Lactobacillus spp and Dental Caries in Young Children

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บทคัดย่อ

ฟันผุเป็นโรคในช่องปากที่พบบ่อยในเด็ก พบว่าเชื้อแลคโตบาซิลลัสมีความเกี่ยวข้องกับการเกิดและการลุกลามของโรคฟันผุ อย่างไรก็ตามความสัมพันธ์ระหว่างสปีชีส์หรือสายพันธุ์ของแลคโตบาซิลลัสกับฟันผุยังไม่ชัดเจน และยังไม่มีข้อมูลในการศึกษาในประชากรไทย จุดมุ่งหมายของการวิจัยนี้คือ 1) เพื่อศึกษาความชุกและความสัมพันธ์ระหว่างโรคฟันผุในเด็กปฐมวัยกับระดับเชื้อแลคโตบาซิลลัสในน้ำลายของเด็กไทย โดยติดตามตั้งแต่อายุ 12-60 เดือน 2) เพื่อศึกษาชนิดสปีชีส์หรือสายพันธุ์ของแลคโตบาซิลลัสในช่องปาก และตรวจสอบว่าสปีชีส์หรือ สายพันธุ์ใดมีความเกี่ยวข้องกับโรคฟันผุ และ 3) เพื่อศึกษาความสามารถที่เกี่ยวข้องกับการก่อให้เกิดฟันผุ ได้แก่ ความสามารถในการเจริญเติบโตของการผลิตกรดของเชื้อแลคโตบาซิลลัสแต่ละสปีชีส์หรือสายพันธุ์ที่พบในช่องปาก โดยทำศึกษาในหลอดทดลอง

เด็กปฐมวัยจำนวน 181 คนที่เข้าร่วมในการศึกษาครั้งนี้ ได้รับการตรวจฟันผุและเก็บตัวอย่างน้ำลาย ที่อายุ 12, 18, 24, 36, 48 และ 60 เดือน ทำการนับจำนวนเชื้อแลคโตบาซิลลัส นำเชื้อแลคโตบาซิลลัสจำนวน 357 สายพันธุ์ที่แยกได้จากเด็ก 59 คน (อายุเฉลี่ย 34.3 $\pm$ 14.6 เดือน) ไปทำการจำแนกชนิดของสปีชีส์โดยวิธี 16S ribosomal RNA genes polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) และวิธี 16S ribosomal RNA gene sequencing ทำการศึกษาจีโนไทป์ของสายพันธุ์แลคโตบาซิลลัสโดยใช้วิธี arbitrarily prime□PCR ผลการศึกษาพบว่าความชุกของการเกิดฟันผุ และค่าเฉลี่ยของจำนวนฟันผุเพิ่มขึ้นอย่างรวดเร็วตามอายุที่เพิ่มขึ้นโดยเด็กอายุ 60 เดือนส่วนใหญ่ (99.3%) มีฟันผุ และมีค่าเฉลี่ยของฟันผุ คือ (□t) 12.47 $\pm$ 4.83 ซี่ และ (□s) 34.19 $\pm$ 19.50 ด้าน พบความสัมพันธ์ทางสถิติระหว่างโรคฟันผุในเด็กปฐมวัยและระดับเชื้อแลคโตบาซิลลัสในน้ำลาย โดยในกลุ่มเด็กที่มีค่าเฉลี่ยของจำนวนฟันผุ (□t/□s)สูง จะพบระดับเชื้อแลคโตบาซิลลัสในน้ำลายสูง จากจำนวนสปีชีส์ทั้งหมด 9 สปีชีส์ของเชื้อแลคโตบาซิลลัสที่แยกได้จากในช่องปากเด็ก, *L. salivarius* พบมากอย่างมีนัยสำคัญในเด็กที่มีความชุกโรคฟันผุในระดับปานกลางถึงสูงเมื่อเทียบกับเด็กที่มีความชุกของโรคฟันผุต่ำ *L. fermentum* เป็นชนิดที่พบมาก

ที่สุดในทุกกลุ่มเด็กที่ศึกษา นอกจากนี้พบว่าในเด็กที่มีความชุกของโรคฟันผุสูงมีแนวโน้มที่จะมีความหลากหลายของจีโนไทป์แลคโตบาซิลลัสมากกว่าเด็กที่มีฟันผุต่ำ

จากการศึกษาอัตราการเจริญเติบโต และการผลิตกรดของเชื้อแลคโตบาซิลลัสในช่องปาก พบว่าความสามารถในการผลิตกรดมีความสัมพันธ์ทางบวกอย่างมีนัยสำคัญทางสถิติกับอัตราการเจริญเติบโต อัตราการผลิตกรดสูงสุดเกิดขึ้นในช่วงระยะเวลาที่มีการเจริญเติบโตอย่างรวดเร็วที่สุด *L. rhamnosus, L. salivarius, L. casei/paracasei* และ *L. plantarum* มีอัตราการเจริญเติบโตและอัตราผลิตกรดสูงสุดในช่วงเวลาที่ 1.5-3 ชั่วโมงในขณะที่ *L. gasseri* และ *L. vaginalis* มี การเจริญเติบโตและการผลิตกรดที่ช้ากว่า

สามารถสรุปได้ว่าเชื้อแลคโตบาซิลลัสมีความเกี่ยวข้องกับการฟันผุในเด็กปฐมวัย ในเด็กที่มีความชุกของโรคฟันผุในระดับปานกลางถึงสูงพบสปีชีส์ของเชื้อแลคโตบาซิลลัสแตกต่างจากกลุ่มโรคฟันผุต่ำโดยพบ *L. salivarius* ได้บ่อยในกลุ่มเด็กที่มีความชุกของโรคฟันผุในระดับปานกลางถึงสูง และเชื้อสปีชีส์นี้มีความสามารถในการเจริญเติบโตและผลิตกรดสูงซึ่งอาจมีความสัมพันธ์กับฟันผุในเด็กไทยมากกว่าเชื้อแลคโตบาซิลลัสสปีชีส์อื่น ๆ

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ABSTRACT

Dental caries is still a common oral disease among children. *Lactobacillus* has 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. The aims of the present thesis were therefore: 1) to investigate the prevalence and the relationship between early childhood caries and salivary lactobacilli levels in Thai children, followed longitudinally from 12 to 60 months of age, 2) to examine the distribution of species and genotypes of oral *Lactobacillus* and investigate whether certain species or genotypes were more related to caries activity than others, and 3) to investigate the potential cariogenic effects of oral *Lactobacillus* species, the *in vitro* growth and acid production of lactobacilli was tested.

A group of 181 children participated in this study. The examinations of caries status and saliva samples collection were performed at the age of 12, 18, 24, 36, 48 and 60 months. The number of lactobacilli was counted and 357 *Lactobacillus* isolates from 59 children (mean age 34.3±14.6 months) were identified to species level by 16S ribosomal RNA genes polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and 16S ribosomal RNA gene sequencing. In addition, the clonal diversity among *Lactobacillus* species was evaluated by using arbitrarily primed PCR. The result showed a rapid increase of caries prevalence and caries score by age, most children (99.3%) at the age of 60 months had caries with a mean number of decayed teeth (dt) of 12.47 ± 4.83 and decayed surfaces (ds) of 34.19 ± 19.50. There was a strong relationship between caries in early childhood and salivary levels of lactobacilli i.e. a higher mean of dt/ds was followed by an increasing numbers of lactobacilli. Among the 9 identified species of oral *Lactobacillus*, *L. salivarius* was more prevalent in children

with a moderate to high caries prevalence compared with children with low caries prevalence. *L. fermentum* was the most predominant species in all study groups. Additionally, 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.

The cariogenic potential of oral *Lactobacillus* species was studied by the *in vitro* growth rate and acid production. A positive and statistically significant correlation was found between the growth rate and the pH decrease among the *Lactobacillus* strains. The highest acid production rate occurred during the period of most rapid growth. *L. rhamnosus*, *L. salivarius*, *L. casei/paracasei* and *L. plantarum* showed the highest acid production rate during the logarithmic growth period, which occurred within 1.5-3 h, while *L. gasseri* and *L. vaginalis* strains were slower to start growing and producing acid.

It concluded that *Lactobacillus* is associated with caries development and progression in early childhood. The presence of various *Lactobacillus* species in children with moderate to high caries prevalence differed from the low caries group, by a higher prevalence of *L. salivarius* in the former group. *L. salivarius* was found to be highly acidogenic and aciduric and may be more asscociated with the caries process in Thai preschool children than other species.

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LIST OF ABBREVIATIONS AND SYMBOLS

AP-PCR	arbitrarily primed polymerase chain reaction
ATCC	American Type Culture Collection
BHI	brain heart infusion broth
bp	base pair
CFU	colony forming units
dmf	for deciduous teeth decayed, missing, filled
DNA	deoxyribonucleic acid
ECC	early childhood caries
ERIC	enterobacterial repetitive intergenic consensus sequences
L.	*Lactobacillus*
MRS broth/agar	de Man, Rogosa and Sharpe broth/agar
OD	Optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA fingerprinting
RFLP	restriction fragment length polymorphism
rRNA	ribosomal RNA
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1. Background and Rationale

Today early childhood caries (ECC) is considered to be a major public health problem among the Thai children. Thitasomakul *et al.*[1] showed a high prevalence of ECC (68.1%) among 18-month old children in southern Thailand. Lactobacilli are considered to be an important risk indicator of dental caries, due to their association with dental caries[2, 3]. They are considered to play a significant role in dental caries progression from their predominance in the opened cavities than in initial lesions[4-6]. High counts of lactobacilli indicate acidic conditions in the oral cavity due to low saliva secretion rate, low buffer capacity, high proportions of strong acid producers and high sugar consumption[7, 8] and may thus be indirect indicators of increased caries risk. However the impact of lactobacilli on caries initiation and progression is less clear.

Microbiological studies revealed that more than twenty *Lactobacillus* species are found in the oral cavity[9-11]; however, most of these species may only be detected transiently and unpredictably[12]. Attempts have been made to search for specific *Lactobacillus* species that are more associated with dental caries than others; the techniques include either biochemical tests or molecular techniques. The species and the number of *Lactobacillus* species reported from previous studies are however disparate [9-11]. Therefore, further investigations on *Lactobacillus* spp associated with caries should be carried out.

The hypothesis inspiring this thesis was that individual species or isolates of oral lactobacilli could be qualitatively different in their association with caries, and thus have different roles in caries development and progression. The ability of bacteria to survive and persist in a particular environment depends, in part, on their ability to modify or express their genes, as a response to variations in local environmental condition or stress[13]. The main characteristics of cariogenic bacteria are thought to be acid production (acidogenicity) and acid tolerance (aciduricity) which enables them to survive and reproduce at low pH[14]. Such characteristics are

found for many oral bacteria especially streptococcal species (e.g. the mutans streptococci), lactobacilli and *Actinomyces*[15-17]. Evaluation of oral bacterial genetics and their characteristics has been mostly performed on mutans streptococci, while few studies have been conducted on oral *Lactobacillus* species[18-21]. Little is known about the aciduric and acidogenic profiles, and the role of these characteristics at species and genotype level of *Lactobacillus* isolates in the caries process.

To disclose the impact of oral lactobacilli in early childhood caries (ECC) or nursing caries, more studies are required. New knowledge could provide the insights in the dental plaque ecology, the relationship between oral bacteria and the disease, and the understanding of the caries progression. This may also provide ways to prevent and control the disease.

2. Literature review

2.1. Lactobacilli

2.1.1. Taxonomy

The members of the genus *Lactobacillus* are gram-positive, rod-shaped, non-spore forming and catalase negative bacteria. Cells are usually long rods but may sometimes be almost coccoid, 0.5-1.2 x 1.0-10.0 μm, commonly in short chains. Normally, they are facultative anaerobes, sometimes microaerophilic, while some species are true anaerobes on isolation[22]. The genus *Lactobacillus* belongs to the Lactic Acid Bacteria, which are able to produce lactic acid as main end-product from carbohydrate fermentation. They are acid-tolerant and may grow at pH as low as 2.5[23]. The genus *Lactobacillus* contains diverse taxa, and currently over 100 species are recognized[24]. More than twenty species have been found in the oral cavity[10]. Historically, the genus *Lactobacillus* has been divided into three groups according to the type of sugar fermentation. Obligately homofermentative lactobacilli, which are the majority of the *Lactobacillus* species, are able to ferment hexoses almost exclusively to lactic acid by glycolysis while pentoses and gluconate are not fermented. Obligately heterofermentative lactobacilli use the 6-phospho-gluconate/ phosphoketolase (6PG/PK) pathway and produce other end products (CO_2,

ethanol) in addition to lactic acid. The third group includes the facultative heterofermentative lactobacilli that ferment hexoses via the glycolysis and pentoses via the 6PG/PK pathway[25].

Nowadays, the identification of lactobacilli is based mainly on DNA-based molecular methods (the comparison of molecular sequences of rRNA genes), and phenotypic molecular methods such as whole cell proteins analysis. Another important taxonomic tool in classification of *Lactobacillus* species is the genome GC content, considering DNA base composition of the genome; they usually show a GC content range from 32% to 56%[24]. According to Fellis and Dellaglio (2007)[24], phylogenetic groups of the genus *Lactobacillus* have been reported as shown in table 1.

Table 1. Phylogenetic grouping of *Lactobacillus* species according to Fellis and Dellaglio (2007)[24].

Phylogenetic groups	Species name
L. delbrueckii group	*L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylotrophicus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasseri, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefiranofaciens, L. kitasatonis, L. psittaci, L. sobrius, L. ultunensis*
L. salivarius group	*L. acidipiscis, L. agilis, L. algidus*, L. animalis, L. apodemi, L. aviarius, L. equi, L. mali, L. murinus, L. nageli, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis, L. vini*
L. reuteri group	*L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. secaliphilus, L. vaginalis*
L. buchneri group	*L. buchneri, L. diolivorans, L. farraginis, L. hilgardii, L. kefiri, L. parabuchneri, L. parafarraginis, L. parakefiri* associated with *L. acidifarinae, L. namurensis, L. spicheri,* and *L. zymae* (which form a robust group)
L. alimentarius-L. farciminis group	*L. alimentarius, L. farciminis, L. kimchii, L. mindensis, L. nantensis, L. paralimentarius, L. tucceti, L. versmoldensis*
L. casei group	*L. casei, L. paracasei, L. rhamnosus, L. zeae*
L. sakei group	*L. curvatus, L. fuchuensis, L. graminis, L. sakei*
L. fructivorans group	*L. fructivorans, L. homohiochii, L. lindneri, L. sanfranciscensis*
L. coryniformis group	*L. bifermentans, L. coryniformis, L. rennini,* not robustly associated with *L. composti*
L. plantarum group	*L. plantarum, L. paraplantarum, L. pentosus*
L. perolens group	*L. perolens, L. harbinensis, L. paracollinoides*
L. brevis group	*L. brevis, L. hammesii, L. parabrevis*

**L. algidus* is not always associated with this group and appears as a distinct line of descent.

2.1.2. Epidemiology

Lactobacilli are almost present everywhere, they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sourdoughs, vegetables, fruits, beverages), oral, gastro-intestinal and genital tracts of humans and animals, and in sewage and plant material. They are usually considered as an innocuous microorganism or even health-promoting for humans, but with some exception they can cause disease, for example, dental caries[22].

The mouth of the newborn baby is usually sterile at birth. Colonization of the microbiota begins immediately following birth when the newborn is exposed to the outside environment, particularly from the mother's vagina and from the first feeding. Before teeth erupt, lactobacilli do not rapidly colonize the oral cavity; only found transiently in the oral microflora[26]. They prefer to colonize the dorsum of the tongue and are released into saliva by the desquamation of the epithelium[12]. There are few data on the source of transmission of lactobacilli to children. Acquisition depends on the successive transmission, which may be from mother or other close caregivers. Molecular typing studies have shown that oral lactobacilli in newborns can be transmitted from vagina or breast milk of their mothers[27, 28]. The organisms may also be derived from water, food and other nutritious fluids[29].

Microbiological studies have revealed that lactobacilli usually comprise less than 1% of the total microbiota in the healthy human mouth[26]. They are more prevalent in saliva and carious dentine in rampant or nursing caries subjects[30, 31]. They are also predominant in the oral cavity of patients with xerostomia[32]. From a longitudinal study in Thai children, these organisms were found in only 8.9% in 3-month old children and the prevalence rapidly increased with age reaching more than 60% in 24-month old children[30]. Similar results have been reported by McCarthy *et al.* (1965)[33] who found that lactobacilli appeared in the oral cavity in 50% of newborns during their first year of life. Factors that influence the prevalence and level of lactobacilli are thought to be the number and the progression of the deep carious lesions[6, 30, 34] and a frequent intake of fermentable carbohydrates[8, 34], which are further discussed in Section 2.2.2 and 2.2.3.

2.1.3. Culture methods and species identification

Growth of *Lactobacillus* is generally enhanced by 5% CO_2. The optimum growth temperature is 30-40^{o}C and optimum pH is between 4.5 and 6.4. They are generally grown on blood agar or on selective medium, Rogosa SL-agar. Colonies on agar media are usually 2-5 mm, convex, opaque, and without pigment. Lactobacilli are chemoorganotrophs, requiring rich and complex media such as De Man, Rogosa, Sharpe (MRS) Agar[26, 35, 36].

Previous classification and identification of lactobacilli has been based mainly on their phenotypic and biochemical characteristics such as Gram staining, fermentation of sugar, biotyping, serotyping and bacteriocin typing. Even though these methods can be applied for subdividing and even specification of *Lactobacillus* isolates, none of them offers an ideal method for clear identification due to poor reproducibility, unreliability of the techniques, extensive logistics for large-scale investigations and poor discriminatory power[37, 38].

Owing to the rapidly developing molecular technology, it is now possible to define the molecular characteristic of microorganisms through the analysis of the universal macromolecule. Nucleic acid analysis is one of the most extensively utilized techniques. This technique is usually performed by the comparison of DNA-DNA homology or 16S ribosomal RNA (rRNA) gene sequences. 16S rRNA gene contains regions, which are highly conserved across all bacterial genera with regions (V1-V9), approximately 1500 bp in length, in which the nucleotide base sequences are highly variable between genera or species. Comparison of 16S rDNA gene sequences from lactobacilli shows that the V1, V2 and V3 regions contain the species-specific information[11, 27, 39, 40]. Therefore, for identification purposes, amplification by PCR and sequencing of less than half of the gene (less than 750 bp) may be sufficient, but whole gene sequencing is still preferable for establishing true phylogenetic relationships[41]. Polymerase chain reaction (PCR) is a technique, which uses a DNA polymerase enzyme to make a huge number of copies of any given piece of DNA or gene. In microbiology, using these techniques to amplify the 16S rRNA gene leads to a new approach for microbial identification and classification[38].

Many molecular typing methods have been applied as tools for *Lactobacillus* species identification, such as restriction fragment length polymorphism analysis (RFLP), arbitrarily primed PCR (AP-PCR), denaturing gradient gel electrophoresis (DGGE) and amplification rDNA restriction analysis (ARDRA). The choice of the most suitable typing method

is based on the ability to identify the particular species, however also rely on other features of the method such as costs, workload and reproducibility of the fingerprints. Consequently, a combination of different techniques is sometimes suitable[37]. Teanpaisan and Dahlén (2006)[42] used a combination of PCR-RFLP and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for identifying at least 13 oral *Lactobacillus* species. It showed that this technique was practical, easy to perform, rapid and reproducible for differentiation of *Lactobacillus* at the species level. Moreover, it has been shown to be appropriate for a wide range of clinical oral isolates[42].

In PCR-RFLP analysis, chromosomal DNA fragments resulting from a selective PCR reaction are digested by restriction enzymes, resulting in the various banding patterns. The discriminatory power of PCR-RFLP methods is high[37, 42], however the method cannot distinguish between *L. casei* and *L. rhamnosus* and between *L. acidophilus* and *L. crispatus*[42]. Although this technique is expensive, more time-consuming and has some limitations, PCR-RFLP is still used for identify oral *Lactobacillus* species due to simple banding patterns that are straightforward to interpret and compare between species.

SDS–PAGE is the most widely used protein analysis technique, which analyses whole-cell protein banding pattern. Proteins are denatured to linear polypeptides, which are bound with sodium dodecyl sulphate (SDS) and form a uniform negative charge density. The polypeptides are migrated and separated in a polyacrylamide gel by electrophoresis according to their size. This technique defines the phenotype rather than the genotype of a particular organism[38]. Although SDS–PAGE may not allow for a sufficient resolution of proteins due to a sometimes complex banding patterns[38], this technique is useful in the discrimination of *L. casei* and *L. rhamnosus*, and of *L. acidophilus* and *L. crispatus*[42]. This method is also inexpensive and rapid.

2.1.4. Genotyping of Lactobacillus species

Genotyping of bacterial isolates is useful in epidemiological studies to track the origin of bacteria and mode of transmission. Genotyping has also been used to discriminate strains associated with disease from strains associated with health. Traditionally classification of bacterial isolates has been based on phenotype characteristics, such as serotype, biotype, phagetype or

antibiotic susceptibility pattern[43]. More recently, techniques have been developed to classify bacterial isolates based on the genotype pattern. Methods for genotyping are able to discriminate intra-species differences with reproducibility higher than the phenotyping methods. Genotyping includes the whole genome while the phenotyping is based on genes that are expressed. Several methods have been used for genotyping of *Lactobacillus* species: plasmid profiling, ribotyping, SDS-PAGE, and arbitrarily primed PCR (AP-PCR).

AP-PCR technique is a molecular method that also is known as randomly amplified polymorphic DNA (RAPD). In this technique, oligonucleotides with a random sequence, commonly 10-base, are used as primers in PCR, which yields strain-specific amplification product patterns. The primers anneal to the complementary or partially complementary sequences in the target DNA (the complete genome) and the DNA between the binding sites on complementary strands can be amplified. In general, AP-PCR fingerprinting allows for differentiation between species and between strains within the same species[25, 28]. Even though AP-PCR technique has fair reproducibility, the potential weakness of the method is compensated by a high discriminatory power[25, 37]. This technique is simple, rapid and feasible for large scale typing of isolates. A recent study reported the success of using AP-PCR with primers ERIC1R and ERIC2 for tracing the transmission of lactobacilli from the mother to the newborn infant[28]. Altogether, these results show it was suggested that this technique is easy to perform and rapid, and it has been proved that AP-PCR with primers ERIC1R and ERIC2 is a potential tool for typing of *Lactobacillus* species.

2.2. Relationship between lactobacilli and dental caries

2.2.1. Early childhood caries

A workshop of the National Institutes of Health (NIH) in 1999 proposed that the term ‘early childhood caries (ECC)’ should be used to describe the presence of one or more decayed (noncavitated or cavitated lesions), missing (because of caries), or filled tooth surfaces of any primary tooth in children up to 71 months of age[44]. This specific form of caries always causes extensive destruction of the deciduous teeth, often very rapidly. The demineralization and cavitation occur at the labial surfaces of maxillary primary incisors, followed by involvement of

the first primary molars[1, 45]. This condition has been described previously as 'nursing caries' or 'bottle caries' and attributed to prolonged frequent bottle feeding, feeding with sugary foods and snacks or putting a child to bed with a soft drink bottle at night[46-49].

The prevalence of ECC in different populations of the world varies between 1% and 80% of preschool children; the proportion of affected children being very high in developing countries and in deprived groups within developed countries[50]. In Thailand, the Ministry of Public Health conducted national oral health surveys of 3-years-old children in 2000-2001 and 2006-2007, respectively[51, 52]. It was found that more than 60% children were affected by dental caries and that the children in the southern region of the country had an even higher prevalence as seen in table 2. Teanpaisan *et al.* (2007)[30] confirmed the high caries development rate among Thai children in the southern region. Thus caries was detected soon after first tooth eruption at the age of 8 months. The progression of caries increased from 4.2% of 9-months-old children with mean caries score (dmf) of 0.14±0.7 to 84.5% of 24-months-old children with a mean caries score (dmf) of 5.35±4.34. Whereas the data from the National Health and Nutrition Examination Survey (NHANES III) indicate that the prevalence of dental caries in US children in the age of 2 to 5 years in 1999–2004 was only 28% and the decayed and filled teeth score was 1.58[53]. Longitudinal studies showed that children who experienced ECC are much more likely to develop further dental problems later in their permanent dentition[54, 55]. This concludes that ECC is a significant health problem among Thai children and it is necessary to find a way to reduce and prevent this problem.

Table 2. The dental caries prevalence in 3-year-old children in different regions of Thailand*.

Region	2000-2001		2006-2007	
	Caries affected children (%)	Mean number of carious teeth	Caries affected children (%)	Mean number of carious teeth
Whole country	65.7	3.6	61.4	3.2
Central	67.7	3.8	69.8	3.6
North	67.0	3.8	56.5	3.1
North-east	70.1	3.9	61.6	2.9
South	71.2	4.0	64.0	4.0

* National Oral Health Survey in 2000-2001 and 2006-2007[51, 52].

2.2.2. Etiology of dental caries

The etiology of caries may involve one or more of the three factors: fermentable carbohydrates (substrate), cariogenic microorganisms, and susceptible tooth surface/host[56]. Fermentable carbohydrates include both sugars and starches; sugars may be sucrose, fructose, lactose, maltose, and glucose. In 1890, the "father" of oral microbiology, W. D. Miller suggested that caries was most likely due to demineralization mainly by lactic acid produced from carbohydrates fermentation of bacteria[57], and also influenced by other acids among the metabolic end products[58]. Diet plays a key role in the development of caries. Cariogenic bacteria such as mutans streptococci and lactobacilli have long been associated with caries prevalence in children, although the etiological relationship is less clear[30, 59, 60]. Study of Teanpaisan *et al.* (2007)[30] has indicated that the earlier mutans streptococci and lactobacilli colonize the oral cavity of children, the higher is their caries experience. Apart from bacteria and fermentable carbohydrates, host/ tooth factors include the factors such as poor oral hygiene[34, 47], reduced salivary secretion rate and buffering capacity [61], and genetic predisposition[62]. In addition to bacteria, fermentable carbohydrates, host and tooth factors other important factors for the development and progression of caries are poor oral hygiene[34, 47], reduced salivary secretion rate and buffering capacity [61], and genetic predisposition[62].

2.2.3. Ecological plaque hypothesis of caries formation

There are two main hypotheses on the role of plaque microflora to disease. The initial hypothesis established for explain the role of bacteria in the etiology of dental disease was the "specific plaque hypothesis" which proposed that only a few species from the diverse composition of organisms in the plaque microflora were responsible for dental diseases, both caries and periodontal disease[63].

Problems can arise with this hypothesis because the association between specific organism and dental disease is not always apparent; either dental disease is diagnosed in the absence of the putative pathogens, or conversely pathogens are present at sites with no evidence of disease[64]. An alternative hypothesis, the "non-specific plaque hypothesis", suggested that dental disease is the outcome of the overall activity of the total oral plaque microflora[63]. However, if the etiology of dental diseases is not totally specific, there is the evidence of a limited subset of bacteria consistently recovered in higher numbers from diseased sites.

Consequently, a third hypothesis (the "ecological plaque hypothesis") which reconciles the key element of the earlier two hypotheses was proposed recently by Marsh in 1994[65]. This hypothesis implies that the dental diseases are due to a shift in the balance in the dental plaque community driven by a change in local environmental conditions (Fig.1). These microbial communities are normally in a state of homeostasis based on a dynamic balance between and among the individual species that are constantly exposed for environmental factors from the surroundings. Such factors include lack of nutrients (starvation), presence of antibacterial peptides (bacteriocins) and the production of metabolic endproducts (e.g. organic acids, amines, ammonia, hydrogenperoxide and sulphur compounds)[64, 66]. The balance is normally maintained by a series of microbial interactions, including synergism and antagonism, however may be destroyed in case the environmental factors are strong or occur frequently. This happens in case of a frequent intake of sugars (especially sucrose) resulting in an increased metabolism of saccharolytic (sugar fermenting) bacteria in the plaque and an overproduction of lactic acid and a pH drop. Frequent pH drops leads to a destruction of the homeostasis and a shift in the balance within the plaque microflora concomitantly with enamel demineralization and carious lesion formation[64, 67].

The environment associated with caries is assumed to be in a more stressful condition than that of health and the dental plaque species may respond to stress in different ways.

The impact of stress on the dental plaque community results in a selection of species and genotypes best suited for the changed environment[67]. The primary colonizers in formation of dental plaque are streptococci, *Neisseria*, *Actinomyces* and *Capnocytophaga*[26]. Paddick *et al.* (2003)[68] reported that the proportions of mutans streptococci and lactobacilli were elevated in the plaque of caries-active subjects, while *A. naeslundii* (claimed as a bacterial species associated with health) isolates formed a significantly greater proportion of the bacteria isolated from caries-free subjects.

Recent molecular analyses have shown that the microflora associated with white spot lesions is more diverse[69]. There initiation of the caries can be promoted by mutans streptococci group[8, 70, 71], 'low-pH' non-MS and Actinomyces[15, 69, 72] while lactobacilli are not considered to be actively involved in caries initiation. Filoche *et al.* (2004)[73] and Shen et al (2004)[74] have shown that lactobacilli are not able to form plaque without the participation of good plaque formers such as *Streptococcus* or *Actinomyces* spp. Caries is most probably a result of combined activities of several species specifically at different stages of the caries process. Moreover, the bacteria associated with caries belong to the members of normal oral microflora, a selection of these resident flora may occur whereby the homeostatic balance of the biofilm is disturbed[69]. The microbial ecological hypothesis of caries must take into consideration.

One important ecological aspect is the interaction between bacteria. Interactions between *Lactobacillus* and mutanstreptococci has been shown to occur *in vitro*[75, 76] as well as *in vivo*[77], implying that *Lactobacillus* may inhibit the growth of mutansstreptococci. Such inhibition may be occur by specific mechanisms (antimicrobial peptides, bacteriocins) or more generally by the acidity produced by the *Lactobacillus*[78]. This interaction may explain the ecological shift that occurs from the incipient ("white spot") enamel lesion predominated by streptococci (e.g. mutans streptococci) and *Actinomyces* spp and with little and no lactobacilli to the deeper dentine cavities predominated by lactobacilli.

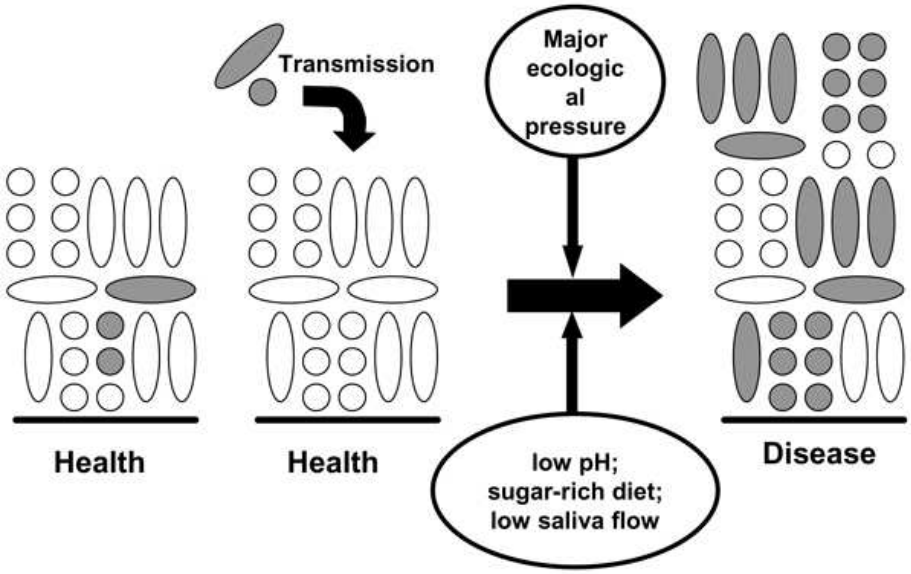

Fig. 1. The ecological plaque hypothesis. Figure was adapted from Marsh (2006)[14].

2.2.6 Lactobacilli, fermentable carbohydrates and dental caries

The relationship between dental caries and the prevalence of lactobacilli has been recognized for several decades. Lactobacilli were previously believed to be the major microorganism responsible for caries development due to their predominance in carious lesions and their cariogenic properties, such as being highly acidogenic and aciduric. However, subsequent research has shown that they are associated ecologically with carious process of the dentine and conditions of the caries cavity and[4, 79, 80], explained by their need for mechanical retention and extreme ability to acid adaptation and tolerance. Lactobacilli are frequently recovered in great numbers on cultivation from established carious lesions[4, 70].

Frequent intake of sucrose-containing products causes frequent pH drops that lead to the reduction of acid-sensitive species. Many predominant bacteria in healthy plaque can tolerate small pH changes, but are inhibited or killed by frequent or extensive exposure to acidic conditions. Acidic conditions caused by frequent pH drops may also favor aciduric bacteria, such as mutans streptococci and lactobacilli. *S. mutans* and lactobacilli gradually increase their proportions in the dental plaque until they finally dominate, and breakdown of the homeostasis had occurred[66, 79, 81]. The metabolism of plaque also changes from a heterofermentative pattern to a homofermentative in which lactic acid is the major end product[82]. If the period below the critical

pH (5.5) of enamel demineralization is prolong or frequently occurring, caries may develop. A semi-closed system is formed in the carious lesion. Under these more acidic conditions, growth of lactobacilli is favored, and they are successfully established and are constantly cultured from the cavitated carious lesions[83]. Chhour *et al.* (2005)[84] reported on the profile of the carious dentine microflora and found a diverse array of lactobacilli which comprised 50% of the total viable count. In general, the prevalence of lactobacilli in caries subjects is high in most population studies[2, 30, 85-87].

Lactobacilli can be primarily detected on retention sites e.g. around fillings, restorations, open cavities and deeper parts of caries lesions[11, 88, 89]. It is reported that lactobacilli were present in 100% of the dentine samples, 70% of the saliva samples and 29% of the plaque samples collected from carious subjects[89]. The salivary lactobacilli may be a result of the hypercontamination from carious dentine. The level of lactobacilli also varies with the carbohydrate intake and frequency[79, 90]. Lactobacilli (as a group) counts alone are poor predictors for future caries on an individual basis, but they may be used either in combinations (models) with other risk factors (indicators) for caries risk assessments[7, 91, 92]. However, it is possible that specific *Lactobacillus* species are more related to the caries process and can be used as predictors for caries risk.

2.2.6 Lactobacillus species and genotype related to caries

More than twenty different *Lactobacillus* species are reported to be isolated from human oral cavity [10], but it is likely that only a few species are present in any one single individual[93]. Rogosa *et al.* (1953)[94], in their classical study on oral lactobacilli, identified 500 strains isolated from saliva from 130 school children by biochemical and culture-based methods. A diverse species composition of *Lactobacillus* was found, and the predominant species were *L. casei* and *L. fermentum*. However, there was no information about the caries condition of the school children investigated. Smith *et al.* (2001)[95] also reported a wide range of *Lactobacillus* species isolated from the patients with dental caries, and the most common species was *L. brevis*.

Several studies using molecular methods verified the results that *Lactobacillus* were recovered more frequently[88] and predominantly from carious dentine[11, 21, 31]. However, the studies reported a wide range of *Lactobacillus* species (table 3). The molecular approaches used in

the study of Byun *et al.* (2004)[11] led to an identification of 18 different phylotypes of lactobacilli in the carious lesions. Quantification by real-time PCR revealed higher mean loads of *L. gasseri* and *L. ultunensis* than of the other prevalent species. This qualitative screening by PCR analysis showed that the members of the *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*) were the most prevalent, followed by *L. salivarius*, *L. gasseri*, *L. ultunensis* and *L. crispatus.*

The studies presented in table 2 show the heterogeneity found for *Lactobacillus* species recovery both in children and adults. The reason for such differences is not known. A similar diversity was found in bacterial species associated with initial caries. van Ruyven *et al.* (2000)[96] found that the bacteria from plaque covering white spot lesions consisted of various species, not only mutans streptococci, non-mutans streptococci and *Actinomyces* but also lactobacilli and *Bifidobacterium*. Interestingly, the samples differed with respect to dominance of particular bacterial species. Similar with *Lactobacillus* species, it may be that any bacterial species can participate in the development of caries as long as they are aciduric and dominant[69]. Another possibility reason to explain the different findings of *Lactobacillus* species may be the difference in identification techniques and alterations in species designation between the studies.

The main purpose for studying the genotypes of cariogenic bacteria is to identify and trace individual genetic variants (clones) of a bacterial species[88] and confirm the transmission among persons[28]. This would be an important tool for the understanding of the caries epidemiology. A finding of pathogenic bacterial clones that are associated with more severe diseased cases may represent clones with higher virulence[75, 97, 98]. Thus, it may be possible that specific clones of a species can be assigned significant biological functions. Most genotypic studies on cariogenic bacteria have been carried out on mutans streptococci, while few studies have been performed on the *Lactobacillus* species[9, 28, 88]. Several studies have shown genetic heterogeneity among *S. mutans* strains[99-101] and lactobacilli strains[9, 88]; however, the relationship between caries activity and the genetic diversity of these bacteria is still controversial. Kreulen *et al.* (1997)[102] reported that children with nursing caries possessed only one single genotype of *S. mutans* while caries-free children harbored numerous genotypes. In contrast, several other studies could not support this finding and demonstrated that subjects with dental caries carried a greater number of genotypes of *S. mutans*[21, 103, 104] and of *Lactobacillus* spp as well[21]. However, this issue has still not been resolved and generally there is limited information on the significance and role of genetic diversity in caries development.

Table 3. *Lactobacillus* species identified in various studies.

Authors	Population	age	No of subjects	Type of samples	Identification technique	*Lactobacillus* species (frequency)
Rogosa *et al.*, 1953[94]	USA	children: school grades 1-7	130	stimulated saliva	biochemical	Percent of subjects: *L. casei* (59%), *L. fermentum* (45%), *L. acidophilus* (21%), *L. brevis* (17%), *L. buchneri* (10%), *L. salivarius* and *L. plantarum*
Milnes and Bowden, 1985[105]	Canada	10-16 months old children	9	plaque from caries susceptible sites	biochemical	Percent of isolates: *L. fermentum*(19%), *L. plantarum*(17%), *L. brevis*(8%), *L. salivarius*(8%), *L. acidophilus*(6%) and *L. casei*(4%)
Smith *et al.*, 2001[95]	Nigeria	adult patients	93	plaque and saliva	biochemical	Percent of isolates: *L. brevis* (25%), *L. fermentum* (19%), *L. casei* (17%), *L. delbrueckii* (15%), *L. plantarum* (9%), *L. acidophilus* (8%) and *L. jensenii* (5%)
Marchant *et al.*, 2001[88]	UK	2-6 years old children	14 C	52 carious lesions	biochemical and gene sequencing	Percent of samples: *L. casei* (38%), *L. fermentum* (34%) and *L. rhamnosus* (24%)
Byun *et al.*, 2004[11]	Australia	adult patients	-	65 carious lesions	real-time PCR	Percent of samples: *L. salivarius* (60%), *L. rhamnosus* (54%), *L. gasseri* (54%), *L. ultunensis* (51%), *L. crispatus* (45%) and *L. casei*/*paracasei* (40%)

Table 3. (continued)

Authors	Population	age	No of subjects	Type of samples	Identification technique	*Lactobacillus* species (frequency)
Munson *et al.*, 2004[21]	UK	adult patients	5 C	carious lesions	gene sequencing	Percent of isolates: *L. gasseri/johnsonii* (26%), *L. rhamnosus* (23%), *L. casei* (21%) and *L. pentosus/plantarum* (15%)
Teanpaisan and Dahlen, 2006[42]	Thai	children and adults	58	saliva	PCR-RFLP and SDS-PAGE	Percent of isolates: *L. fermentum* (49%), *L. rhamnosus* (32%), *L. salivarius* (6%), *L. casei* (6%), *L. acidophilus* (4%) and *L. plantarum (1%)*
Dal Bello and Hertel, 2006[106]	Germany	healthy adults	3	saliva	PCR-DGGE	*L. gasseri*, *L. paracasei*, *L. rhamnosus* and *L. vaginalis**
Caufield *et al.*, 2007[9]	USA	women	6 C	saliva	gene sequencing	*L. vaginalis, L. fermentum* and *L. salivarius**
Svec *et al.*, 2009[107]	Czech Republic	2-6 years old children	40C	carious lesions	biochemical and rep-PCR	Percent of isolates: *L. fermentum* (29%), *L. rhamnosus* (20%), *L. casei*/*paracasei* (10%), *L. gasseri* (10%), *L. salivarius* (10%) and *L. plantarum* (6%)
Almstahl *et al.*, 2010[108]	Sweden	adult patients	6 RT, 3 pSS	plaque	PCR-RFLP and SDS-PAGE	Percent of isolates: *L. fermentum* (30%), *L. rhamnosus* (22%), *L. casei* (20%), *L. paracasei* (8%), *L. salivarius* (3%), *L. acidophilus* (3%) and *L. gasseri* (0.9%)

C= caries active subjects, RT= subjects with radiation-induced hyposalivation, and pSS= subjects with primary Sjogren's syndrome

* frequency of isolates was not presented

2.2.6 Caries-associated characteristics of Lactobacillus

2.2.6.1 Ability to grow and survive

The transmission, acquisition, and establishment as well as the ability to survive and multiply in the plaque biofilm are essential stages in the colonization process of the resident plaque bacteria. Like all other organisms, bacteria can affect each other's reproduction simply by using shared and limiting resources. By dividing rapidly, a colonizer can obtain a larger share of such resources and reduce the availability of nutrients for other members of the population[109]. Growth is a reflection of the adaptation of the organisms to the environmental stress. Oral cavity is an open system; the strains or species best fitted in the environment have the best chance to colonize and persist. The growth of *Lactobacillus* is influenced by a variety of factors such as access of fermentable carbohydrates[110], and interaction with other bacteria[74]. However, *in vitro* growth rate of oral *Lactobacillus* species has scarcely been compared.

2.2.6.2 Acidogenicity and Aciduricity

The ability to produce acid (acidogenicity) and to thrive in a low-pH environment (aciduricity) is essential virulence factors for an organism to be 'cariogenic'[79, 90, 111]. These virulence factors were applied to differentiate the more cariogenic microorganisms from those that are less cariogenic[19, 69, 110, 112]. Lactobacilli can generate the lowest pH from fermentable carbohydrates. Increased caries progression is associated with increased proportions of organisms with higher rates of acid production and greater ability to metabolise and grow at low pH (aciduricity)[15, 67]. These abilities are more advantageous in excess of sugar, either glucose or sucrose. Recent work indicated that, oral bacteria such as mutans streptococci may adapt *in vitro* to great changes in pH[17, 113]. Such adaptation may involve bacterial growth, acidogenesis, or the minimal pH at which acidogenesis can occur[67]. Lactobacilli can metabolize many different sugars; including glucose, sucrose, lactose, sorbitol and xylitol and produce lactic acid as the major end product[114]. Based on sugar fermentation patterns, two broad metabolic categories of lactobacilli were catagorised: homofermentative and heterofermentative[115].

Under conditions of excess glucose and limited oxygen, homofermentative lactocacilli catabolize one molecule of glucose to two molecules of lactic acid via the Embden-

Meyerhof (EMP) pathway (Fig. 2). This process yields two moles of ATP per glucose consumed. Obligately homofermentative lactocacilli includes *L. acidophilus, L. delbrueckii, L. crispatus, L. gasseri* and *L. salivarius.* They produce possess fructose 1,6-biphosphate-adolase but lack phosphoketolase, therefore neither gluconate nor pentoses are fermented[115].

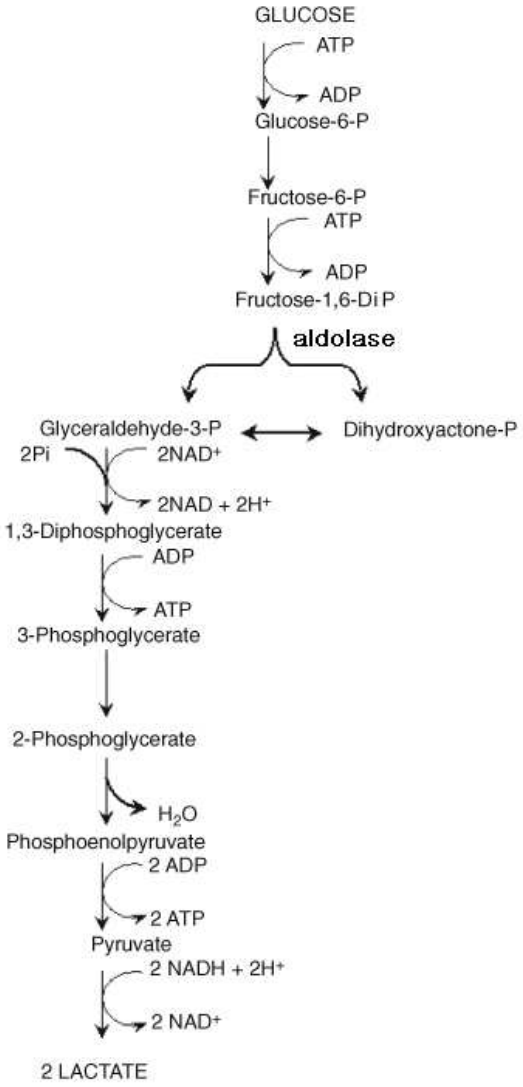

Fig. 2. The pathway of homolactic acid fermentation (the Embden-Meyerhof pathway)[115]

Heterofermentative lactobacilli utilize the phosphoketolase pathway (pentose phosphate pathway) to dissimilate sugars. The reaction proceeds as follows (Fig.3), with one molecule of glucose converted to one molecule of lactic acid, one molecule of ethanol, and one molecule of carbon dioxide. Obligately heterofermentative lactocacilli includes *L. brevis, L.*

fermentum and *L. oris*. In addition, *Lactobacillus* species that are almost exclusively fermented to lactic acid by EMP pathway and also possess phosphoketolase, are grouped as facultatively heterofermentative. These species include *L. casei, L.paracasei, L. plantarum, rhamnosus* and *L. vaginalis*[115].

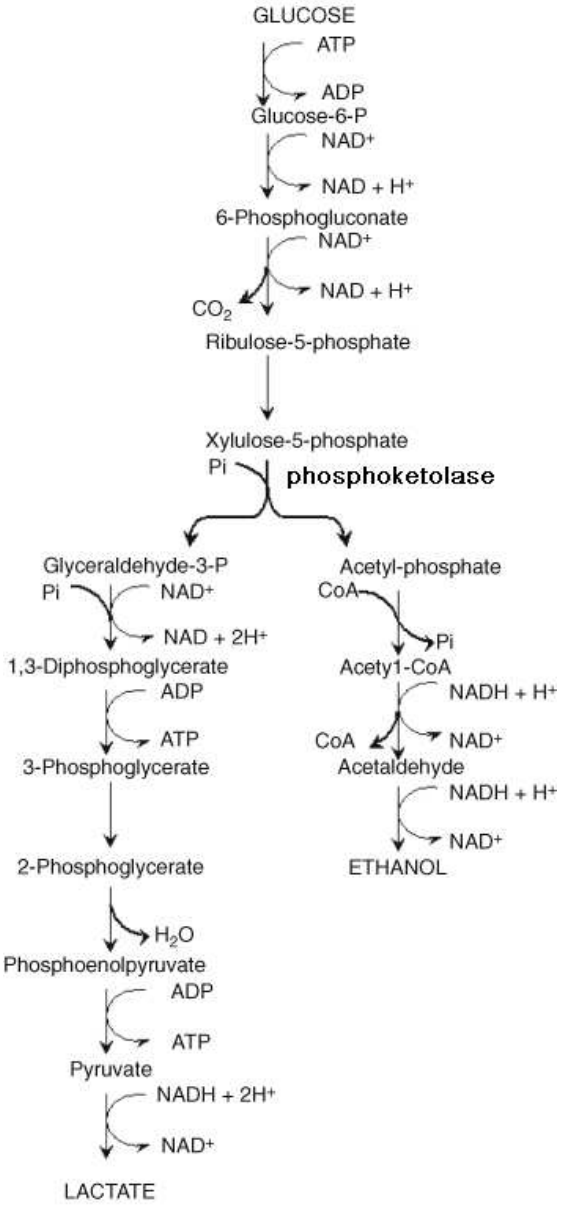

Fig. 3. The pathway of heterolactic acid fermentation[115]

Lactobacilli are highly aciduric [19, 90, 116] and can continue to produce acids even at a very low pH[17, 117]. Svensater et al. (1997)[17] tested the ability of oral bacteria to tolerate acid, in which they found that the death threshold pH for *L. casei* strains varies from 4.5 to 2.3. Badet *et al.* (2001)[19] reported that the final pH of the lactobacilli in a glucose containing broth dropped to 3.65 (*L. salivarius*) and 2.2 (*L. plantarum*) within 24 hours. All tested strains were still alive after this 24-h culture, even when the pH reached 2.2.

Although several studies have reported on the acid production and acid tolerance of *Lactobacillus* species[19, 112, 114, 116, 118], not many species of oral lactobacilli were included. Large variations both between species and between strains have been found among lactobacilli[19, 112, 114], and there were few studies in the relation between the acid production and caries prevalence. Another study reported, however that mutans streptococci strains isolated from caries subjects were more aciduric and acidogenic than those isolated from the caries-free subjects[98]. It may be suggested that the cariogenic environment *in vivo* is important in the expression of genes and in influencing the aciduric and acidogenic characteristics of mutans streptococcus species and strains. It is logical to extend this prediction to include *Lactobacillus* species and strains as well.

3. Objectives

Despite decades of research on oral lactobacilli and their role in the caries process, there are numerous questions that need to be answered before we understand the relation between the bacteria and the disease process completely. More basic information is required on the colonization pattern and virulence characteristics of *Lactobacillus* species. The general objective of this doctoral thesis was to investigate the impact of oral *Lactobacillus* species in early childhood caries using comprehensive molecular, clinical, laboratory and statistical methodology.

The specific objectives of this study were:

1. to study the prevalence and the relationship between early childhood caries and salivary lactobacilli levels in Thai children,

2. to identify and investigate the distribution of the oral *Lactobacillus* at the species and strain level and determine their possible association with early childhood caries,
3. to determine the number of species and clonal types of *Lactobacillus* detected within the individual, between individuals, and to compare the intraindividual clonal diversity with caries prevalence,
4. to examine the *in vitro* differences in growth rate and acid producing capacity (acidogenicity) between species and genotypes of *Lactobacillus* strains isolated from early childhood caries cases.

CHAPTER 2

MATERIALS AND METHODS

The present study was conducted as part of a larger research project: A longitudinal study of factors influencing development, occurrence of oral disease and impact on general health of children in Thepa district of Songkhla province, Thailand[1, 47]. It was focused on an epidemiological survey in which the relation between lactobacilli and dental caries prevalence was determined. The aim was to identify the species or genotype relation of *Lactobacillus* to ECC and whether individual *Lactobacillus* species may play a role in the dental caries progression. For evaluation of ecological factors such as growth capacity and acidogenicity, *Lactobacillus* strains isolated from subjects of the epidemiological ECC study were used.

1. Subjects

From totally 795 children who attended a longitudinal study on factors influencing development, occurrence of oral diseases of children in Thepa district, Songkhla province, Thailand[1, 47], a group of 181 children were invited to participate in a study on salivary levels of lactobacilli. Based on our preliminary study in 12 month-old children, the sample size was calculated using a formula illustrated below.

$$n = [Z^2_{\alpha/2}NP(1-P)]/[Z^2_{\alpha/2}P(1-P)+Nd^2]$$

$$= 180$$

n = number of subjects

N = number of total subjects = 795

P = prevalence of salivary lactobacilli detection of subjects at 12 month-old = 0.19

d = the distance, in either, from the population proportion = 5%

$\alpha = 0.05$, $Z_{\alpha/2} = 1.96$

Dental examination and saliva sample collection were made for all eligible subjects at the ages of 12, 18, 24, 36, 48 and 60 months old. The subjects were randomly selected. The parents agreed to let their child joining the project and consented to the child's clinical examination and microbial sampling. Due to unwillingness of the parents to participate in the study, inconvenience, the family moved out of the study area or uncooperativeness of the child during the period, some children were unable to take part in all the examinations. The number of children who participated at each occasion is shown in Table 4.

The study protocols were approved by the Ethics Committee on Human Subject Research, Ministry of Public Health, Thailand.

Table 4. Number of children attending the examinations at each age.

Examination age	Children attending	
	the main study (n)	saliva sample collection (n)
12 months	595	92
18 months	493	129
24 months	546	141
36 months	667	138
48 months	683	144
60 months	702	146
Total invited	795	181

2. Oral examination

The examination of dental caries status of the subjects was performed by five calibrated dentists using WHO probe (no. 621) and mouth mirror under natural light. The chair-side assistants performed recording of the clinical data. The children were examined in supine position; children less than three years of age were in the knee-to-knee position. The prevalence of dental caries was obtained using a scoring system adapted from the WHO's criteria, 1997[119]. The dental status of each examined surface was categorized as decayed, missing and filled

teeth/surfaces (dmft/dmfs). The surfaces were recorded as decayed when they presented detectably softened floor, undermined enamel or softened wall area and/or the opacity adjacent, or the area providing evidence of undermining or demineralization. Missing teeth were scored when the tooth was extracted due to caries, as judged by the examiner after interviewing the subjects or their parents.

The standardization of the examiners was performed. The range of the Cohen's Kappa coefficients of overall intra-examiner standardization ranged from 0.75 and 0.91 and the overall inter-examiners coefficients ranged from 0.68 to 0.89[1].

3. Microbiological examination

3.1. Bacterial sampling and culture

Bacterial sampling was performed using the modified spatula method[120]. A 1.8 mm-wide wooden spatula was inserted into the mouth to moisten it with saliva. Any excess of saliva was removed by withdrawal of the spatula between closed lips. Each side of spatula was then placed directly on the surfaces of Rogosa SL agar (Difco) in the Petri dishes (Nunc, Copenhagen, Denmark) for recovery of lactobacilli. Two predetermined spatula pressed areas, approximately 1.5 cm^2 of each area, were performed for each child. The plates were transported to the laboratory within 6 hours and incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37ºC for 72 h. The colony numbers (colony forming units, CFU) of lactobacilli, average colonies per spatula pressed area, were counted.

3.2. Isolation of *Lactobacillus* strains

The colonies were collected from the plates, which contained a numbers of lactobacilli of 5 CFU or more /1.5 cm^2. A random sampling technique was used for the agar plates of all subjects by selecting at least 4 colonies with either the same or different colony appearance. A tentative identification of the lactobacilli was performed, based on their growth on Rogosa SL agar, a colonial morphology, the gram-stained microscope morphology (gram-positive non-

sporulating rod) and with a negative catalase reaction[24]. A total of 357 isolates were obtained from 59 children aged 24-60 months (mean age = 34.3±14.6 months). None of the children was selected more than once during the entire examination period. After pure culture, all isolates were kept in -80°C in skim milk until used.

3.3. DNA extraction

The putative *Lactobacillus* isolates were grown for 24-48 hours on MRS agar. Lactobacilli cells were harvested and washed twice in 1 ml sterile water. All DNA samples were extracted using a genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan), according to the manufacturer's protocol for gram-positive bacteria. The DNA suspensions were kept at -20ºC until used.

3.4. Identification of *Lactobacillus* species

Three hundred and fifty-seven *Lactobacillus* isolates were identified to species level by the combined methods of restriction fragment length polymorphism analysis of 16S rRNA genes (PCR-RFLP) and the whole-cell proteins analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the method of Teanpaisan and Dahlen (2006)[42]. Initially 14 type strains of *Lactobacillus* were included in the panel: *L. acidophilus* ATCC 4356, *L. brevis* ATCC 14869, *L. casei* ATCC 393, *L. crispatus* ATCC 33820, *L. curvatus* ATCC 25601, *L. delbrueckii* ATCC 9649, *L. fermentum* ATCC 14931, *L. gasseri* ATCC 33323, *L. paracasei* CCUG 32212, *L. plantarum* ATCC 14917, *L. reuteri* CCUG 33624, *L. rhamnosus* ATCC 7469, *L. salivarius* ATCC 11741, *Olsenella* (formerly *Lactobacillus*) *uli* CCUG 31166. The isolates that did not fit to the panel listed above were identified by 16S rRNA gene sequencing (see below). Clinical isolates of *L. mucosae*, *L. oris* and *L. vaginalis* were subsequently found by gene sequencing. Thus, *L. mucosae* CCUG 43179, *L. oris* CCUG 37396 and *L. vaginalis* CCUG 31452 were included in the panel.

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

The confirmation of identification results from PCR-RFLP of 16S rRNA genes was performed by sequencing of 16S r RNA genes of 3-4 strains from each *Lactobacillus* species.

3.4.1. Restriction fragment length polymorphism analysis of 16S ribosomal RNA genes polymerase chain reaction (16S rRNA genes PCR-RFLP)

Briefly, the 16S-rRNA genes were amplified by PCR using the universal primers 8UA (5'-A□A□TTT□ATCCT□□CTCA□-3') and 1492R (5'-TAC□□□TACCTT□TTAC□ACTT-3')[121]. The PCR 50-µl reaction mixture contained 100 ng of DNA template, 1.0 µM of each primer, 5 µl 10x buffer with 2.0 mM MgCl2, 1.0 U of Taq DNA polymerase and 0.2 mM of each dNTP. Amplification proceeded using a □eneAmp PCR System 2400 (Applied Biosystems, Foster, CA, USA) 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 (New England Biolab, Ipswich, MA) according to the manufacturer's instruction. Digestion products were run on 7.5% polyacrylamide gel, stained with silver staining. A DNA ladder of EZ load® 100 bp Rulers (Bio-Rad, Hercules, CA) was used as a size marker. A scanner (Canon N676U; Canon Inc, Tokyo, Japan) was used for digitalization of the gels. Banding patterns of clinical *Lactobacillus* strains were compared with the reference strains (Fig. 4).

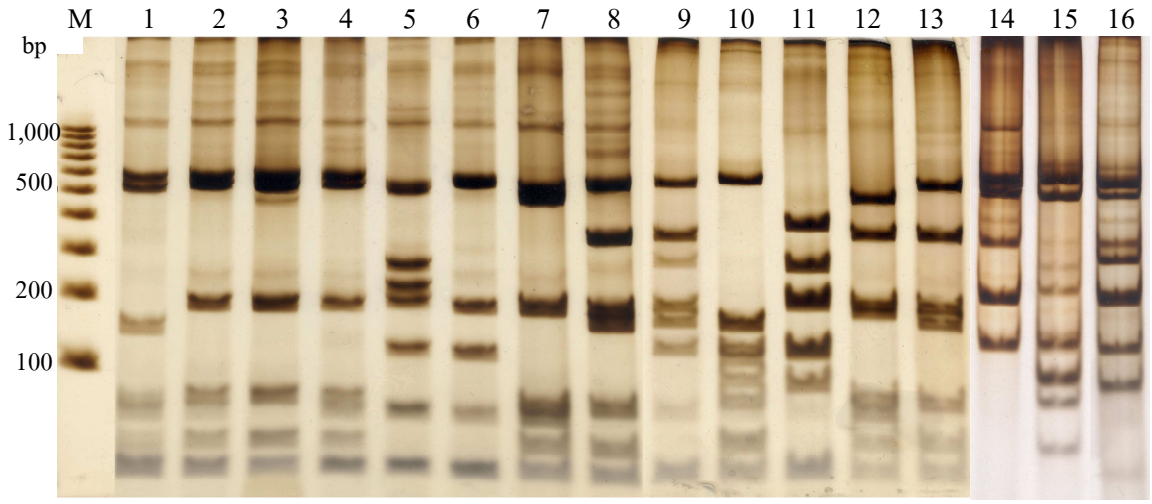

Fig. 4. 16S rRNA genes PCR-RFLP patterns of 16 type strains of *Lactobacillus* digested with HpaII. Lanes: 1, *L. curvatus* (ATCC 25601); 2, *L. casei* (ATCC 393); 3, *L. paracasei* (CCU□32212); 4, *L. rhamnosus* (ATCC 7469); 5, *L. fermentum* (ATCC 14931); 6, *L. plantarum* (ATCC 14917); 7, *L. salivarius* (ATCC 11741); 8, *L. acidophilus* (ATCC 4356); 9, *L. delbrueckii* (ATCC 9649); 10, *O. uli* (CCU□31166); 11, *L. reuteri* (CCU□ 33624); 12, *L. gasseri* (ATCC 33323); 13, *L. crispatus* (ATCC 33820); 14, *L. mucosae* (CCU□ 43179); 15, *L. oris* (CCU□ 37396); 16, *L. vaginalis* (CCU□ 31452); M, Molecular size markers (100 bp DNA Ladder, Bio-Rad). Figure was adapted from Teanpaisan and Dahlen (2006)[42].

3.4.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The discrimination between *L. casei* and *L. rhamnosus*, and between *L. acidophilus* and *L. crispatus*, which could not be differentiated by the PCR-RFLP pattern, were performed using 12% SDS-PA□E[42]. The 24-48 hour-old cultures growing on Rogosa plates were harvested, washed, and resuspended in distilled water. The suspensions were sonicated for 20 seconds with a cell disrupter (Vibra cell™, Sonics & Materials INC, Newtown, USA) in order to lyse the bacterial cell wall. Equal volumes of sonicated cells and SDS sample buffer (0.125 M Tris buffer pH 6.8, 4% SDS, 10% ß- mercaptoethanol, 20% glycerol, 0.002% bromophenol blue) were mixed and boiled for 5 minutes. The samples were electrophoresed on 12% polyacrylamide separating gels added with a stacking gel solution (4%) to form grids for sample inoculation. □els

were placed in an electrode chamber and filled with SDS electrophoresis buffer. Electricity was supplied by the Model 200/2.0 power supply (Bio-Rad, Hercules, CA), set at 200 v and run for 45 minutes. After electrophoresis, staining was accomplished with a solution of Coomasie blue for 30 minutes and destained with an acetone-based solution for 3 hours. A scanner (Canon N676U; Canon Inc, Tokyo, Japan) was used for digitalization of the gels. Banding patterns of clinical *Lactobacillus* strains were compared with the reference strains (Fig. 5).

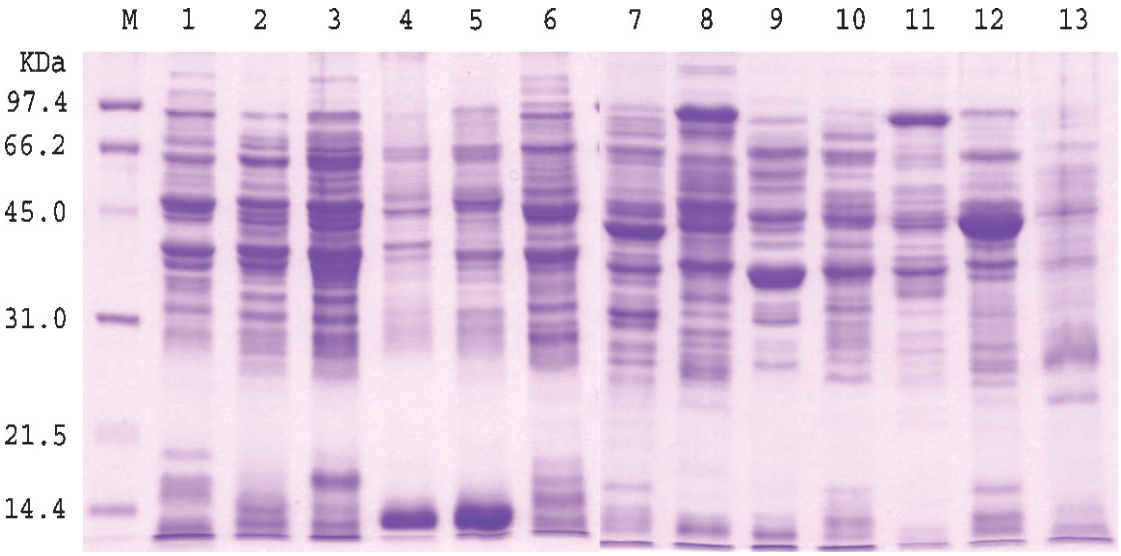

Fig. 5. SDS-PA□E protein profiles of 13 type strains of *Lactobacillus*. Lanes: 1, *L. curvatus* (ATCC 25601); 2, *L. paracasei* (CCU□31610); 3, *L. paracasei* (CCU□32212); 4, *L. plantarum* (ATCC 14917); 5, *L. rhamnosus* (ATCC 7469); 6, *L. salivarius* (ATCC 11741); 7, *L. acidophilus* (ATCC 4356); 8, *L. crispatus* (ATCC 33820); 9, *L. delbrueckii* (ATCC 9649); 10, *L. gasseri* (ATCC 33323); 11, *L. fermentum* (ATCC 14931); 12, *L. reuteri* (CCU□33624); 13, *O. uli* (CCU□31166). M, Molecular size markers (Bio-Rad). Figure was from Teanpaisan and Dahlen (2006)[42]

3.4.3. Sequencing of 16S rRNA genes

Sequencing was performed using ABI PRISM Big Dye Terminator Kit and ABI PRISM 377 genetic analyzer (Applied Biosystems, Foster City, CA, USA). In a 50-µl volume, PCR mixture consisted of 500 ng template, 0.8 µl of Terminator Ready Reaction Mix (Applied

Biosystems), and 3.2 pmol of primers. PCR was performed at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes for a total of 25 cycles using GeneAmp® PCR System 2400 (Applied Biosystems, Foster City, CA, USA). Alignment analysis of percent homology for the obtained sequences was performed by the blast programs (http://www.ncbi.nlm.nih.gov/BLAST).

3.5. Genotyping of *Lactobacillus* strains

After identification, 3 or more isolates of the same *Lactobacillus* species of the same child (totally 304 isolates) were collected for genotyping using arbitrarily primed polymerase chain reaction method (AP-PCR) with the primers; ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGC-3')[28]. The reaction mixture in a 50 µl-reaction mixture contained 100 ng of DNA template, 1.0 µM of each primer, 5 µl 10x buffer with 2.0 mM $MgCl_2$, 1.0 U of Taq DNA polymerase and 0.2 mM of each dNTP. The amplification was performed in a GeneAmp® PCR System 2400 (Applied Biosystems, Foster, CA, USA). The mixture was subjected to 35 cycles of denaturation at 95°C for 1 minutes; ramping 1 to 35°C in 3 minutes; annealing at 35°C for 1 minutes; extension at 74°C for 2 minutes and a final extension at 74°C for 5 minutes. AP-PCR products were run on a 7.5% polyacrylamide gel, and stained with silver staining. A 100-bp EZ load® DNA ladder (Bio-Rad, Hercules, CA) will be used as a size marker in the gel. A scanner (Canon N676U; Canon Inc, Tokyo, Japan) was used for digitalization of the gels. The AP-PCR fingerprints were analyzed by side-by-side visual comparison. Only isolates processed at the same time, starting with PCR reaction mixing step up to electrophoresis in the same gel, were used for the final assessment of similarity or dissimilarity. Fingerprints were considered similar when all major bands were exactly alike, and minor bands had no more than two differences.

3.6. Growth and acid producing characteristics of *Lactobacillus* strains

3.6.1. Bacterial strains and culture conditions

A total of 39 *Lactobacillus* strains, 29 clinical isolates and 10 type strains were selected for further characterization. The clinical isolates were chosen at random from the

collection of *Lactobacillus* isolates in Materials and methods 3.4, one to four isolates of each species. Each tested strain was isolated from a different child to avoid a possible clonal relationship between strains. Type strains used in this study are listed in Table 5.

Initially, strains were grown as starter cultures anaerobically (80% N_2, 10% H_2, and 10% CO_2) in filter sterilized (pore size 0.22 μm, Nalgene, NY, USA) de Man Rogosa and Sharpe (MRS) broth (Lab M, Bury, UK) at 37^{o}C for 16-18 hours, which brought them into exponential growth phase. From these, cells were harvested by centrifugation at 3000 rpm for 5 minutes at 4^{o}C, washed twice in phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) and inoculated into fresh, pre-warmed MRS broth (50ml) containing 2% (w/v) glucose, pH 7.0, to give an optical density of 1.0 at 650 nm using a spectrophotometer (Pharmacia Ltd, Milton Keynes, UK). Cultures were then incubated in an anaerobic chamber (miniMacs Anaerobic Workstation, Don Whitley Scientific Ltd, West Yorkshire, UK) for 24 h.

Table 5. *Lactobacillus* type strains used in this study

Bacterial strain	Origin / relevant property	Reference
L. casei ATCC 393	Cheese	Byun *et al.* (2004)[11]
L. fermentum ATCC 14931	Fermented beets	Byun *et al.* (2004)[11]
L. gasseri ATCC 33323	Human (from unknown tissue)	Byun *et al.* (2004)[11]
L. mucosae CCU□43179	Pig small intestine	Sakamoto *et al.* (2006)[122]
L. oris CCU□37396	Human saliva	Sakamoto *et al.* (2006)[122]
L. paracasei CCU□32212	Milk products	Teanpaisan *et al.* (2009)[123]
L. plantarum ATCC 14917	Pickled cabbage	Byun *et al.* (2004)[11]
L. rhamnosus ATCC 7469	Not known	Byun *et al.* (2004)[11]
L. salivarius ATCC 11741	Human saliva	Byun *et al.* (2004)[11]
L. vaginalis CCU□31452	Human vagina	Ahrne *et al.* (1998)[124]

3.6.2. Measurement of cell growth and pH

Two milliliters from each culture was collected and measured for growth and pH at the start (0) and after 1.5, 3, 5, 7 and 24-hour inoculation. Bacterial growth was determined by measuring the OD of cultures at 650 nm (OD_{650}). The number of cells was calculated from the standard curve between OD and number cells of *Lactobacillus*. The growth of each strain was expressed as the growth rate constant which was determined from the slope of a logarithmic line of best fit through the data points for the exponential phase of growth of the culture according to the formula:

$$\text{growth rate constant} = (\log 10\ N_2 - \log 10 N_1) * 2.303 / t_2 - t_1,$$

where N_1 and N_2 are number of bacterial cells at time point 1 (t_1) and time point 2 (t_2), respectively. In each case purity and viability of each *Lactobacillus* strain was assessed at the final sampling time (24-hour) by plate counting on MRS agar anaerobically for 48 hours.

Acid production was studied by recording pH change using a pH electrode and meter (Hanna pH 211, Hanna Instrument, Bedfordshire, UK) during the incubation period. Hydrogen ion $[H^+]$ values were converted from pH according to the formula:-

$$[H+] = (10^{pH})^{-1}$$

The rate of acidification by each strain (acid production rate) was determined from the change in H^+ (δH^+) divided by the average number bacterial cells per hour at the logarithmic growth period, which was calculated from the following equation:

$$\text{acid production rate} = (\delta H^+)/[(\frac{N2 - N1}{2}) * t_2 - t_1],$$

where N_1 and N_2 are number of bacterial cells at time point 1 (t_1) and time point 2 (t_2), respectively.The overall acidogenicity of each stain was expressed as the “pH area”, which is the integrated area limited by the pH curve and the line of pH 7, as described by Moynihan *et al.*, 1998 [16]. The “pH area” indicates, therefore, how much the medium was acidified by the bacteria within a certain period of time and was calculated using ImageJ software. Also, the final pH reached was recorded for each strain. Each strain was tested twice, using separately grown cultures. All measurements were performed in triplicate.

4. Statistical Analysis

Due to very low number of missing (m) and filled (f) teeth among the subjects, so only the number of decayed teeth/surfaces (dt/ds) was further analyzed in this study. The children were divided into 3 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, 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 association between the number of lactobacilli and caries score was evaluated with the Kruskal-Wallis test. Chi-square test was used to test differences in the level of salivary lactobacilli and caries score. In the study on species and clonal diversity of *Lactobacillus* in children with different caries score, the distribution of *Lactobacillus* species and genotype was calculated as a percentage. The differences in frequencies of each *Lactobacillus* species between caries groups were compared using the Fisher's exact probability test. The frequency distribution of AP-PCR types of *Lactobacillus* species in different caries groups was compared with the chi-square test. The analyses were performed with the SPSS statistical program (SPSS Inc., Chicago, IL). The differences were considered significant when $p<0.05$.

The following parameters were used to characterized growth and acid production by *Lactobacillus* isolates: the growth rate constant, maximum OD increased, acid production rate, time to pH 5.5 and the final pH at 24-hour were used as evaluated parameter. The average value of each parameter is presented as mean $\pm$ standard error (SE). The correlation between growth and pH change was assessed using Pearson's correlation coefficient at the significant level $p<0.05$. The analyses were performed with the SPSS statistical program (SPSS Inc., Chicago, IL, USA).

CHAPTER 3

RESULTS

This chapter presents a summary of the thesis results, based on one published paper (Paper I), one submitted manuscript (Paper II), and two related published paper (Paper III and Paper IV). The papers are shown in appendices A-D.

Paper I: Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlén G. *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. ***Mol Oral Microbiol*** 2010;25(2):157-64.

Paper II: Piwat S, Teanpaisan R, Dahlén G, Thitasomakul S, Douglas CWI. Acid production and growth by oral *Lactobacillus* spp *in vitro*. ***Arch Oral Biol*** (submitted)

PaperIII: Teanpaisan R, Thitasomakul S, Piwat S, Thearmontree A, Pithpornchaiyakul W, Chankanka O. Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children. ***Int Dent J*** 2007; 57(6):445-51.

Paper IV: Thitasomakul S, Thearmontree A, Piwat S, Chankanka O, Pithpornchaiyakul W, Teanpaisan R, et al. A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants. ***Community Dent Oral Epidemiol*** 2006; 34(6):429-36.

1. **Lactobacilli in ECC** (part of results presented in the paper III and IV)

1.1. ECC in children of 12-60 months of age

Of the 181 Thai children examined at regular interval from 12 to 60 months of age, the number of children with caries and the number of decayed teeth/ surfaces significantly increased with age ($p<0.01$). The prevalence of caries increased from 25.6% at the age of 12 months to 86.0% at the age of 24 months and nearly all children (99.3%) at the age of 60 months had caries (Fig. 6). The progression of caries of the subjects was rapidly increased. The mean dt for children at age of 12 months was 0.92 $\pm$ 1.76 and increased to 9.47 $\pm$ 5.47 at the age of 36 months and then further increased to 12.47 $\pm$ 4.83 for the 60 months old children. However, caries surfaces index (ds) increased substantially through the entire study, from mean ds of 1.27 $\pm$ 2.71 for children at the age of 12 months to 34.19 $\pm$ 19.50 at the age of 60 months (Fig. 7).

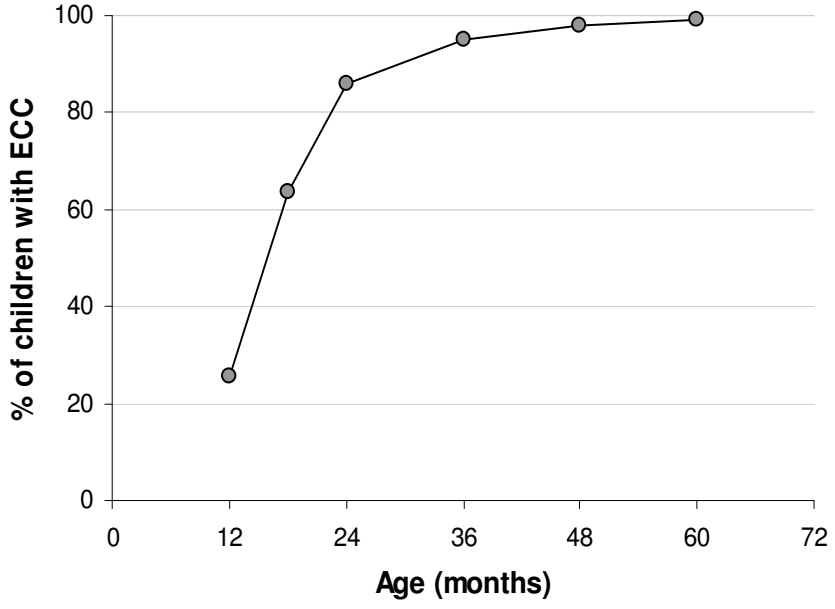

Fig. 6. Prevalence children with ECC, from the age of 12 to 60 months.

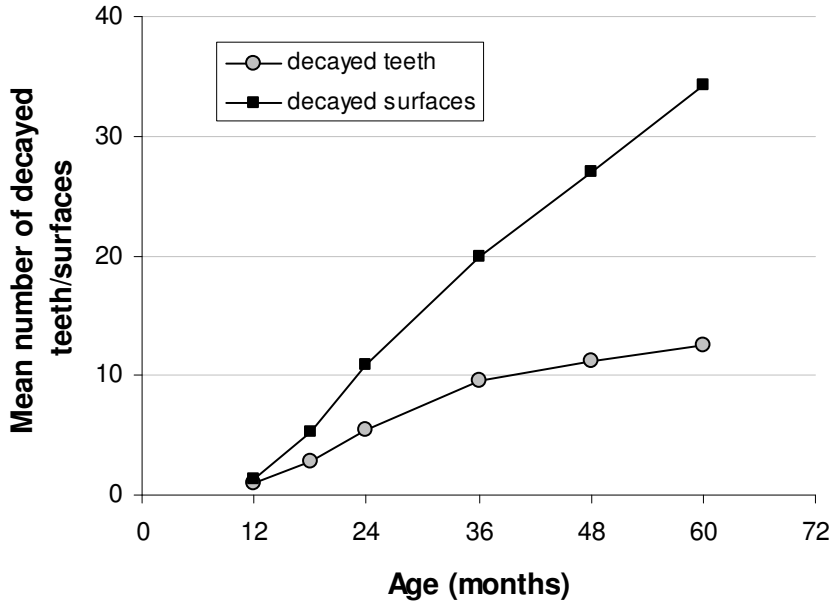

Fig. 7. Mean number of decayed teeth/surfaces in children, from the age of 12 to 60 months.

1.2. Oral colonization of lactobacilli in young children

Oral colonization by lactobacilli was studied in 181 children. Saliva samples were collected annually from the age of 12 to 60 months old. Lactobacilli were isolated from 19% of 12-month-old children. The colonization frequency increased significantly with age ($p<0.01$). More than 70% of children were colonized by lactobacilli at the age of 60 months. Two-thirds of the colonized children harbored a high level of lactobacill (> 10 CFU/1.5 cm^2) (Fig. 8).

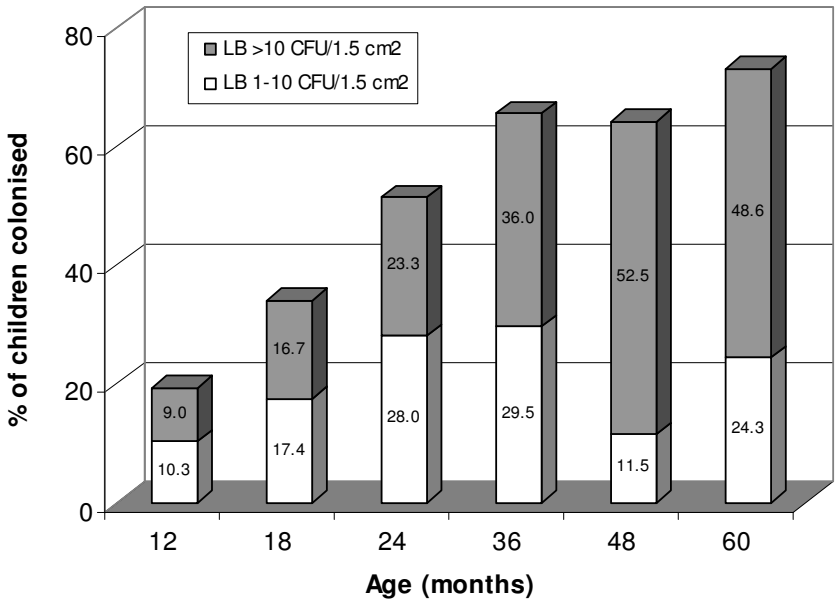

Fig. 8. Percent of lactobacilli (LB) colonized children, from the age of 12 to 60 months.

1.3. Level of lactobacilli in ECC

The correlation between salivary levels of lactobacilli and the number of decayed teeth and surfaces is shown in table 6. The number of decayed teeth and surfaces was significantly associated with the level of lactobacilli at all ages ($p<0.05$) except for the age of 12 months. There were a significantly higher mean of decayed teeth and decayed surfaces with an increasing numbers of lactobacilli ($p<0.05$).

Table 6. Mean carious teeth/surfaces in relation to the lactobacilli count (total N = 181)

Age (months)	Lactobacilli (CFU/1.5cm^2)				p_value (Kruskal Wallis Test)
		0	1 to 10	> 10	
12	n	63	8	7	
	Mean dt	0.79 ± 1.66	1.25 ± 2.38	1.71± 1.89	0.196
	Mean ds	1.16 ± 2.70	1.75 ± 3.62	1.71± 1.89	0.240
18	n	87	23	22	
	Mean dt	2.30 ± 2.71[a]	2.78 ± 2.09	4.91 ± 4.37[a]	0.010
	Mean ds	4.29 ± 6.53[a]	5.52 ± 5.79	9.36 ± 9.85[a]	0.011
24	n	73	42	35	
	Mean dt	3.90 ± 3.48[a, b]	6.02 ± 4.48[a]	7.57 ± 4.32[b]	<0.001
	Mean ds	7.74 ± 8.70[a, b]	11.69 ± 10.26[a, c]	16.71 ± 11.40[b, c]	<0.001
36	n	48	41	50	
	Mean dt	5.94 ± 4.34[a, b]	9.90 ± 4.86[a, c]	12.52 ± 5.01[b, c]	<0.001
	Mean ds	10.83 ± 9.85[a, b]	19.29 ± 11.82[a, c]	29.06 ± 17.33[b, c]	<0.001
48	n	50	16	73	
	Mean dt	6.96 ± 4.55[a, b]	11.38 ± 4.87[a, c]	14.19 ± 3.89[b, c]	<0.001
	Mean ds	13.92 ± 13.47[a, b]	28.63 ± 20.76[a,]	35.67 ± 16.24[b]	<0.001
60	n	39	35	70	
	Mean dt	9.87 ± 5.36[a, b]	13.06 ± 4.24[a]	13.63 ± 4.28[b]	<0.001
	Mean ds	24.62 ± 20.36[a, b]	37.06 ± 19.65[a]	38.10 ± 17.26[b]	<0.001

*Cells with the same alphabetical symbol indicate significant difference (Mann-Whitney U test, $p<0.05$).

dt: decayed teeth, ds: decayed surfaces, n: number of children

2. *Lactobacillus* species and genotypes in ECC (Paper I)

2.1. *Lactobacillus* species distribution in oral cavity of young children

Lactobacilli were differentiated to species using the combination methods of PCR-RFLP and SDS-PAGE. The species frequency analysis of lactobacilli was performed from those 59 children with a predominant presence of lactobacilli (more than five colonies of lactobacilli per 1.5 cm^2). A total of nine *Lactobacillus* species were detected from 357 isolates (Table 7). There were large intra- and inter-individual variations in frequencies of *Lactobacillus* species. *L. fermentum* (83% of the children) were the most frequently isolated species, followed by *L. salivarius* which was found in 25% of the children, while *L. casei/paracasei*, *L. plantarum*, *L. rhamnosus*, *L. oris*, *L. gasseri*, *L. mucosae* and *L. vaginalis* were presented in a low frequency.

2.1.1. Lactobacillus *species distribution in ECC*

The distribution of *Lactobacillus* species in children with high (dt >4) or low (dt 0-4) caries prevalence is shown in Table 7. *L. salivarius* was the only species found in significantly higher numbers in the high caries prevalence 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 prevalence. *L. fermentum* was the most frequently found (more than 80% of children in each group) species in all groups. *L. plantarum* and *L. mucosae* were found only in the 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.

Table 7. Distribution of *Lactobacillus* isolated from children in low caries-group (dt ≤ 4) and children in moderate to high-caries group (dt > 4) (From Paper I, Table 2)

Species	All subjects		Low-caries group		Moderate to high-caries group	
	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* (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)

*Fisher's exact test: p=0.01.

2.1.2. *Lactobacillus* species diversity in ECC

Most children (79.6%) harbored only one or two species of *Lactobacillus* with the maximum of five species detected in one individual. The number of *Lactobacillus* species was not statistically different between the low caries (dt 0-4) and moderate to high caries groups (dt >4) (p=0.76) (Fig. 9), however the children who harbored 3-5 species tended to have more caries (Table 8).

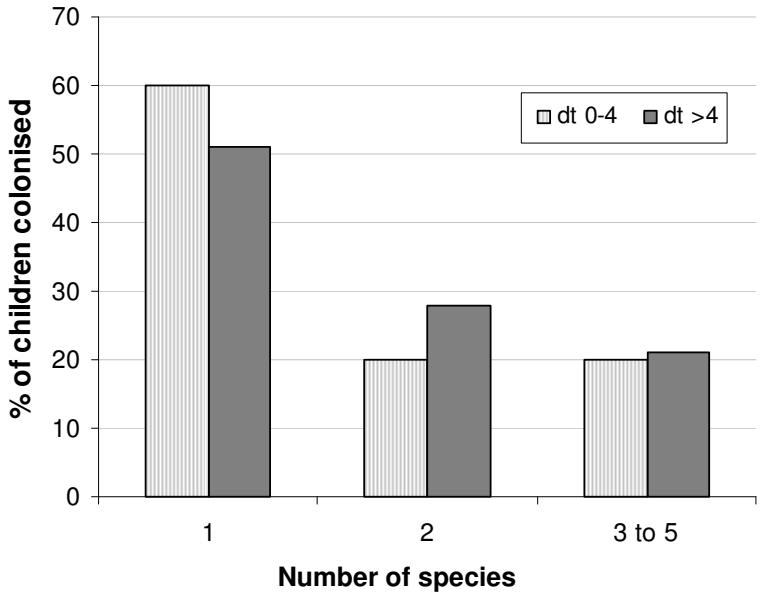

Fig. 9. Percent of children colonized by various number of *Lactobacillus* species compared between the low caries (dt 0-4) and moderate to high caries (dt >4) groups. Pearson Chi-square test: p=0.76.

Table 8. Mean number of decayed teeth by the number of species harbored (total N = 59)

No. of species	No. of children	Mean dt ± SD	Mean ds ± SD
1	32	6.47 ± 4.74	14.38 ± 12.46
2	15	7.53 ± 5.45	17.07 ± 16.10
3-5	12	10.50 ± 7.45	28.25 ± 25.09

Kruskal-Wallis test: p=0.307

2.2. Clonal diversity of *Lactobacillus* in ECC

All *Lactobacillus* species from 56 children, totally 304 isolates were further investigated by AP-PCR. The numbers of subjects and isolates of each species are shown in Table 9. One to five different clonal types of *Lactobacillus* species could be detected in a single child. When comparing isolates from unrelated subjects, identical AP-PCR pattern was not detected. Generally, isolates from each individual showed a distinct genotypic pattern (Fig. 10).

Table 9. Genotypes of 304 *Lactobacillus* strains (with genotype = 1 or > 1) of 56 children (From Paper I, Table 3)

		No. of children (%) / isolates (%)		
Species (No. of children/ isolates)	No. of genotypes	Low-caries group	Moderate-caries group	High-caries group
L. fermentum (38/ 180)*	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# (10/ 45)	1	2 (75)/ 11 (57.9)	4 (100)/ 15 (100)	3 (100)/ 11 (100)
	> 1	1 (25)/ 8 (42.1)	0	0

*Chi-square test only for *L. fermentum*: p = 0.02.

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

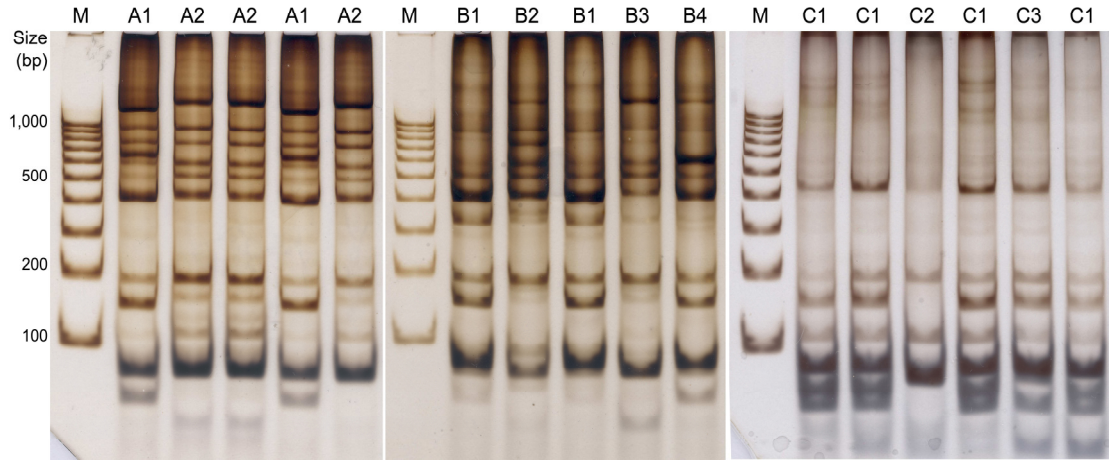

Fig. 10. Arbitrarily primed polymerase chain reaction profiles of 16 *Lactobacillus fermentum* isolates from saliva of three children (A–C) with high caries, which showed A1–A2, B1–B4, and C1–C3 genotypes, respectively. M, molecular size markers-100 base pairs (bp) DNA Ladder; Bio-Rad, Hercules, CA. (From Paper I, Fig. 1)

By comparing the frequency distribution of AP-PCR types of each *Lactobacillus* species and caries score of the subject, it was noted that children who had high caries score tended to be colonized by more than one genotype. This distribution was statistically significant for *L. fermentum* ($p<0.05$). However, it was not possible to perform statistical analyses on the distribution of the other species because too few isolates were recovered (Table 9).

3. *Lactobacillus* species – growth rate and acid production (Paper II)

A total of 39 *Lactobacillus* strains, 29 clinical isolates and 10 type strains were examined. The number of tested strains, the designation of type strains and clinical association of the *Lactobacillus* strains are shown in Table 10.

Table 10. Distribution of *Lactobacillus* strains (total = 39) evaluated in the study, the designation of type strains and the number of selected clinical strains with caries score (From Paper II, Table 1)

Species	Number of tested strains	*Lactobacillus* strains			
		Type strains	Number of clinical strains from children with		
			caries free	dt 2 to7	dt >10
L. casei / paracasei	9	*L. casei* ATCC 393, *L. paracasei* CCUG 32212	2	2	3
L. fermentum	4	*L. fermentum* ATCC 14931	1	0	2
L. gasseri	3	*L. gasseri* ATCC 33323	0	1	1
L. mucosae	5	*L. mucosae* CCUG 43179	0	1	3
L. oris	4	*L. oris* CCUG 37396	0	1	2
L. plantarum	4	*L. plantarum* ATCC 14917	0	1	2
L. rhamnosus	4	*L. rhamnosus* ATCC 7469	1	2	0
L. salivarius	4	*L. salivarius* ATCC 11741	0	1	2
L. vaginalis	2	*L. vaginalis* CCUG 31452	0	1	0

dt = Number of decayed teeth

3.1. Growth rate of *Lactobacillus* species *in vitro*

The growth rate of oral lactobacilli is presented by the increase of OD and the growth rate constant as shown in Fig. 11 and Table 11. Growth rate constant were calculated during the period of most rapid growth. *L. casei/paracasei*, *L. fermentum, L. plantarum*, *L. rhamnosus* and *L. salivarius* had exponential growth during 1.5- to 5-h period. Some species such as *L. vaginalis, L. mucosae, L.oris* and *L. gasseri* had delayed rapid growth period.

L. plantarum and *L. salivarius* had highest growth rate. They grew more rapidly than the other species at the exponential growth phase, having growth rate constants of 0.59±0.05 and 0.53±0.04 h^{-1}, respectively (Fig. 11). The species which reached a higher optical density than the others were *L. plantarum, L. salivarius*, *L. rhamnosus* and *L. casei/paracasei* (Table 11). *L. gasseri*, *L. oris*, *L. vaginalis* and *L. mucosae* were the slowest growers in our system, with growth rate constants about 0.21-0.20 h^{-1}.

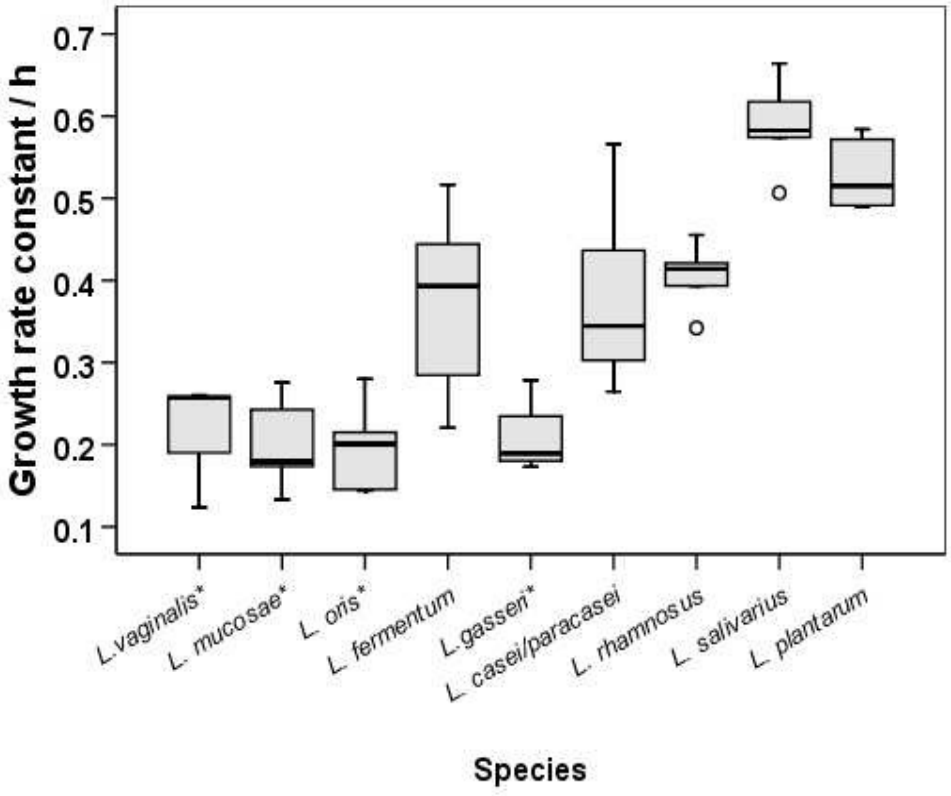

Fig. 11. Growth rate constant at exponential phase of *Lactobacillus* species. Results were calculated during 1.5- to 5-h period unless * calculated during 3- to 7-h period.

Table 11. Growth and acid production characteristics by *Lactobacillus* species (From Paper II, Table 2)

Lactobacillus species (n)	Growth characteristics	Acid production characteristics	
	Max OD increase ± SE	Time to pH 5.5 (h) ± SE	Final pH at 24-h± SE
L. casei/paracasei (9)	1.02 ± 0.04	2.87 ± 0.18	4.02 ± 0.06
L. fermentum (4)	0.82 ± 0.05	4.18 ± 0.47	4.58 ± 0.02
L. gasseri (3)	0.88 ± 0.03	5.70 ± 1.40	4.13 ± 0.08
L. mucosae (5)	0.72 ± 0.04	4.10 ± 0.27	4.62 ± 0.03
L. oris (4)	0.60 ± 0.06	5.50 ± 1.35	4.65 ± 0.04
L. plantarum (4)	1.18 ± 0.02	2.98 ± 0.34	3.89 ± 0.03
L. rhamosus (4)	1.12 ± 0.05	2.27 ± 0.06	3.89 ± 0.02
L. salivarius (4)	1.17 ± 0.01	2.45 ± 0.12	3.92 ± 0.04
L. vaginalis (2)	0.86 ± 0.11	16.39 ± 4.40	5.02 ± 0.28

3.2. Acid production of *Lactobacillus* species

The acid production characteristics of the *Lactobacillus* species are shown in Fig. 12 and Table 11. There were marked differences in acidogenicity among the species, *L. plantarum* and *L. salivarius* had highest acid production rate and also highest growth rate among the logarithmic growth period. However *L. plantarum*, *L. salivarius*, *L. rhamnosus* and *L. casei/paracasei* were able to produce acid to a critical pH of 5.5 faster than the other species, within 2.2-3 h. *L. gasseri* and *L. vaginalis* strains were lower acid producer. However, the final pH at 24-h was not much different among the species.

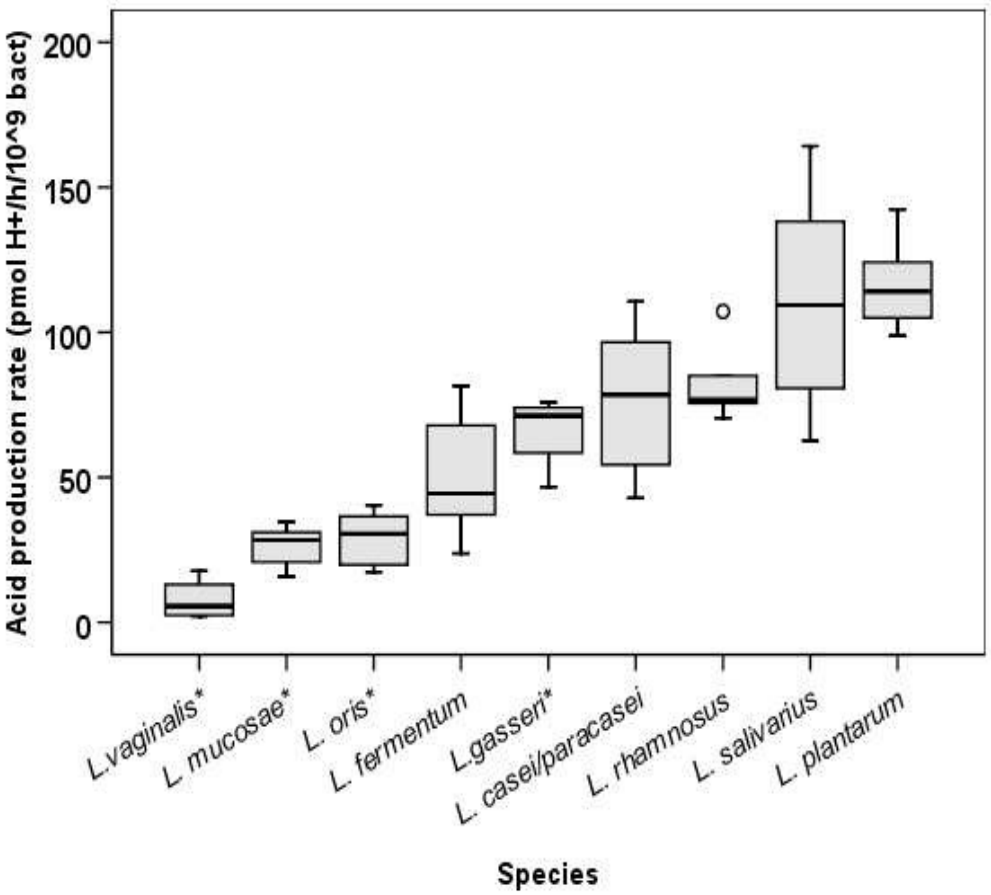

Fig. 12. Acid production rate at exponential phase of *Lactobacillus* species. Results were calculated during 1.5- to 5-h period unless * calculated during 3- to 7-h period.

3.3. Relation between growth rate and acid production in *Lactobacillus* species

The rate at which the *Lactobacillus* species acidified their growth medium paralleled with their growth rate (Fig. 11, 12 and 13). There were positive and statistically significant correlation between the growth and the pH decrease among the *Lactobacillus* strains (Pearson correlation coefficients r = 0.86-0.999, all significant at $p<0.01$). As expected the highest rate of acid production occurred during the period of most rapid growth. From the Fig. 11 and 12, even the ability to produce acid of *L. gasseri* was high; its growth rate was quite low. Conversely for *L. fermentum*, the low acid production rate with high growth rate was presented.

The overall acidogenicity from the combination of acid production together with the growth ability were analysed by using "pH area". The "pH area" was greater for *L. salivarius*,

L. rhamnosus, *L. plantarum* and *L. casei/paracasei* and was particularly different after 24 h incubation (Fig. 14) and thus they were termed the "strong acidogenic" group. Also, these species were still viable after 24 h of cultivation, even when the pH had dropped as low as 3.9. Although the species other than the strong acidogenic group also dropped the pH below the critical pH, the rate of acid production was always lower and the time to reach the critical pH was markedly extended. From the overall data, these bacterial species can be divided to the other two groups; a moderate acidogenic group i.e. *L. fermentum*, *L. gasseri*, *L. mucosae* and *L. oris*, and a weakly acidogenic group i.e. *L. vaginalis*.

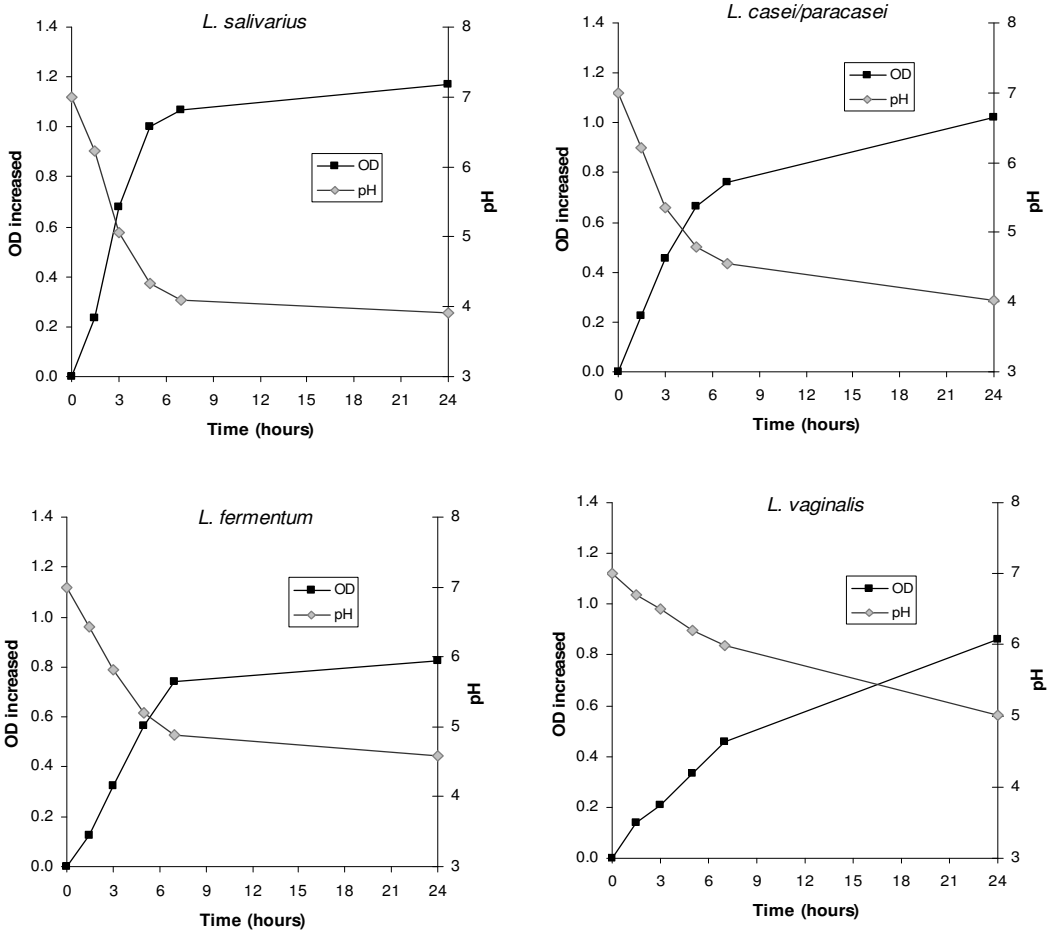

Fig. 13. Growth and acidification as a function of time for *L. salivarius*, *L. casei/paracasei*, *L. fermentum* and *L. vaginalis*. Bacterial growth is demonstrated by the increasing of OD at 650 nm.

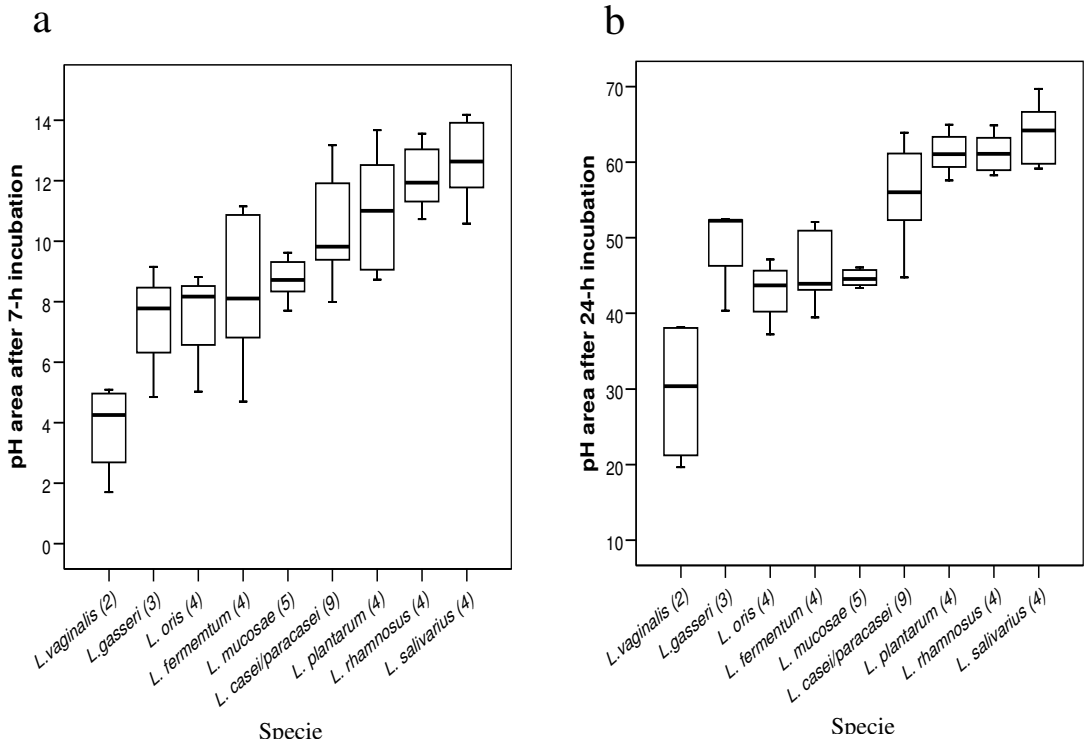

Fig. 14. Acidogenicity of *Lactobacillus* species expressed by "pH area" after 7-h (a) and 24-h (b) incubation period.

CHAPTER 4

DISCUSSION

The oral ecosystem is complex, more than 700 species of bacteria have been identified[125]. *Lactobacillus* is part of the normal oral microflora, however it has been recognized for decades as a major contributor in the caries process. In order to determine the various types of oral *Lactobacillus* in preschool children, and identify their association to dental caries and virulence characteristics, more information is needed regarding the numbers and identity of the *Lactobacillus* species present in the children with various levels of caries severity. Moreover the growth rate and acid production of the *Lactobacillus* strains were studied.

1. *Lactobacillus* association with ECC

In this study ECC and oral lactobacilli were studied in children from 12 to 60 months. The longitudinal study was conducted to follow the prevalence of dental caries and salivary level of lactobacilli during 5 years of children´s early life, which provided a clear understanding of the caries progression over time. Moreover longitudinal data of caries progression would indicate an appropriate access-time for ECC prevention. The children of this study lived in a rural area and most of them had no dental treatment. Therefore only the parameter dt/ds, implying untreated carious cavities/teeth only and no fillings and extractions, was used for analysis. The study of Wright *et al*. (1992)[126] showed that the removal of carious teeth or restoration of carious lesion resulted in a decreased of number of lactobacilli in saliva. It implies that dt/ds score could more truely represent the oral lactobacilli level than the score which include missing and filled teeth and surfaces (dmft/dmfs).

The caries prevalence of our children increased rapidly by age, from 25.6% of children at the age of 12 months to 86.0% at 24 months old. This is in accordance with a previous epidemiological study in Thailand where the prevalence of ECC among 15-19-month-old children in Suphan Buri province was 83%[49]. Thitasomakul *et al.* (2006)[1] reported that the teeth of Thai

children generally developed caries at 3–6 months after tooth eruption into the oral cavity and new carious lesions occurred continuously over time. Also the number of carious teeth was increasingly high. The mean number of carious teeth was 0.92 $\pm$ 1.76 at the age of 12 months, increased to 9.47 $\pm$ 5.47 at the age of 36 months. These data were higher than the findings from other countries, where the caries prevalence was 33.7% of Australian children aged 4–5 years (dmft 1.4 ± 2.8)[127], 32% of British children aged 4–5 years (dmft 1.4 ± 2.8)[128] and 56.5 % of Korean children aged 6-59 months (dmft 3.67 ± 1.4)[129]. Our preschool children exhibited early caries development with high caries progression; unfortunately there is no specific policy on ECC prevention and the early dental checkups or dental treatment can not thoroughly cover the children in this age group especially in the rural area. In this study, most children had no dental treatment; despite of high coverage of national health insurance scheme for all Thai children. National Health Survey from Health Systems Research Institute reported that only 2-3% of Thai preschool children can utilize dental health care service due to their low accessibility[130]. Caries preventive programs usually start in kindergarten with 3-year-old or older children. It has been suggested that these children should obtain an intensive preventive program much earlier, may be within the first year of life or before their birth.

Strong associations between the lactobacilli level and caries score in preschool children have been reported[2, 85]. A similar association was demonstrated in the present study. The children with high levels of lactobacilli were more likely to have dental caries than children with lower levels. The statistical associations were found in all ages except for the age of 12-months. This may be explained by the fact that lactobacilli are late colonizers and they may contribute to the demineralization of the teeth once lesions had occurred[8, 87, 131]. As the age increased, the more open cavities occurred, and the significant associations between lactobacilli and dental caries were found.

2. *Lactobacillus* species distribution in ECC

Lactobacillus species distribution was performed from 59 preschool children, 357 *Lactobacillus* isolates (paper I). This study included a greater number of subjects and strains than

previous reports[88, 105], and included not only children with high caries scores but also a moderate and a low caries group. A limitation of the study was the unexpected high caries prevalence (86.0%) at the age of 24 months, leaving only five caries-free children with sufficient number of lactobacilli isolates 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 shown in table 12, appendix E), these subjects were included in the low-caries group to gain sufficient numbers of children for statistical analyses.

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[42]. By this way of species classification in the present study, nine different *Lactobacillus* species were found in saliva samples from the children. Byun *et al.* (2004)[11], using culture-independent methods with 16S rDNA sequence analysis and real-time PCR, found 18 different phylotypes of lactobacilli in carious dentine in permanent teeth. About 50% of these were novel and it was concluded that diversity among lactobacilli was much greater than previously thought. The present study might have underestimated the diversity of lactobacilli by relying on culture on Rogosa agar plates; however the predominant species can be presented.

When the distribution of *Lactobacillus* species in the group with low caries prevalence and the group with moderate to high caries were compared, interestingly, *L. salivarius* was found to be more associated with caries than the other lactobacilli in the present study. Caufield *et al.* (2007)[9] 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[132]. *L. salivarius* is also aciduric and is reported to survive 4 h incubation at pH 2.5[23]. 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[12, 79]. Matsumoto *et al.* (2005) [133] showed that *L. salivarius* could adhere to saliva-coated hydroxyapatite *in vitro*, therefore it is possible that *L. salivarius* can be efficiently to be incorporated into dental plaque. Fitzgerald *et al.* (1981)[134] reported that *L. salivarius* isolates from human dental plaque could induce severe caries in the fissures of molars in gnotobiotics rats receiving either the glucose-containing or sucrose-containing diet. Pham *et al.* (2009) detected the

effects of sucrose and the *L. salivarius* strain W24 on oral bacterial communities derived from the saliva of four individuals by comparing the patterns of the DGGE profiles of the bacterial communities. In the presence of sucrose, *L. salivarius* lowered the pH more than the pH of the oral bacteria exposed to sucrose without *L. salivarius*. In addition, a significant reduction of band pattern of the DGGE profiles of the communities tested with sucrose alone was found[135]. Therefore, the close association of *L. salivarius* with caries prevalence found in the present study, together with other evidence, indicates that *L. salivarius* might play a significant role in the caries process. However, it should be borne in mind that most studies were based on *in vitro* investigation. Further *in vivo* studies are needed to clearify the role of *L. salivarius* in caries process.

The relation between *L. fermentum* and the caries process is not clear. Some studies have found *L. fermentum* to be the most predominant species in saliva of caries-free subjects[18, 136], while others have reported a high prevalence of *L. fermentum* in subjects with caries[9, 88, 105]. In the present study, *L. fermentum* was the most common species in saliva of both the low-caries and the moderate to high-caries groups. *L. fermentum* is frequently found in the human gut[137] and commonly isolated from human milk[27, 138]. It is also utilized in the food fermentation process[139]. Possibly food-associated lactobacilli survive within the normal resident microflora of the human mouth. The detection of *L. fermentum* in a large proportion both in healthy and caries group suggest that this species might be able to colonize and survive in both environments, and so may have less impact on the caries process than other species.

Other species that have been associated with caries are *L. casei*, *L. paracasei* and *L. rhamnosus*[88, 89], sometimes called *L. casei* group. Several studies have reported a predominance of these species in carious lesions or in caries active subjects[11, 21, 88]. This group is facultatively heterofermentative and produce large amounts of lactic acid like other lactobacilli. These species is most common application in fermented food industrial, specifically for dairy production. Cariogenic properties such as being highly acidogenic and acid tolerant were also reported for these species[17, 118, 140], thus their role in caries progression cannot be ignored. However, *L. casei*, *L. paracasei* and *L. rhamnosus* were infrequently found in the present study, and may only weakly be related to caries in our study subjects. *L. gasseri*, *L. plantarum* and *L. vaginalis* were found in a

relatively low prevalence in the present study. These species are seldom reported in the literature may thus be regarded uncommon in the oral cavity.

The species composition of the oral *Lactobacillus* was found to be subject-specific. A consistent finding of one or several *Lactobacillus* species that are more specifically related to caries has not been demonstrated as summarized in table 3, Chapter 1. The reason for this is not known, however, different populations, geography, race, age or habits may be involved.

3. Species and clonal diversity of *Lactobacillus*

Genotypic studies of oral *Lactobacillus* species are limited, and only two studies report the relationship to caries[9, 88]. Marchant *et al.* (2001)[88] showed on 39 *Lactobacillus* isolates 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. Similarly, Caufield *et al.* (2007)[9] reported genetic heterogeneity among 180 isolates of salivary lactobacilli from six women with active caries. However, these studies were performed only in the caries subjects. In our study, strains isolated from preschool children with various caries score were used to analyze whether there is an association between the number of genotypes and caries activity.

To assess the genotypic identity of the *Lactobacillus* strains and to reveal the intra-species variability, we used AP-PCR with ERIC primers (ERIC-PCR) set. ERIC-PCR gave high discrimination with the polymorphic AP-PCR patterns reflecting differences within the species at the subspecies level. Marchant *et al.* (2001)[88] and Matsumiya *et al.* (2002)[28] showed that ERIC-PCR methodology was efficient and practical for discriminating genotypes within species of *Lactobacillus*. In our study, we found a genetic heterogeneity among our subjects, up to five different clonal types of *Lactobacillus* species could be found in a single child. None of the subjects shared the same genotype with someone else. If such hypothesis that food-associated *Lactobacillus* strains are able to colonize in the oral cavity was true, it may be possible that subjects in the same geographical area habor the same clone of those bacteria. This was not found in this study. The knowledge about the source of oral *Lactobacillus* should be more elucidated.

When the association between the number of species/genotypes of *Lactobacillus* and caries activity were considered, it was found that most children (79.6%) harbored only one or two species of *Lactobacillus*. A few children who harbored 3-5 species tended to have more caries. Furthermore the high-caries children were found to be frequently colonized with more than one genotype. Similar results have been reported in the studies of *S. mutans*[103] showing caries-active subjects to harbor a larger number of genotypes of *S. mutans*. The reason why a greater species and genotypic diversity was found in subjects with high caries is unknown. In some respect, it has previously been postulated that environmental stress in the oral cavity could lead to a reduced number of species or genotypes and favor those that are best adapted to colonize and proliferate in a particular environment[141]. While Beighton *et al.* (1996)[34] reported that high sugar availability could lead to the growth of various *Lactobacillus* clones compared with less favorable conditions. From the review of Bowden and Hamilton (1998) [67], the authors concluded that the wider phenotypic and genotypic diversity, the better they survive the environmental fluctuations in the oral cavity. Moreover, a frequent intake of fermentable carbohydrates and acidic environment may not be a stress but a favorable condition for most lactobacilli and the promotion of *Lactobacillus* strains colonization and persistence can occur. The simultaneous action of multiple *Lactobacillus* phenotypes and genotypes with possibly differing cariogenic potential may increases the risk of caries[103].

4. Growth rate and acid production ability of *Lactobacillus* strains

The ability to grow and to produce acid at a fast rate, in addition to their aciduric properties provides an environmental advantage for cariogenic bacteria when excess sugar is presented[69, 141]. Lactobacilli are one of the major bacterial groups thought to be involved in the progression of caries by cavitation into the dentine[10] and high counts of lactobacilli indicate a low pH environment often as result of a frequent sugar consumption[142]. This is probably due to the fact that the majority of the *Lactobacillus* species are able to derive almost all of their energy from the fermentation of glucose, they utilize sugar and convert to lactic acid depending on species[143]. In this study, a positive correlation between the growth rate and the pH decrease of the *Lactobacillus*

strains was found in general, although the rate of growth and acid production of each species differed. Both growth and acid production ability influence the ability of *Lactobacillus* to acidify their environment. The ability to produce acid may related to their sugar fermentation patterns. The species with the lowest acid production rate including *L. vaginalis*, *L. oris*, *L.mucosae* and *L. fermentum* are obligately heterofermentative[115], which can convert only one molecule of lactic acid from one molecule of glucose. However, the species in same fermentative group may produce acid in a different rate, as found in *L. salivarius* and *L. gasseri*. Although these two species are hemofermentative, *L. salivarius* had higher acid production rate than *L. gasseri*.

The majority of *Lactobacillus* strains were able to metabolize glucose to generate a final pH below 5.5, the critical pH for the demineralization of enamel and dentine. The group of "strong acidogenic" species, (*L. salivarius*, *L. rhamnosus*, *L. casei/paracasei* and *L. plantarum*), were able to reach pH 5.5 within only 2.2-3 h (mean 2.64 h) compared with a mean of 4.7 h for the "moderate acidogenic group (*L. fermentum, L. gasseri, L. mucosae, L. oris*). Moreover, the strong acidogenic species grew faster than the other species in the culture system used here and it is tempting to speculate that they may also grow to a higher proportion in plaque.

Our previously mentioned result showed that one of the "strong acidogenic" species, *L. salivarius*, was the predominant species associated with caries in present study. The present findings further support a role for *L. salivarius* as a cariogenic species and is in accordance with findings of Pham *et al.*(2009)[135], which showed that *L. salivarius* could significantly lower the pH and change the community profiles of oral biofilms in the presence of sucrose. The other members of the "strong acidogenic" group, (*L. rhamnosus*, *L. casei/paracasei* and *L. plantarum*) have also been associated with caries; however the predominant species in various populations differ[10, 88, 89]. The review of Badet and Thebaud (2008)[10] described that regardless of the sampling method or the identification technique, the carious site or the age of sampled subjects used to search for the *Lactobacillus* species in carious lesions, most species found belong to the casei group of *Lactobacillus*. These included *L. rhamnosus, L. casei, L. paracsei, L. salivarius, L. plantarum, L. fermentum, L. brevis* etc. Our results give a deeper understanding in which they are strong acid producers and aciduric. Thus, when an imbalance situation appears in oral cavity, these organisms will take a chance in overgrowth to produce the lower pH. And by that, dental caries could occur. However, it was not found any specific clonal type and caries activity. Cariogenicity

in vivo is an outcome of many factors, including ability to colonize and compete with other microorganisms in addition to their acidogenic and aciduric characteristics[67]. A study of the cariogencity of individual strains *in vivo* will be required to confirm the importance of the "strong acidogenicity" group of lactobacilli.

In this study, the "moderate acidogenic" species, *L. fermentum,* was commonly isolated in both the low- and moderate to high-caries groups, while the other species in this group were found less frequently (*L. gasseri*, *L. oris* and *L. vaginalis*). Although *L. fermentum, L. gasseri*, *L. oris* and *L. vaginalis* were not able to present a great acidic challenge, they were nonetheless able to drop the final pH to below the critical pH and may thus contribute to the caries process. However, the presence of strains with high acidogenicity is presumably more important in faster rates of caries progression than strains with lower acidogenicity.

Variation in acidogenicity among strains of the same species was also noticeable, particularly during the first 7 h of incubation. It is not unexpected to find variations within the *L. casei/paracasei* group, since these two species may possess a different acidogenicity. However, we were not able to differentiate *L. casei* from *L. paracasei* using our identification scheme. However, others have reported a strain-to-strain variation in growth rate and ability to produce acid among strains within species of other bacteria e.g. *S. sanguinis* and *Actinomyces* spp[15]. The importance of this propertyis not clear at this stage, but it may also reflect strain-to-strain variation in cariogenicity. This is in accordance with the results obtained from Khoo *et al.* (2005)[98], who reported that strains of mutans streptococci isolated from caries subjects were more acidogenic than those isolated from caries-free subjects. In this study, it seemed that species identity related more strongly to acidogenicity than to caries status of the individual from whom the strain was isolated. However, the different numbers of strains tested in the low caries and high caries groups may have an effect to this difference, due to the study reported here might contain too few caries-free subjects to be able to draw statistically valid conclusions about the true relationship between caries status and relative acidogenicity of the *Lactobacillus* isolates.

A number of studies have suggested that *Lactobacillus* play an important role in caries progression. In this study, attempt has been made to search for unique type of species or of genotypes involving in caries process and search for the characteristics which related to caries. The question may be raised if our results truly reflect the situation in vivo. We realized that the

data obtained from in vitro experiments are somewhat different from the natural intra-oral habitat. The complex environmental conditions in the oral cavity may limit the value of extrapolations from the in vitro experiment. It should be bare in mind that the findings in the present study were based on the in vitro investigations, and other factors should be considered for interpretation in vivo.

CHAPTER 5

SUMMARY AND CONCLUSION

The present studies were performed to characterize oral *Lactobacillus* in the relation to caries status of Thai preschool children. The caries prevalence of the children in this study increased rapidly with age. Finally, at the age of 60 months nearly all children (99.3%) had caries and caries had developed in more than half of their deciduous teeth (dt 12.47±4.83). There was a strong relationship between caries and salivary lactobacilli level. The children with high levels of lactobacilli significantly had more dental caries than children with lower levels.

The assessment of children's oral *Lactobacillus* was achieved in this thesis by using the molecular methods and inclusion of numerous children. In addition, the characteristics i.e. growth rate and acid production of the *Lactobacillus* were investigated. The species frequency analysis of *Lactobacillus* was performed on 357 isolates from 59 children,. Nine *Lactobacillus* species were detected; *L. fermentum* (83% of the children) was the most frequently isolated species, followed by *L. salivarius* (25% of the children), while *L. casei/paracasei*, *L. plantarum*, *L. rhamnosus*, *L. oris*, *L. gasseri*, *L. mucosae* and *L. vaginalis* were presented in a low frequency. A significant association between a *Lactobacillus* species and dental caries in children was found only for *L. salivarius*, while *L. fermentum* predominated in all subject groups.

By clonal analysis, a genetic heterogeneity among the subjects was found and up to five different clonal types of *Lactobacillus* species were detected in a single child. The children with high caries were found to be frequently colonized with more than one genotype. Besides *Lactobacillus* diversity at species level, different genotypes among strains of a species were also detected e.g., *L. fermentum* and *L. salivarius*. That the genetic heterogeneity of the species found in high-caries children suggests that a frequent intake of fermentable carbohydrates and a resulting low pH environment may be such a favorable condition that promotes various *Lactobacillus* strains to colonize and persist in the oral cavity.

Cariogenic characteristics, including acid production and growth rate were determined. All tested *Lactobacillus* were acidogenic, although there were differences between

species. The rate of acid production is consistent with their growth rate. For example, *L. rhamnosus*, *L. salivarius*, *L. casei/paracasei* and *L. plantarum* showed the highest acid production rate and the lowest final pH reached comparing with other species. These species, classified as the "strong acidogenic", were also aciduric due to their tolerance to pH as low as 3.9. The other species can be divided into two groups; a moderate acidogenic group i.e. *L. fermentum*, *L. gasseri*, *L. mucosae* and *L. oris*, and a weakly acidogenic group i.e. *L. vaginalis*.

More information about the composition and patterns of oral *Lactobacillus* and their relationship to caries in young children could help to increase our understanding about the roles these microorganism in caries development and lead to define a risk group of bacteria. The findings of marked cariogenic characteristics of *L. salivarius* together with their significant presence in high caries children reported here raise the possibility that *L. salivarius* possesses cariogenic characteristics and may be a specific *Lactobacillus* pathogen related to caries in Thai children.

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APPENDICES

Appendix A: Paper I

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

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

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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).

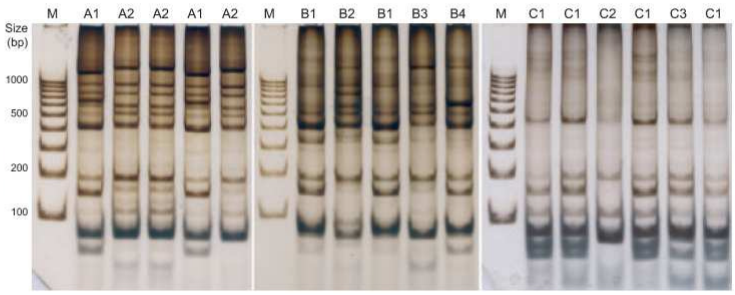

Figure 1 Arbitrarily primed polymerase chain reaction profiles of 16 *Lactobacillus fermentum* isolates from saliva of three children (A–C) with high caries, which showed A1–A2, B1–B4, and C1–C3 genotypes, respectively. M, molecular size markers – 100 base pairs (bp) DNA Ladder; Bio-Rad, Hercules, CA.

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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Appendix B: Paper II

Acid production and growth by oral *Lactobacillus* spp *in vitro*

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Abstract

The ability of bacteria to grow and produce acid at the tooth surface is an important cariogenic factor. Our previous study in Thai preschool children indicated that certain species of *Lactobacillus* may play a more important role in caries development than others. The aim of the present study was to analyze the acid producing and growth abilities of 39 oral clinical strains and type strains of *Lactobacillus*, representing 9 species including *L. casei/paracasei*, *L. fermentum*, *L. gasseri*, *L. mucosae*, *L. oris*, *L. plantarum*, *L. rhamnosus*, *L. salivarius* and *L. vaginalis*. Overnight, anaerobically grown bacterial cells were inoculated in MRS broth containing 2% glucose at pH 7. Acid production and growth were measured at 1.5, 3, 5, 7 and 24 h. Most *Lactobacillus* species were able to lower the pH below the critical pH for tooth demineralization (pH 5.5) within 7 h. *L. salivarius*, *L. rhamnosus*, *L. casei/paracasei* and *L. plantarum* grew more rapidly and reached an optical density higher than other species and they produced more acid than the others. *L. vaginalis* showed the lowest rate of growth and acid production. These findings showed that all the strains were acidogenic but could be categorized into three groups, strong, moderate and weakly acidogenic. Four species, *L. salivarius*, *L. rhamnosus*, *L. casei/paracasei* and *L. plantarum*, were the strongest acid producers and suggests that they may play a more important role in caries development than the others.

Key words: Acid production, growth, oral, *Lactobacillus*

Introduction

Lactobacillus species are a large group of gram-positive, facultative and anaerobic bacteria which are acid-producing and are widely acknowledged as being cariogenic pathogens [Tanzer et al., 2001; Teanpaisan et al., 2007; van Houte, 1994]. Our previous study of *Lactobacillus* species in young children also indicated the association between the oral lactobacilli and dental caries and although a variety of *Lactobacillus* species were isolated from the subjects, only *L. salivarius* was found to be more associated with caries than other lactobacilli [Piwat et al., 2011]. However, it is not known whether this species is truly more cariogenic than other species of lactobacilli.

Caries develops as a result of imbalance between de- and remineralization of enamel and dentine and the critical pH for enamel to be demineralized is considered to be approximately 5.5 [Larsen and Pearce, 1997]. Acidogenesis is one of the two most important virulence factors that differentiate the more cariogenic microorganisms from those that are less cariogenic and lactobacilli can generate the lowest pH from fermentable carbohydrates [Badet et al., 2001; Klinke et al., 2009; Matsuyama et al., 2003; Takahashi and Nyvad, 2008]. Moreover, increased caries progression is associated with increased proportions of organisms with higher rates of acid production and greater ability to metabolise and grow at low pH (aciduricity) [Bowden and Hamilton, 1998; van Houte et al., 1996]. Lactobacilli can metabolize many different sugars; including glucose, sucrose, lactose, sorbitol and xylitol, to lactic acid [Haukioja et al., 2008] and they are aciduric [Badet et al., 2001,. However, in similarity to non-mutans streptococci, lactobacilli show heterogeneity in acidogenicity between species and strains [de Soet et al., 2000; Klinke et al., 2009; Matsuyama et al., 2003]. Although, several studies have reported on the acid production ability of *Lactobacillus* species, not many species of oral lactobacilli were included [Klinke et al., 2009; Matsuyama et al., 2003; Moynihan et al., 1998]. Consequently, the aim of the present study was to analyze the growth and acid producing capabilities of oral lactobacilli with particular focus on clinical isolates obtained from preschool children in Thailand.

Materials and Methods

Bacterial Strains and Culture Conditions

A total of 39 *Lactobacillus* strains, 29 clinical isolates and 1 type strains were examined. The number of tested strains, the designation of type strains and clinical association of the *Lactobacillus* strains are shown in Table 1. ach tested strain was isolated from a different child to avoid a possible clonal relationship between strains and were chosen at random from our collection. The details of isolation and identification of the clinical *Lactobacillus* strains have been described in a previous study [Piwat et al., 2 1]. riefly, strains were isolated from saliva of children on Rogosa SL agar and identified according to 16S-rRNA gene profiles by restriction fragment length polymorphism analysis (PCR-RFLP) and protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAG) [Teanpaisan and Dahlén, 2 6]. The strains were stored at -8 °C until used.

Initially strains were grown as starter cultures anaerobically (8 % N2, 1 % H2, and 1 % CO2) in filter sterilized (pore size .22 µm, Nalgene, NY, USA) de Man, Rogosa and Sharpe (MRS) broth (Lab M, ury, UK) at 37oC for 16-18 h, which brought them into exponential growth phase. From these, cells were harvested by centrifugation at 3 rpm for 5 min at 4oC, washed twice in phosphate buffered saline (P S; Oxoid, asingstoke, UK) and inoculated into fresh, pre-warmed MRS broth (5 ml) containing 2% (w/v) glucose, pH 7. , to give an optical density of 1. at 65 nm using a spectrophotometer (Pharmacia Ltd, UK). Cultures were then incubated in an anaerobic chamber (miniMacs Anaerobic Workstation, Don Whitley Scientific Ltd, UK) for 24 h.

Measurement of cell growth and pH

Two milliliters of each culture was removed and measured for growth and pH at the start ( ) and at 1.5, 3, 5, 7 and 24 h after inoculation. acterial growth was monitored by measuring the OD of cultures at 65 nm (OD65). The growth of each strain was expressed as the growth rate constant which was determined from the slope of a logarithmic line of best fit through the data points for the exponential phase of growth of the culture according to the formula:-

growth rate constant = (ln OD2 - ln OD1)/ t2-t1,

where OD1 and OD2 are OD value at time point 1 (t1) and time point 2 (t2), respectively.

In each case purity and viability of each *Lactobacillus* strain was assessed at the final sampling time (24 h) by plate counting on MRS agar anaerobically for 48 h.

Acid production was studied by recording pH change using a pH electrode and meter (Hanna pH 211, Hanna Instrument, UK) during the incubation period. The rate of acidification by each strain (acid production rate) was determined from the change in pH (δpH) divided by the OD65 per hour at the logarithmic growth period. The overall acidogenicity of each stain was expressed as the "pH area", which is the integration of the area bounded by the pH curve and the line of pH 7, as described by [Moynihan et al., 1998]. The "pH area" indicates, therefore, how much the medium was acidified by the bacteria within a certain period of time and was calculated using ImageJ software. Also, the final pH reached was recorded for each strain. ach strain was tested twice, using separately grown cultures. All measurements were performed in triplicate.

Statistical Analysis

The average value of each parameter is presented as mean ± standard error (S). The correlation between growth and pH change was assessed using Pearson's correlation coefficient at the significant level p< .1. The analyses were performed with the SPSS statistical program (SSPS Inc., Chicago, IL, USA).

Results

The growth and acid production from glucose among oral lactobacilli varied between species (fig. 1 and table 2). *L. salivarius*, *L. rhamnosus* and *L. plantarum* grew more rapidly than the other species, having growth rate constants of .21, .19 and .18 h^{-1}, respectively. They reached stationary phase by the 5th hour and reached a higher optical density than the other species (fig. 1a). *L. gasseri*, *L. oris* and *L. vaginalis* were the slowest growers in our system, with growth rate constants of .1 , .18 and .18 h^{-1}, respectively.

The rate at which the *Lactobacillus* species acidified their growth medium was consistent with their growth rate (fig. 1). There were positive and statistically significant correlation between the growth and the pH decrease among the *Lactobacillus* strains (Pearson correlation coefficients r = .86-.999, all significant at p<.1). As expected the highest rate of acid production occurred during the period of most rapid growth, which was within 1.5-3 h for most species, although *L. gasseri* and *L. vaginalis* strains took longer to start growing quickly (fig. 1). Table 2 shows the acid production characteristics of the *Lactobacillus* species studied and there were marked differences in acidogenicity among them. *L. rhamnosus*, *L. salivarius*, *L. casei/paracasei* and *L. plantarum* showed the highest acid production rate at the logarithmic growth period. They were able to drop the pH to the critical pH (5.5) within 2.2-3 h and reach a lower minimal pH sooner than the other species. The "pH area" was greater for these species and was particularly different after 24 h incubation (fig. 2) and thus they have been termed the "strong acidogenic" group. Also, these species were still viable after 24 h culture, even when the pH had dropped as low as 3.9. Although the species other than the strong acidogenic group also dropped the pH below the critical pH, the rate of acid production was always lower and the time to reach the critical pH was markedly extended. From the overall data, these bacterial species can be divided to the other two groups; a moderate acidogenic group i.e. *L. fermentum*, *L. gasseri*, *L. mucosae* and *L. oris*, and a weakly acidogenic group i.e. *L. vaginalis*.

Discussion

A positive correlation between the growth and the pH decrease of the *Lactobacillus* strains was found in general, although the rate of growth and acid production of each species differed. The ability to grow and to produce acid at a fast rate, in addition to their aciduric properties provides an environmental advantage for cariogenic bacteria when excess sugar is presented [Bradshaw and Marsh, 1998; Takahashi and Nyvad, 2008]. Lactobacilli are one of the major bacterial groups thought to be involved in the progression of caries [Badet and Thebaud, 2008] and high counts of lactobacilli often indicate a high sugar consumption [Bowden, 1997]. This is probably due to the fact that the majority of the *Lactobacillus* species are able to derive almost all of their energy from the homolactic fermentation of glucose, with 85-90% of the sugar utilized being converted to lactic acid [Carlsson, 1989]. In this study the majority of strains of lactobacilli were able to metabolize glucose to generate a final pH below 5.5, the critical pH for the demineralization of enamel and dentin. However, the group of "strong acidogenic" species, (*L. salivarius, L. rhamnosus, L. casei/paracasei* and *L. plantarum*), were able to reach pH 5.5 within only 2.2-3 h (mean 2.64 h) compared with a mean of 4.7 h for the "moderate acidogenic group (*L.fermentum, L.gasseri, L.mucosa* and *L.oris*). Moreover, the strong acidogenic group grew faster than the other species in the culture system used here and it is tempting to speculate that they may also grow to a higher proportion in plaque.

Indeed our earlier study showed that one of the "strong acidogenic" group, *L. salivarius*, is the predominant species associated with caries in Thai children [Piwat et al., 2011]. The present findings further support a role for *L. salivarius* as a cariogenic pathogen and is in accordance with findings of Pham et al.[2009], which showed that *L. salivarius* could significantly lower the pH and change the community profiles of oral biofilms in the presence of sucrose. The other members of the "strong acidogenic" group, (*L. rhamnosus, L. casei/paracasei* and *L. plantarum*) have also been associated with caries, even if the predominant species among various populations differed [Badet and Thebaud, 2008; Marchant et al., 2011; Nancy and Dorignac, 1992]. Cariogenicity in vivo is a product of several factors, including ability to colonize and compete with other microorganisms as well as acidogenicity and aciduricity [Bowden and Hamilton, 1998]. A study of the cariogencity of individual strains will be required to confirm the importance of the "strong acidogenicity" group of lactobacilli.

In our previous study, the "moderate acidogenic" species, *L. fermentum*, was commonly isolated in both the low- and moderate to high-caries groups, while the other species in this group were found less frequently (*L. gasseri, L. oris* and *L. vaginalis*) [Piwat et al., 2011]. Although in this study *L. fermentum, L. gasseri, L. oris* and *L. vaginalis* were not able to present a great acidic challenge, they were nonetheless able to drop the final pH to below the critical pH and so may contribute to the caries process. However, the presences of the strains with high acidogenicity are presumably more likely to be important in higher rates of caries progression than strains with lower acidogenicity.

Variation in acidogenicity among strains of the same species was also noticeable, particularly during the first 7h of incubation (Table 2, Fig. 2). It is not unexpected that we found variation within the *L. casei/paracasei* group, since these two species may possess the different acidogenicity but we were not able to differentiate *L. casei* from *L. paracasei* using our identification scheme. However, others have reported similar strain-to-strain variation in growth and ability to produce acid among strains of other bacteria e.g. *S. sanguinis* and *Actinomyces* spp [van Houte et al., 1996]. The importance of this is not clear at this stage, but it may also reflect strain-to-strain variation in cariogenicity. Khoo et al. [2005] reported that strains of mutans streptococci isolated from caries subjects were more acidogenic than those isolated from caries-free subjects. In this study, it seemed that species identity related more strongly to acidogenicity than to caries status of the individual from whom the strain was isolated. However, unfortunately, the study reported here contained too few caries-free subjects to be able to draw statistically valid conclusions about any relationship between caries status and relative acidogenicity of the *Lactobacillus* isolates. The study in the future will elucidate the unclear point mentioned.

In conclusion, this study has demonstrated that oral *Lactobacillus* species can be categorized into three groups according to their acidogenicity (strong, moderate and weak). It is speculated that the strong acidogenic species may present a greater acidogenic challenge in vivo and so contribute to a faster caries progression rate than the less acidogenic species.

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Legends:

Table 1. Distribution of *Lactobacillus* strains (total = 39) evaluated in the study, the designation of type strains and the number of selected clinical strains with caries score

Table 2. Growth and acid production characteristics by *Lactobacillus* species

Fig. 1. Growth and acidification of *Lactobacillus* species after 3-, 7- and 24-h incubation in MRS broth containing 2% glucose. (a) Bacterial growth shown as the increase of OD650 from the initial at OD650 = 1.0. (b) Decrease of pH from initial (7.0) of pH 7. Number of strains tested is in parenthesis.

Fig. 2. Acidogenicity of *Lactobacillus* species expressed by the area bounded by the pH curve and a horizontal line of pH 7 (pH area) after 7-h (a) and 24-h (b) incubation period. Note the dissimilar y-axis.

Table 1. Distribution of *Lactobacillus* strains (total = 39) evaluated in the study, the designation of type strains and the number of selected clinical strains with caries score

Species	Number of tested strains	*Lactobacillus* strains			
		Type strains	Number of clinical strains from children with		
			caries free	dt 2 to7	dt >1
L. casei / paracasei	9	*L. casei* ATCC 393, *L. paracasei* CCUG 32212	2	2	3
L. fermentum	4	*L. fermentum* ATCC 14931	1		2
L. gasseri	3	*L. gasseri* ATCC 33323		1	1
L. mucosae	5	*L. mucosae* CCUG 43179		1	3
L. oris	4	*L. oris* CCUG 37396		1	2
L. plantarum	4	*L. plantarum* ATCC 14917		1	2
L. rhamnosus	4	*L. rhamnosus* ATCC 7469	1	2	
L. salivarius	4	*L. salivarius* ATCC 11741		1	2
L. vaginalis	2	*L. vaginalis* CCUG 31452		1	

dt = Number of decayed teeth

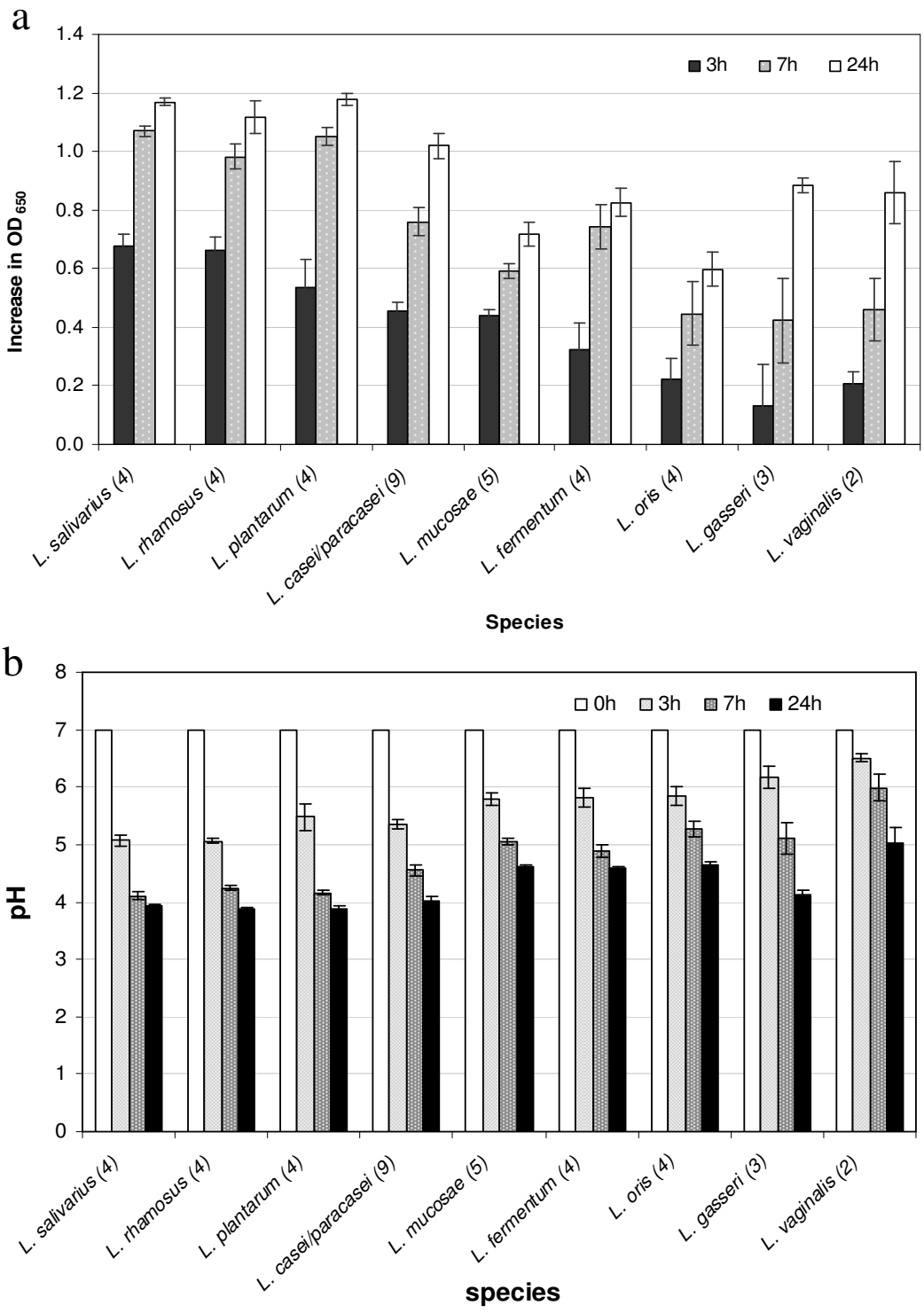

Fig. 1. Growth and acidification of *Lactobacillus* species after 3-, 7- and 24-h incubation in MRS broth containing 2% glucose. **a**) Bacterial growth shown as the increase of OD_{650} from the initial at OD_{650} = 1.0. **b**) Decrease of pH from initial (0 h) of pH 7. Number of strains tested is in parenthesis.

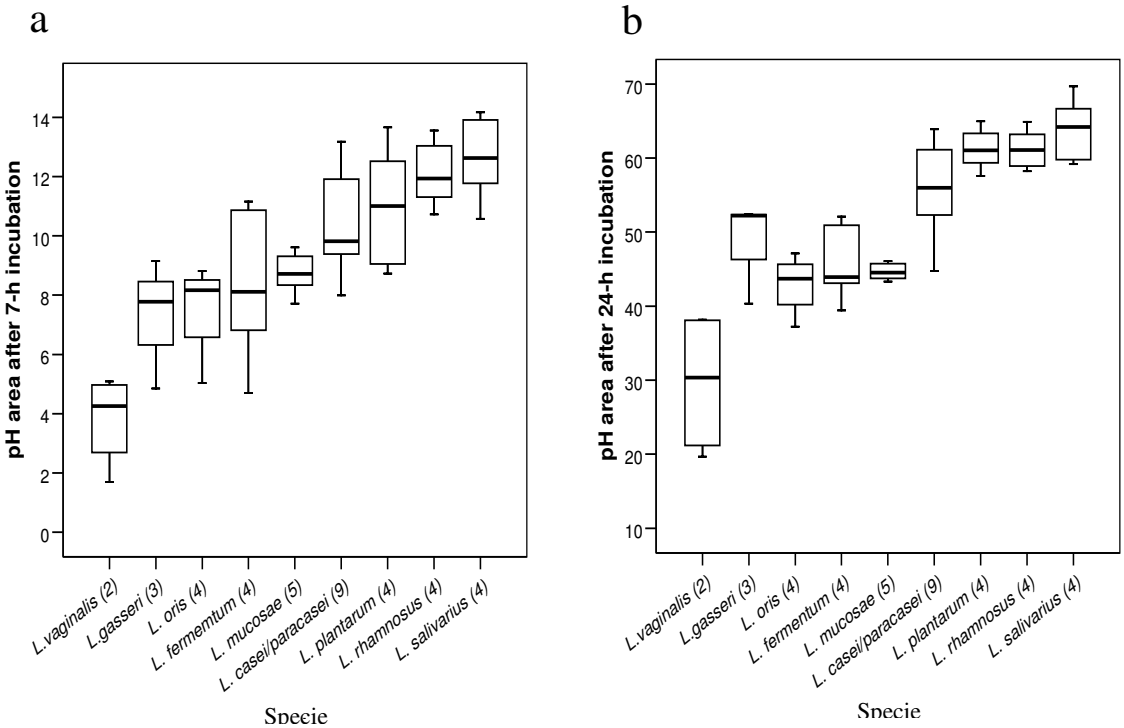

Fig. 2. Acidogenicity of *Lactobacillus* species expressed by the area bounded by the pH curve and a horizontal line of pH 7 (pH area) after 7-h (**a**) and 24-h (**b**) incubation period. Note the dissimilar y-axis and the number of strains tested is in parenthesis.

Table 2. Growth and acid production characteristics by *Lactobacillus* species

Lactobacillus species (n)	Growth characteristics		Acid production characteristics		
	Growth rate constant* (h^{-1}) ± S□	Max OD increase ± S□	Acid production rate# (OD^{-1} h^{-1}) ± S□	Time to pH 5.5 (h) ± S□	Final pH at 24-h ± S□
L. casei/paracasei (9)	.14 ± .1	1.2 ± .4	.41 ± .2	2.87 ± .18	4.2 ± .6
L. fermentum (4)	.12 ± .2	.82 ± .5	.34 ± .5	4.18 ± .47	4.58 ± .2
L. gasseri (3)	.1 ± .2	.88 ± .3	.27 ± .4	5.7 ± 1.4	4.13 ± .8
L. mucosae (5)	.15 ± .1	.72 ± .4	.4 ± .3	4.1 ± .27	4.62 ± .3
L. oris (4)	.8 ± .2	.6 ± .6	.37 ± .4	5.5 ± 1.35	4.65 ± .4
L. plantarum (4)	.18 ± .1	1.18 ± .2	.39 ± .5	2.98 ± .34	3.89 ± .3
L. rhamosus (4)	.19 ± .2	1.12 ± .5	.47 ± .2	2.27 ± .6	3.89 ± .2
L. salivarius (4)	.21 ± .1	1.17 ± .1	.42 ± .3	2.45 ± .12	3.92 ± .4
L. vaginalis (2)	.8 ± .1	.86 ± .11	.18 ± .2	16.39 ± 4.4	5.2 ± .28

* $(\ln OD_2 - \ln OD_1)/ t_2 - t_1$

δpH / OD/ h

Appendix C: Paper III

Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children

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Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children

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Objective: To study the acquisition of mutans streptococci (MS) and lactobacilli in relation to dental caries development in 3-24 month old Thai children. **Methods:** Salivary samples were collected from 169 children using sterile wooden tongue depressors at the ages of 3, 9, 12, 18 and 24 months. The blades were pressed onto selective media for MS or lactobacilli. After incubation, the colony forming units of MS/lactobacilli were counted. Dental status was recorded from 9 months old using modified WHO criteria. **Results:** The number of children with caries and number and severity of decayed teeth significantly increased with age. The presence of MS/lactobacilli was detectable at an early age and the cumulative prevalence of MS/lactobacilli increased with age. Children who were colonised early by MS or lactobacilli showed a higher number of decayed teeth than of those who were colonised later. The children with no MS/lactobacilli had significantly fewer decayed teeth and there was a significant correlation between MS/lactobacilli level and tooth decay. **Conclusions:** This longitudinal study shows early colonisation of the mouths of Thai children by MS/lactobacilli and where there are persistently high levels of the bacterias increased risk of development of dental caries.

Key words: Mutans streptococci/lactobacilli, dental caries, young children

Mutans strepcocci (MS) are considered to be causative agents of dental caries[1,2]. The degree of colonisation by these organisms correlates with the prevalence of dental caries in children and experimental animals[3,4]. Moreover, MS are found to be more closely associated with the initiation of caries than any other organisms[1,5].

Unlike MS, lactobacilli have been claimed to be related to the progression of caries and also to be an indicator of the content of fermentable carbohydrates in the diet[6]. Positive correlations have been shown to exist between lactobacilli counts and caries prevalence and activity[7-9].

Based on such findings, MS and lactobacilli have been recommended and used as predictors of caries in an attempt to identify individuals with a high risk of acquiring the disease. The time of MS/lactobacilli acquisition may also be important since caries risk is found to be dependent on early colonisation of organisms[10]. Some studies have found MS in children at or before their first birthday[11-13], while others report later acquisition[14,15]. However, similar information on lactobacilli is sparse.

Thailand has a high and an increasing prevalence of dental caries among young children; however, there are relatively few data on the time of acquisition of MS/lactobacilli. Therefore, the objective for the present study was to determine the basal MS/lactobacilli carriage among Thai children age 3-24 months, and the

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relationship of time of acquisition and level of colonisation to dental caries development.

Material and Methods

Subjects

The present study was a longitudinal, observational, community-based study. It was designed in association with the national Prospective Cohort Study of Thai Children (PCTC), which aims to follow infants from birth to 24 years of age. The study site, Thepa district, Songkhla province has a population of 66,990. Of its seven sub-districts, six could be categorised as rural, and the other sub-district is urban, although 90% of the population live in the rural areas. A total of 1,076 children born between November 2000 and October 2001 were selected as the study population. For dental examination, 795 children were recruited by cluster sampling, which covered all seven sub-districts and among these, 25% (198 children) were randomly selected for bacterial study. Details of the study site and the recruitment of the study sample have been published elsewhere[16]. This study was approved by the National Ethical Committee, at the Ministry of Public Health. All eligible guardians of the infants in the study area were invited to join the PCTC and provide their consent for the infants to participate in a series of assessments including the oral health study.

Oral examination

The dental examination started at the age of 9 months with further examinations at 12, 18, and 24 months. The clinical examination of dental status was conducted by five dentists using standardised methods at health centres or hospital depending on convenience for the subjects' carers. The range of the Cohen's Kappa coefficients of intra-examiner calibration ranged from 0.75 and 0.91 and the inter-examiners' coefficients ranged from 0.68 to 0.89. The clinical examinations were carried out using a WHO probe (#621) under natural light, and a scoring system adapted from the WHO's criteria, 1997. All teeth present were examined for dental status using this coding system as follows:

d_1 = initial decay/decay limited in enamel; the lesion demonstrates whitish/yellowish opaque with/without micro-cavity but no softened floor/wall

d_2 = decay to dentine; cavitated lesion is seen to extend beyond enamel that certainly catches the probe with softened floor/wall of determined enamel

d_3 = decay involving pulp; a deep lesion with probable pulpal involvement or deep lesion with present/history of spontaneous pain/fistula opening.

Bacteriological examination

Salivary samples from each child were collected by inserting a sterile wooden tongue depressor into the oral cavity until the blade was visibly moistened. The blade was then pressed immediately onto contact petri dishes that contained Mitis Salivarius Bacitracin agar[17] and Rogosa agar[9], which are selective media for MS and lactobacilli, respectively. The plates were incubated in an anaerobic jar at 37°C for 72h, after which the number of colony forming units (CFU) of MS and lactobacilli within the impression were determined based on their typical morphological appearance under a microscope[9,17].

Data analysis

The data of dental status is described as prevalence of dental caries (number and percentage of children with decayed teeth, mean and sd of decayed teeth), number of erupted teeth and severity of decay (d_1, d_2 and d_3). The prevalence of bacterial presence is presented as cumulative prevalence according to the age of the children at examination. The count of MS and lactobacilli was used as the main independent variable. The presence of 1CFU/1.5cm^2 was taken as positive presence of bacteria. The number of MS and lactobacilli were categorised as: 0CFU/1.5cm^2, 1-50CFU/1.5cm^2, and >50CFU/1.5cm^2. The mean age of first detectable acquisition of each organism was used as a cut-off point to dichotomise the children as either early or late colonisation. The Mann-Whitney-U test was used to determine the association of the mean number of decayed teeth with early or late bacterial colonisation. All related variables were further used for logistic regression analysis, the odds ratios and their 95% confidence interval were calculated. During the logistic regression analysis the dependent variables, number of decayed surfaces, was dichotomised at different cut-off points according to the prevalence of decayed surfaces and age. At the age of 9 months the presence of $\geq$ 1 decayed surface was counted as belonging to the decayed group while decay-free children were set as the reference group. At the age of 12 months the decayed group was defined as children with $\geq$ 2 decayed surfaces, while at 18 and 24 months of age, $\geq$ 4 decayed surfaces defined the decayed group. The low decay-group, children with < 2 decayed surfaces at the age of 12 months and children with < 4 decayed surfaces at the age of 18 and 24 months, was set as a reference group. A critical level of significance of all analyses was set at $p<0.05$. The analyses were carried out using SPSS statistical software (SPSS, release 10.0 for Windows)

Results

Of 198 recruited children, 29 (14.6%) failed to attend because either the family moved out of the study area or because the children were uncooperative. Of the 169 children who participated in the bacterial study, 52 children completed 5 salivary examinations whereas 62, 36, 12 and 7 children had 4, 3, 2, and 1 salivary examinations, respectively.

The prevalence of decayed teeth at different age intervals is shown in *Table 1*. The number of children with caries and the number and severity of decayed teeth significantly increased with age.

The cumulative prevalence of MS/lactobacilli detection is shown in *Figure 1*. By the age of 24 months, 86.98% and 66.86% of children harboured MS and lactobacilli, respectively. The temporal pattern of colonisation by MS differed from that of the lactobacilli. The lactobacilli were found more frequently during the first year of age than MS. The frequency of detection of lactobacilli was higher than MS from base line to 9 months of age but MS was detected with a higher frequency between 18-24 months.

Of the 169 children studied, MS was detected in 147 and lactobacilli in 113. The mean ages of first detection were 16.74±6.71 months and 13.88±8.43 months

Table 1 Dental caries status of children at different ages

Age in months	Mean number of erupted teeth	Number (%) of children		Mean ± SD of decayed teeth (surfaces):			
		without decayed teeth	with decayed teeth	Total	d_1	d_2	d_3
9	2.55±2.24	136 (95.8)	6 (4.2)	0.14±0.70 (0.20±1.05)	0.13±0.64 (0.18±0.98)	0.01±0.17 (0.01±0.17)	0.00±0.00 (0.00±0.00)
12	5.99±2.70	94 (70.1)	40 (29.9)	1.05±1.84 (1.57±3.38)	0.90±1.54 (1.26±2.32)	0.15±0.64 (0.31±1.71)	0.00±0.00 (0.00±0.00)
18	10.56±3.66	55 (36.9)	94 (63.1)	2.81±3.09 (5.34±7.16)	1.89±2.16 (3.36±4.24)	0.87±1.68 (1.81±4.17)	0.05±0.29 (0.17±1.06)
24	16.80±2.14	24 (15.5)	131 (84.5)	5.35±4.34 (10.89±10.42)	2.65±2.37 (4.35±4.19)	2.43±2.67 (5.52±6.73)	0.26±0.91 (1.01±3.50)

d_1 = initial decay/decay limited in enamel
d_2 = decay to dentine
d_3 = decay involving pulp

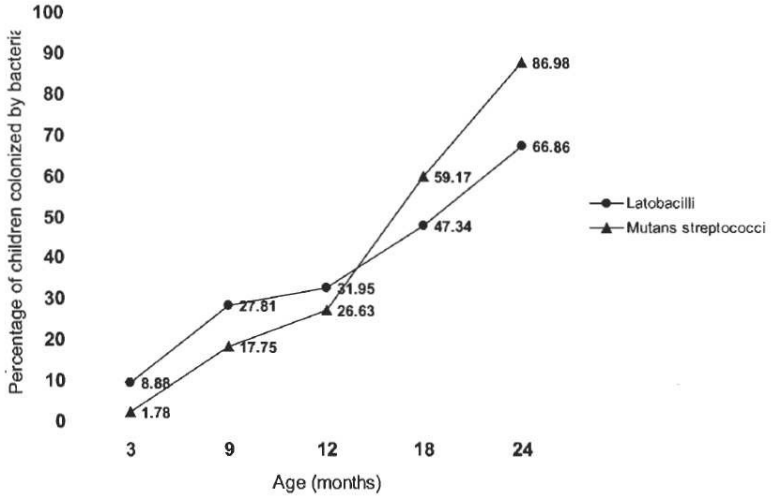

Figure 1. Cumulative prevalence of MS/lactobacilli at different ages

for MS and lactobacilli, respectively. Children who had detectable MS/lactobacilli before the population mean age of when MS/lactobacilli were detected had a higher number of decayed teeth at all ages, which was statistically significant by 24-month of age (*Table 2*). Moreover, children who harboured both MS and lactobacilli tended to have higher numbers of decayed teeth, while children who had none or only one of the two had a significantly lower number of decayed teeth at age of 12, 18, 24 months (*Table 3*).

When the main variables were analysed at once (*Table 4*), MS was found to be the factor significantly associated with caries at ages of 12, 18, 24 months. The odds ratios of developing caries at these ages when having MS > 50CFU/1.5cm^2 were 13.0, 8.8 and 7.5, respectively. The lactobacilli count was associated with the number of decayed teeth only at the age of 24 months and children who harboured lactobacilli counts of 1-50 CFU/1.5 cm^2 and of > 50 CFU/1.5 cm^2 had significantly higher risk of developing caries (odds ratios of 3.1 and 13.3, respectively).

Discussion

Longitudinal data on the development of the oral microflora in young children are still scarce. This study based on a community cohort focuses on the time for MS and lactobacilli colonisation in relation to dental caries development in 3-24 month old Thai children. Dental caries related to socio-economic factors in the same group of children has been reported separately[16].

In this study the rate of caries development was extremely high in comparison to other studies performed with 24-month old children. In our study's caries was detected soon after first tooth eruption at 8 months of age and more decayed teeth were found the older the children became. For example, 4.2% of 9 month old children had a mean total caries score of 0.14±0.7, whereas 84.5% of children at 24 months old had a mean total caries score of 5.35±4.34 and most of these were in categories of d1 and d2. In the study of Roeters *et al.*[18] only 5.9% of children had the mean dmfs of 0.14 ± 0.72, and in another study 26.3% of Japanese children showed a mean dmft of 3.19 ± 0.77 [19].

The number of children with detectable MS entering this investigation was also higher than previous studies[18,19]. However, the result of the present study is consistent with earlier reports which have indicated the prevalence of MS colonisation increases with age and number of teeth[11,15,19-21]. Most studies have reported that MS first appears in the mouth at the time of tooth eruption, explained by the assumption that MS require non-shedding surfaces for colonisation[22,23]. However, our findings would suggest that MS present prior to tooth eruption in 4% of Thai children. This is in keeping with other studies that have noted the presence of MS in predentate infants, although on small numbers of children[24-26]. This could be explained by the find-

Table 2 Decayed teeth among early- and late- detection of MS/lactobacilli at different ages

Age in months	Mean ±SD of decayed surfaces of children (number) whom found MS			Mean ±SD of decayed surfaces of children (number) whom found lactobacilli		
	≤ 16 m	> 16 m	p-value	≤ 13 m	> 13 m	p-value
9	0.31±1.28 (52)	0.15±0.94 (81)	0.408	0.38±1.48 (52)	0.10±0.70 (81)	0.198
12	2.00±3.19 (49)	1.26±3.57 (74)	0.241	2.21±4.71 (43)	1.17±2.43 (82)	0.181
18	6.53±8.67 (47)	4.63±6.19 (89)	0.186	6.87±9.44 (47)	4.38±5.50 (90)	0.100
24	13.90±12.85 (48)	9.24±8.38 (91)	0.026	13.08±12.36 (50)	9.47±8.86 (89)	0.048

Table 3 Mean of decayed teeth and presence of MS or lactobacilli at different ages

Age in months	Mean ±SD of decayed surfaces and children (number) who presented of			
	MS only	lactobacilli only	both MS/lactobacilli	no MS/lactobacilli
9	0.13±0.50 (16)	0.33±1.41 (18)	0.00±0.00 (6)	0.05±0.45 (78)
12	2.50±4.20 (14)	1.71±3.73 (7)	2.33±2.07 (6)	0.75±7.95[a] (51)
18	6.00±2.49 (48)	4.14±5.49 (7)	9.50±3.91 (28)	2.51±4.16[b] (49)
24	9.05±8.89 (65)	9.10±0.50 (10)	16.41±10.99[d] (56)	3.05±4.67[c] (10)

a, statistically significant difference of no MS/lactobacilli group to MS only group at 12 months
b, statistically significant difference of no MS/lactobacilli group to both MS/lactobacilli and to MS only groups at 18 months
c, statistically significant difference of no MS/lactobacilli to both MS/lactobacilli, to MS only and to lactobacilli only groups at 24 months
d, statistically significant difference of both MS/lactobacilli to MS only and to lactobacilli only groups at 24 months

Table 4 Logistic regression analyses, odds ratios (95%CI), on risk of getting decay* by MS count, lactobacilli count and the age of bacterial colonisation

Age in months	Variable		n	β	Odds ratios (95%CI)
9	Lactobacilli	0	86		
	(CFU/1.5cm²)	1-50	26	0.87	2.39 (0.20-28.41)
		> 50	5	-6.42	0.002 (0.00-9.9E+49)
	MS	0	93		
	(CFU/1.5cm²)	1-50	18	0.39	1.49 (0.13-17491)
		> 50	7	-6.36	0.002 (0.00-5.9E+59)
12	Lactobacilli	0	58		
	(CFU/1.5cm²)	1-50	7	0.65	1.92 (0.41 - 8.94)
		> 50	13	7.67	2148.16 (0.00 - 3.44E+46)
	MS	0	63		
	(CFU/1.5cm²)	1-50	12	-13.92	0.00 (0.00 - 1.37E+56)
		> 50	78	2.57	13.01 (2.89 - 58.52)**
18	Colonisation age of lactobacilli	early (≤ 13 months)	47		
		late (> 13 months)	90	0.25	1.28 (0.52 - 3.14)
	Colonisation age of MS	early (≤ 16 months)	47		
		late (> 16 months)	89	0.29	1.34 (0.54 - 3.32)
	Lactobacilli	0	55		
	(CFU/1.5cm²)	1-50	27	0.49	1.63 (0.53 - 5.03)
		> 50	49	0.75	2.12 (0.49 - 9.19)
	MS	0	87		
	(CFU/1.5cm²)	1-50	31	0.15	1.16 (0.41 - 3.25)
		> 50	14	0.22	8.79 (3.1 - 24.77)**
24	Colonisation age of lactobacilli	early (≤ 13 months)	50		
		late (> 13 months)	89	-3.69	0.69 (0.25 - 1.91)
	Colonisation age of MS	early (≤ 16 months)	48		
		late (> 16 months)	91	-0.75	0.93 (0.37 - 2.34)
	Lactobacilli	0	25		
	(CFU/1.5cm²)	1-50	20	1.13	3.09 (1.27 - 7.54)**
		> 50	101	2.59	13.29 (1.53 - 115.31)**
	MS	0	73		
	(CFU/1.5cm²)	1-50	53	0.07	1.070 (0.28 - 4.12)
		> 50	24	2.01	7.46 (2.54 – 21.89)**

*, The cut-off points to dichotomise the dependent variable for age 9, 12, 18 and 24 months was 1, 2, 4 and 4 decayed surfaces, respectively (see text).
** $p<0.05$

ing that *Streptococcus mutans* has the ability to adhere to keratinised and non-keratinised human oral epithelial cells[27]. Therefore, the colonisation of MS can occur before tooth eruption.

Unlike studies on MS, cohort studies on the presence of lactobacilli in young children are sparse. Our study appears be the first to determine the presence of lactobacilli and the caries prevalence pattern in children in the age range 3-24 months, since previous studies have been restricted to children of 2 years or older[18,28]. It is generally quoted that lactobacilli are late colonisers of the mouth[14,29,30]. Lactobacilli are assumed to be preferentially recovered from carious lesions, although usually after those lesions were colonised by the MS[31,32]. Some data has suggested that lactobacilli colonisation may be favoured by prior colonisation by the MS. Indeed, lactobacilli seem to be rarely found alone and are often isolated together with MS from plaque and saliva[29]. In contrast, the present study showed that lactobacilli were found in up to 7.2% of the children at 3 months of age and 40% of those children had lactobacilli at a high level (>100 CFU, data not shown). Therefore, early acquisition of lactobacilli is possible. However, differences between reports of lactobacilli acquisition in infants may reflect true differences between the populations being studied or may reflect differences in sampling methods.

The early detection of lactobacilli in this study may be explained by the finding that maternal microflora may be transferred to the infant during childbirth and then subsequently survive in the oral cavity[33]. Those authors found that the oral cavity in 40% of the infants was colonised by *Lactobacillus* spp. already at birth. The number of the colonised infants declined to 2% during first two weeks, however, the number increased to 10% one month later. In another study using the genotyping method[34], it was reported that one-quarter of infants acquired vaginal lactobacilli from their mother at birth, but that the acquired lactobacilli do not remain for long and are replaced by other genotypes from milk or unknown sources after birth. Our findings are in agreement with those results, and showed that 7.2% of children harboured lactobacilli at an early age. An increasing rate of lactobacilli in a second period (18 to 24 months) may derive either from environmental sources or relate to caries progression.

It is not clear whether the bacterial species detected are part of the indigenous flora at any point in time or whether they are transient strains. It has been generally assumed that the persistence of bacterial strains in the mouth is associated with their capacity not only to adhere and grow, but also to withstand a range of stresses, such as the presence of the other competing bacteria and inhibiting substances. A certain strain can be more adapted, once it can cope with physical, chemical, biological and environmental challenges, to dominate and to become established permanently, while other strains are not suited to the host and thus form a transient population. In the past, the method of identifying strains of lactobacilli to species level was limited; therefore, little information is available concerning which species of lactobacilli dominate in the oral cavity. We have recently proposed a PCR method of 16S rRNA and SDS-PAGE to identify oral lactobacilli[35]. Further studies of species and genotyping of lactobacilli will allow us to understand the persistence and colonisation in early childhood.

Many reports have shown that dental caries is strongly associated with the number of salivary MS and lactobacilli[3,7-9,18,19], and suggested that the earlier the colonisation of *S. mutans*, the higher the caries risk[10]. The present findings strongly support this but extend the findings to include lactobacilli. Children who developed caries tended to acquire MS/lactobacilli at a high level and/or at an earlier age than those who had a low caries experience. Thus, caries risk was associated with the age and level of acquisition of MS/lactobacilli. Furthermore, our study has confirmed that the number of MS is the primary risk factor for caries initiation, while lactobacilli were more significant as carious lesions progress. It was found that MS was significantly associated with clinically detectable caries from 12 months old; whereas, lactobacilli were only of significance by the age of 24 months (*Table 4*). Of course, by at the age of 24 months more children had decayed teeth developed to involve dentine (*Table 1*). However, the effect of early- or late-bacterial colonisation was eliminated when the bacterial count was included into the model (*Table 4*). This showed that the bacterial count was a more important contributor to caries than the age of colonisation. Thus, to reduce caries risk, measures to control the bacterial number would be more effective than measures to delay the time of colonisation.

In conclusion, data from our longitudinal study have shown that the presence of MS/lactobacilli among Thai children was detectable at an early of age and that there was a positive association between salivary MS/lactobacilli and the prevalence and severity of dental caries. Acquisition of MS/lactobacilli at high level is a significant risk factor for caries development; and so appropriate preventive programmes are urgently needed for such high risk children.

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Appendix D: Paper IV

A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants

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COMMUNITY DENTISTRY AND ORAL EPIDEMIOLOGY

A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants

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Abstract – *Objective:* To examine the rate and pattern of early childhood caries (ECC) development and to investigate the transitional changes of the carious lesions during a follow-up period of 3–9 months. *Methods:* A longitudinal observational community-based survey of 599 children, 9–18 months old. The children's dental examinations were first carried out at the age of 9 months with re-examination at 12 and 18 months by five dentists using standardized methods. The affected rates of dental caries were determined for prevalence, incidence density for risk of caries per person (ID_p) and risk by surface (ID_s). Changes in dental status over time were explored from unerupted (U) to sound (S), including enamel caries (D1), dentine caries (D2) and caries involving pulp (D3) by computing transitional probabilities. *Results:* The prevalence of caries was 2.0%, 22.8% and 68.1% among 9-, 12- and 18-month olds, respectively. The ID_p observed for newly affected children 9–12 and 12–18 months old was 10.32 and 15.70 persons/100 person-months, respectively. The ID_s for children 9–12 months old was 2.17 newly affected surfaces/100 surface-months whereas it was 2.22 surfaces/100 surface-months for children 12–18 months old. The buccal surface of maxillary incisors was the most affected (44.9%) followed by lingual, mesial and distal surfaces, respectively. The transitional probability of caries progression ranged between 1.79% and 15.38% during the follow-up period from 9 to 12 months old. It was 3.43–39.60% from 12 to 18 months old. *Conclusions:* An extremely high caries-affected rate was found among the study children even before the age of 18 months. The buccal surface of the maxillary incisors was the most affected. The teeth acquired caries at 3–6 months after initial eruption and carious lesions developed continuously over time.

Key words: early childhood caries; epidemiology; incidence density; prevalence; Thailand; transitional probability

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It is widely accepted that deciduous teeth are important, as they can influence the growth and development of children (1, 2). The most obvious oral disease among young children is dental caries, now known as early childhood caries (ECC) (3–5). The etiology of ECC is known to be multi-factorial (6–8). In developed countries, ECC is most prevalent among infants from deprived groups (9–11). The ECC development in children under 3 years of age could lead to long-term caries risk (12, 13). Among children with a high prevalence of ECC, especially in developing countries, ECC mostly develops early in life (14–16). Most of the previous studies of ECC development have been cross-sectional in design and restricted to a single observation of subjects to determine caries prevalence. Although they contained some descriptions of the incremental rate of caries development, the prevalence was calculated at different chronological ages, and the time-period of observation of each individual tooth was not included in the analyses. A longitudinal study is better able to follow the changes of dental caries progression, which addresses clearly understanding the rate of caries

progression of each individual tooth and the probability of transitional changes over time in order to find an appropriate access-time for ECC prevention. The aims of the present study were to examine the rate and pattern of ECC development and to investigate the transitional changes of the carious lesions during a follow-up period from 9 to 18 months of age.

Materials and methods

The study was a longitudinal observational community-based study. It was designed in association with the national child health research project, Prospective Cohort Study of Thai Children (PCTC). The PCTC intends to follow infants from birth to 24 years of age, and study changes in their physical and behavioral development. Among the five study regions participating in the PCTC, the present study was confined to a southern cohort, which is located in Thepa district of Songkhla province, Thailand.

Population and sample

The study site in Thepa district, Songkhla province is located about 1000 km south of Bangkok. It has a population of 66 990. Of its seven sub-districts, six could be categorized as rural. The other sub-district is urban. Ninety percent of the population lives in the rural areas. They are predominantly Muslims (68%), 32% are Buddhists. The average annual household income is 68 931 Baht (1681 US$). Forty-five percent of the population is employed in rubber plantations, rice growing and fishing. The remaining, 20% are employees of non-agricultural businesses, 19% are farmers working on their own land and 16% are merchants and government officers. The fluoride concentration of drinking water in this area is low, ranging from 0.1 to 0.2 ppm (17). Each sub-district is served by one to two of the total 11 health centers and a district hospital. Dental care is only available at the district hospital; all dental services are provided by two general dental practitioners and two dental nurses. A total of 1076 children born between November 2000 and October 2001 and registered at one of these health centers or at the district hospital were set as the study population. During the sampling process, seven health centers and the hospital were selected to cover all of the seven sub-districts. The 795 infants registered at these faculties were recruited. This study has been approved by the National Ethical Committee, at the Ministry of Public Health. All eligible guardians of the infants in the study area were invited to join the PCTC and provide their consent for the infants to participate in a series of assessments including the oral health study.

Clinical examination

Dental examination appointments were made for all eligible subjects at the ages of 9, 12, and 18 months old. A second appointment was arranged within 1 month if the first appointment was missed. As a result the children were examined at a ±1-month interval around the intended examination age. The examination took place at the health centers, the hospital or in the village depending on convenience for the subject's caretakers. The subjects were examined in a knee to knee position. The teeth were examined with a WHO probe (no. 621) under natural light using a scoring system adapted from the WHO's criteria, 1997. The dental status of each examined surface was categorized as:

U = Unerupted surface; no part of the surface emerges to the oral cavity.

S = Normal enamel surface/texture and no restoration.

D1 = Initial caries/caries limited in enamel; the lesion demonstrates whitish/yellowish opaque with/without micro-cavity but no softened floor/wall.

D2 = Caries to dentine; cavitated lesion is seen to extend beyond enamel that certainly catches the probe with softened floor/wall of undermined enamel.

D3 = Caries involving pulp; a deep lesion with probable pulpal involvement or deep lesion with present/history of spontaneous pain/swelling/fistula opening.

The dental status data were collected by five dentists using standardized methods. Prior to the data collection, all examiners and recorders participated in a meeting to discuss the process of data collection and to study the dental examination criteria. Later a practical standardization was carried out at the university daycare center. The standardization was performed at tooth surface level. The range of the Cohen's Kappa coefficients of overall intra-examiner standardization ranged from 0.75 and 0.91 and the overall inter-examiners coefficients ranged from 0.68 to 0.89. The Kappa coefficients of intra-examiner standardization, only in surfaces with S and D1, ranged from 0.66 to 0.79 whereas the inter-examiner coefficients fell

between 0.49 and 0.78. The level of reliability was maintained over the study period.

Statistical analysis

The data were checked for accuracy and entered in the computer using the SPSS® statistical program (SPSS Inc., Chicago, IL, USA). Frequency counts and cross-tabulations of the data were used to check for errors in data entry. Analysis first resulted in the reporting of descriptive statistics. The rates of dental caries were presented in prevalence, incidence density and transitional probability of carious lesions from one stage to another. The prevalence was the number of persons with carious teeth divided by the population at a specified time. The incidence was the number of new caries-affected teeth in a defined population, within a specified period of time. Incidence in the present study was calculated as incidence density for risk of caries of a person (ID_p) and of a tooth surface (ID_s) summarized by the formula below:

$$ID_p = \frac{\text{Number of new caries-affected subjects}}{\text{Total person} - \text{time at risk for having at least one caries lesion (person} - \text{month)}}$$

$$ID_s = \frac{\text{Number of new carious surfaces}}{\text{Total surface-time at risk (surface-month)}}$$

Person-time in ID_p and surface-time in ID_s was calculated by summing the observation-time of individuals/surfaces having no caries. For simplicity, we assumed that the teeth had a uniform hazard rate within each follow-up interval. For teeth that were present as sound at t_0 and t_1, the time at risk was $= t_1 - t_0$. For teeth which were absent at t_0, the time of eruption was assumed to be $(t_1 - t_0)/2$. For teeth that were present as sound at t_0 and became carious teeth at t_1, the onset of caries was assumed to take place at $(t_1 - t_0)/2$. Similarly if the teeth were absent at t_0 and became carious teeth at t_1, the eruption time was calculated to be at $(t_1 - t_0)/2$ whereas the onset of caries was assumed to be at $t_0 + 3/4(t_1 - t_0)$. In other words, the caries-free duration of these teeth was one-fourth of the interval. When a carious lesion was detected that person/surface was considered to be a new case for the period. It was then excluded from the at-risk status.

In addition to the ID analysis, we employed transitional probabilities computation dividing each tooth into five stages as unerupted tooth (U), sound tooth (S), enamel caries (D1), dentine caries (D2) and caries involving pulp (D3) in a series from U > S > D1 > D2 > D3. In this analysis, all the durations were ignored and the calculation was carried out for the whole mouth and broken down by tooth type, that is, incisors, canines and molars. The distribution of carious surfaces is presented in bar charts.

Results

Of the 795 recruited subjects, 196 (24.7%) were absentees. This was due to the unwillingness of their parents to participate in the study, inconvenience, the family moved out of the study area or the uncooperativeness of the children. Out of 599 attending the dental examination, 42 (7.0%) had only one examination; therefore this data was used only for the prevalence analysis. The remaining 557 (93.0%) were used for incidence density analysis. For transitional analysis only, 406 (67.8%) subjects who completed all follow ups were used (Table 1).

The prevalence of caries, which included all D1, D2, and D3 was 2.0%, 22.8%, and 68.1% among 9, 12 and 18 month olds, respectively. The average number of teeth in children of 9, 12 and 18 months was 2.2 ± 2.1, 5.5 ± 2.6 and 10.4 ± 3.6 teeth/child, respectively. The number of caries-affected teeth was 0.05 ± 0.39, 0.73 ± 1.6, and 2.82 ± 2.69 teeth/child, respectively. The incidence of caries, D1, D2 and D3, affected persons (ID_p) observed from the age of 9 to 12 months was 10.32 persons/100 person-months of observation whereas it was 15.70 person/100 person-months from the period of 12 to 18 months. The rate of new caries-affected tooth surfaces (ID_s) in the period from 9 to 12 months was 2.17 surfaces/100 surface-months of observation, whereas the ID_s in the period from 12 to 18 months was 2.22 surfaces/100 surface-months (Table 2).

Figure 1 shows the percentage of unerupted, sound and decayed lesions by tooth surfaces and tooth types. Decayed surfaces included all D1, D2 and D3. At the age of 9 months a few carious surfaces (0.3%) were observed on the buccal surface of upper incisors. At 12 months, 10% of the erupted surfaces were affected by caries. The majority of the lesions were located on the upper incisors, where 60% of the buccal surface and 30% of lingual surfaces were affected. By 18 months, 35.0% of the teeth and 14.9% of the surfaces were affected by caries. Buccal surfaces were the most affected surfaces (44.9%), followed by lingual

Table 1. Distribution of children by examination ages

Examination age (months)			Children attending examination		
			N	%	Data analysis
9	12	18	406	67.78	Incidence density, transitional probability of caries attacked and prevalence of dental caries
9	12		75	12.52	Incidence density and prevalence of dental caries
9		18	62	10.52	Prevalence of dental caries
9			23	3.84	Prevalence of dental caries
	12	18	14	2.34	Incidence density and prevalence of dental caries
	12		5	0.83	Prevalence of dental caries
		18	14	2.34	Incidence density and prevalence of dental caries
		Total	599	100.00	

Aggregate totals for 9, 12 and 18 months = 566, 500 and 496, respectively.

Table 2. Frequency, incidence and rates of risk of caries-affected children/surfaces

	Observation period (months)	
	9–12	12–18
N (persons)	463	396
No. of new caries-affected cases	105	249
Person-months of observation	1 017.25	1 585.50
ID_p/100 persons at risk	10.32	15.70
N (surfaces)	11 315.00	23 159.00
No. of new caries-affected surfaces	504.00	2 107.00
Surface-months of observation	23 271.00	94 774.50
ID_s/100 surfaces at risk	2.17	2.22

(24.2%), mesial (20.0%) and distal surfaces (8.9%). The only posterior teeth present were first molars, 10.7% of them were carious teeth. Occlusal surfaces were the most affected surfaces (50.7%), followed by buccal (39.6%), lingual (8.4%) and distal surfaces (1.3%).

Table 3 shows the transitory changes in dental status over the 9- to 12- and 12- to 18-month periods. The calculation was based on a total number of 20 deciduous teeth. The dental status of the majority of cases in both periods remained unchanged while the probability of one-step progression from 9 to 12 months ranged between 12% and 18% but doubled at second follow-up at 18 months. The major transitory change with a two-step progression was observed from S at 12 months to D2 at 18 months (8.38%). A three-step progression was rare.

The transitional probability calculation by tooth type shows that from 9 to 12 months, 69.8% and 44.2% of maxillary and mandibular incisors had erupted, whereas the percentage of erupted canines and molars of both jaws ranged between 0.9% and 2.6%. Among newly erupted maxillary incisors, 9.5% had changed from U to D1 whereas 0.6% had changed to D2. In respect of sound teeth (S) at 9 months, 68.0% were still sound teeth whereas 27.1% and 4.9% had changed to D1 and D2, respectively. Of D1 teeth at 9 months, 77.8% had no progression, whereas 22.2% had changed to D2. For mandibular incisors, 0.9% of unerupted teeth were later seen to be D1 whereas 3.1% with normal enamel surface developed to D1.

The transitory changes during the second follow-up period at 12–18 months were in line with those from 9 to 12 months (Table 4). The majority of transitional changes were observed among maxillary and mandibular incisors. One-step changes among incisors ranged from 24.1% to 78.1%. Maxillary incisors suffered the highest caries attack rate. One-step caries progressions, S to D1 and D1 to D2, were observed in approximately 40% of the cases. The probabilities of two-step progressions among maxillary incisors were also high; and the transitions from U to D1 and S to D2 were 28.0% and 15.8%, respectively.

Discussion

The results of this study on prevalence of dental caries correspond with the results of many previous epidemiological studies (14–16). However, detailed dental caries development measurements in particular deciduous teeth have previously been less available. The rate of caries in the study population was extremely high. The prevalence of ECC rose sharply from 2.0% at 9 months to 68.1% at 18 months. These results raise similar concerns from a previous epidemiological study in Thailand where the prevalence of ECC among children in Suphan Buri province was 83% (14). As previously stated, early eruption may pose a higher risk of

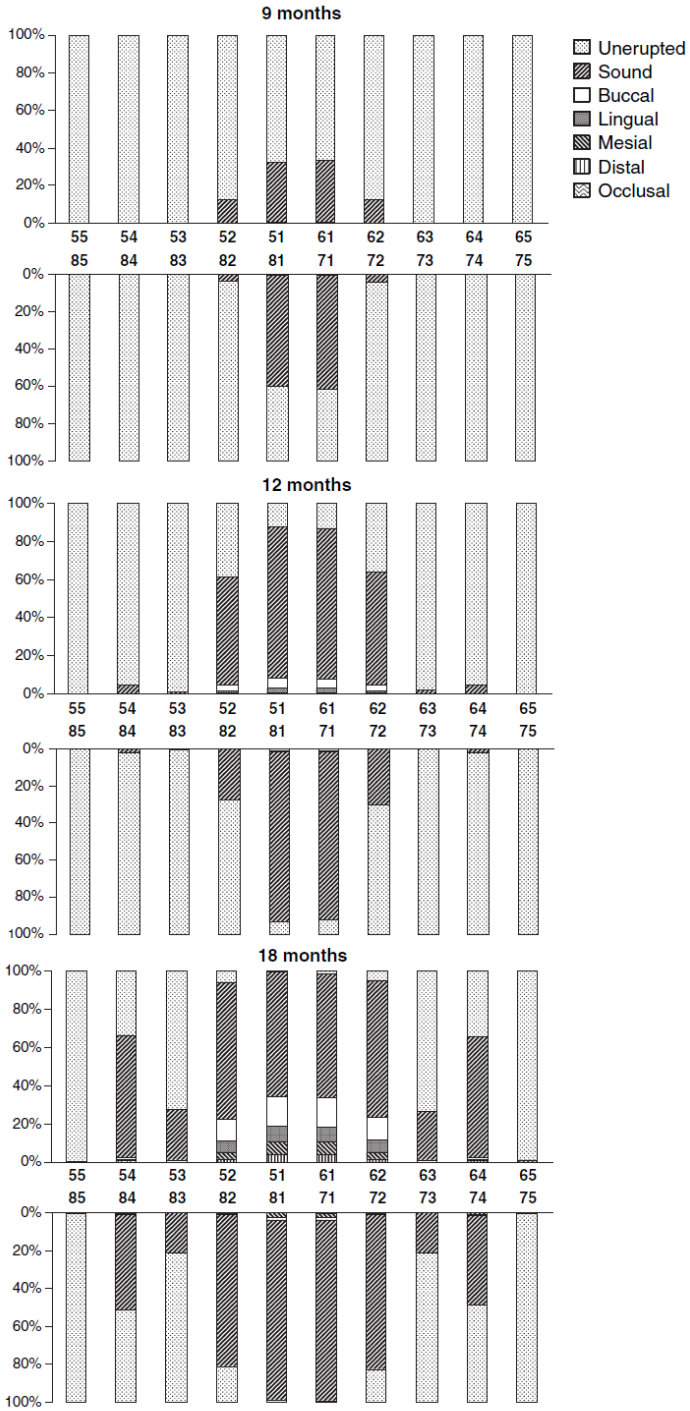

Fig. 1. The distribution of carious surfaces at the ages of 9, 12 and 18 months.

ECC (18). However, in this study population the average age of first tooth eruption was 8.53 months, whereas the average age of first tooth eruption among children in Bangkok is 7.5 months (19). Thus, tooth eruption did not occur early but the caries attacked rate was still very high.

Table 3. Transitional probability (%) of dental status of the whole mouth observed in the same children aged 9–12 and 12–18 months ($N = 406$)

Transitional status	Observation period (months)	
	9–12	12–18
U → U	79.96	60.39
U → S	18.13	35.51
U → D1	1.79	3.43
U → D2	0.11	0.67
U → D3	0.00	0.00
S → S	84.93	67.99
S → D1	12.86	23.62
S → D2	2.20	8.38
S → D3	0.00	0.00
D1 → D1	84.61	55.60
D1 → D2	15.38	39.60
D1 → D3	0.00	2.00
D2 → D2	100.00	77.42
D2 → D3	0.00	22.58
D3 → D3	0.00	0.00

U, unerupted; S, sound; D1, enamel caries; D2, dentine caries; D3, caries involving pulp.

Therefore, the higher risk is not explained by early eruption. By the age of 9 months an average of 2.2 teeth had already erupted; among these 0.05 teeth were affected by caries. The increasing number of caries-affected teeth was considerably higher than the increasing number of newly erupted teeth in the same period. This implies that the caries process starts as soon as the teeth emerge into the oral cavity. This is of significant interest and needs further investigation as a factor.

A cohort study is suitable for the calculation of an incidence rate and better characterizes the incremental rate of disease events. Incidence density was used in the study, as this more precisely estimates the rate of disease occurrences as it accounts for the varying times of follow up where the prevalence and incidence rates do not (20, 21). This is of importance as the length of follow up was not consistent for all participants. Some participants lost contact during the follow-up period, and some entered the study later than others. Moreover, the length of time between the two examinations was also different (3 versus 6 months) for the two periods.

The incidence density of caries-affected children sharply increased from 9 to 18 months. Among children unaffected by caries at 9 months, 22.7% were affected at the age of 12 months. Further, for those who were caries free at 12 months, 62.9% had acquired caries 6 months later at the 18-month follow up. This shows that susceptibility to caries in study children occurred in the first 3–6 months after the teeth had erupted into the oral cavity. To counteract the high caries rate among this high-risk population, a preventive program must be implemented early in life. To obtain the greatest benefit from the preventive program, it should commence at the time of tooth eruption or even before.

The analysis of carious surface distribution revealed that buccal surfaces of maxillary incisors were most affected. Lingual surfaces were next most affected, followed by mesial and distal surfaces. This confirmed the results of previous studies of caries manifestation of ECC (12, 13, 22). The high rate of caries attacks on buccal surfaces of maxillary incisors is known to be associated with both biological mechanisms and psychosocial behaviors (7, 8, 23). The mandibular teeth were less affected which is consistent with the findings of previous studies (14, 16).

Table 4. Transitional probability (%) categorized by tooth types observed in the same children aged 12–18 months ($N = 406$)

Transitional status	Maxillary teeth			Mandibular teeth		
	Incisors	Canines	Molars	Incisors	Canines	Molars
U → U	10.40	64.01	65.53	19.39	74.94	73.56
U → S	5.52	33.97	31.39	78.09	24.31	24.98
U → D1	28.00	1.90	2.50	1.65	0.76	1.32
U → D2	6.40	0.13	0.58	0.33	0.00	0.13
S → S	43.06	84.62	79.07	92.23	1.00	73.68
S → D1	41.12	15.38	16.28	6.63	0.00	15.79
S → D2	15.82	0.00	4.65	1.14	0.00	10.53
D1 → D1	52.73	0.00	0.00	76.67	0.00	0.00
D1 → D2	43.64	0.00	0.00	10.00	0.00	0.00
D1 → D3	2.27	0.00	0.00	0.00	0.00	0.00
D2 → D2	75.86	0.00	0.00	100.00	0.00	0.00
D2 → D3	24.14	0.00	0.00	0.00	0.00	0.00

U, unerupted; S, sound; D1, enamel caries; D2, dentine caries; D3, caries involving pulp.

Analysis of the transitional changes probability of caries susceptibility for the whole mouth shows that among study children there is a tendency for all teeth to demonstrate a rapid rise in caries susceptibility. These results demonstrate that new lesions develop not only as the child ages but more importantly, that the progression of caries lesions continues in the child's early years. Although the observation period of the subjects was limited to only 18 months of age, it is noteworthy to observe that during the 6-month follow up, a third of the enamel caries (D1) developed to be dentine caries (D2) and a fifth of dentine caries (D2) developed further to involve pulp tissue (D3). Based on this pattern, we would expect to see some of these D3 lesion teeth to be missing in the near future. As this study was carried out among children of 9–18 months, unerupted teeth accounted for the majority of the probability.

Maxillary teeth are more susceptible than mandibular teeth. Analysis by tooth type demonstrated that a rapid rise in caries susceptibility occurred in a rather short time as seen with maxillary incisors where around 9% of those unerupted teeth (U) at 9 months were enamel caries (D1) affected at 12 months. Although the nature of all caries development cannot be inferred from the present analysis, investigation is certainly needed to identify important associated factors in this rapid caries progression. A previous cross-sectional study among pre-school children in southern Thailand found that the high caries rate was related to oral hygiene, consumption of sweet milk, use of non-fluoride toothpaste, whether mothers or caretakers examined the child's teeth and socioeconomic status (24). Early colonization with mutans streptococci seems also to be an important risk factor as shown in a study by Bratthall et al. (25). It demonstrated that children in Bangkok who had oral streptococcus mutans colonization were more caries affected than remote hill tribe children who were mutans-free. The major route of the mutans streptococci transmitted to the children is from mothers (26, 27). Therefore, preventing cariogenic bacteria transmission from mothers to children would prevent ECC (28, 29).

An extremely high caries-affected rate was found among study children. The buccal surfaces of the maxillary incisors were the most affected. Generally, the teeth acquired caries at 3–6 months after initial eruption into the oral cavity and the carious lesions developed continuously over time. By the age of 18 months, a large proportion of children were affected by caries. Further investigation to identify risk factors of ECC development is needed.

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Appendix E: Additional information

Table 12. Distribution of *Lactobacillus* isolated from caries free children and children with dt 1-4

Species	Low-caries group (dt 1-4)		Caries free group		Children with dt 1-4	
	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)
L. fermentum	17 (85)	74 (59.7)	4 (8)	13 (48.2)	13 (86.7)	61 (62.9)
L. salivarius	1 (5)	2 (1.6)			1 (6.7)	2 (2.1)
L. casei/paracasei	5 (25)	14 (11.3)	1 (2)	3 (11.1)	4 (26.7)	11 (11.8)
L. mucosae						
L. rhamnosus	2 (1)	4 (3.2)	1 (2)	3 (11.1)	1 (6.7)	1 (1)
L. oris	3 (15)	6 (4.8)			3 (2)	6 (6.2)
L. gasseri	3 (15)	14 (11.3)			3 (2)	14 (14.4)
L. plantarum						
L. vaginalis	2 (1)	1 (8.1)	1 (2)	8 (29.6)	1 (6.7)	2 (2.1)
Total	2 (1)	124(1)	5 (1)	27 (1)	15 (1)	97 (1)

VITAE

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ducational Attainment

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Scholarship Awards during nrolment

1. The Royal Golden Jubilee Ph.D. Grant (RGJ) (grant No. PHD/ 91/2549). The Thailand Research Fund (TRF), Thailand, 2 6-2 9.
2. The National Research Council of Thailand, Thailand, 2551.
3. The Swedish Research Council (grant No. 348-2 7-665), Sweden, 2 7.

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List of Publication and Proceeding

1. Dahlen G, Konradsson K, riksson S, Teanpaisan R, Piwat S, Carlen A. A microbiological study in relation to the presence of caries and calculus. ***Acta Odontol Scand*** 2 ; 68(4):199-2 6.
2. Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlén G. Lactobacillus species and genotypes associated with dental caries in Thai preschool children. ***Mol Oral Microbiol*** 2 ; 25(2):157-64.
3. Kiatwateeratana T, Kintarak S, Piwat S, Chankanka O, Kamaolmatyakul S, Thearmontree A. Partial pulpotomy on caries-free teeth using enamel matrix derivative or calcium hydroxide: a randomized controlled trial. ***Int Endod J*** 2 9; 42(7):584-92.

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