L. rhamnosus* (Nancy & Dorignac, 1992; Marchant *et al.*, 2001), were infrequently found in this study and were not related to caries in our subjects. The prevalence of *L. gasseri*, *L. plantarum* and *L. vaginalis* was relatively low and they may be uncommon in the oral cavity.

Genotypic studies of oral *Lactobacillus* species are limited, and only two studies reported on relationship to caries (Marchant *et al.*, 2001; Caufield *et al.*, 2007). Marchant *et al.* (2001) showed in a genotypic study on 39 *Lactobacillus* strains isolated from carious dentine of three children that diverse genotypes of *Lactobacillus* species were found within and between carious lesions in the same child as well as between children. Furthermore, Caufield *et al.* (2007) reported genetic heterogeneity among 180 isolates of salivary lactobacilli from six women with active caries. Our study using AP-PCR, with ERIC primers set to reveal the *Lactobacillus* intra-species variability, gave high discrimination with the polymorphic AP-PCR patterns reflecting differences within the species at the subspecies level. Marchant *et al.* (2001) and Matsumiya *et al.* (2002) showed that ERIC-PCR methodology was efficient and practical for discriminating genotypes within species of *Lactobacillus*. We found a genetic heterogeneity among 304 *Lactobacillus* strains from 56 children, and neither individual was colonized with the same genotypes. The high-caries prevalence children were found to be frequently colonized with more than one genotype compared with the low-caries group, and from one up to five genotypes could be found in individuals. The reason why a greater genotypic diversity was found in subjects with high caries is unknown. It has previously been postulated that environmental stress in the oral cavity could lead to a reduced number of genotypes that are best adapted to colonize and proliferate in a particular environment (Bowden & Hamilton, 1998). Conversely, high sugar availability in the carious environment could lead to growth of increased numbers of different *Lactobacillus* clones compared with less supportive conditions (Beighton *et al.*, 1996). There is, however, limited knowledge available

regarding the importance of genetic diversity and the impact of such diversity on the ecology of the oral microflora.

In conclusion, this study showed that salivary *Lactobacillus* isolated from Thai preschool children exhibited wide species and genotype heterogeneity. *L. salivarius* was predominant in children with high levels of caries, which may indicate an association with the cariogenic process. *L. fermentum* on the other hand was the most predominant species in all study groups. Children with high caries levels were often colonized with more than one clonal type. Further studies of the biological properties of these bacteria are necessary to determine their role in caries processess.

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