

Gut Microbiota of Children Living in Rural South Thailand and Urban Singapore

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ABSTRACT

Background: An imbalanced prevalence of allergic diseases occurs in the region of South East Asia. It has been suggested that a change in lifestyle associated with improved hygiene and modernization has altered the composition of human gastrointestinal microbiota, and hence susceptibility to allergy.

Methods: This cross-sectional study was designed to investigate the differences between fecal microbiota in children living in areas with contrasting socioeconomic development. Fecal samples from 73 young children (age 3.0 ± 0.5) from rural Thailand and 69 age-matched children from urban Singapore were collected and studied using selective culture. Clinical data were also collected using modified ISAAC questionnaires, aiming to identify the key differences in the demographic as well as clinical features between the two study groups.

Results: The two contrasting populations studied differed significantly in multiple lifestyle factors such as family size, antibiotic use and sources of drinking water in the households. Rural children harbored significantly higher counts of lactic acid bacteria (LAB) [7.1 (6.4, 8.3) vs 6.0 (5.3, 7.0) logCFU/g, $p < 0.001$], coliforms [8.9 (7.3, 10.2) vs 6.9 (5.7, 7.7) logCFU/g, $p < 0.001$] as well as staphylococci [5.3 (4.8, 6.3) vs 4.3 (3.6, 5.0) logCFU/g, $p < 0.001$] than their urban counterparts. However, enterococcal counts did not differ between the two groups. No single lifestyle factor could be identified to have caused such differences.

Conclusions: Certain fecal microbial counts were higher in rural children compared with urban children in South East Asia. Several contrasting home environmental conditions and practices were also identified. These may serve as a basis for future investigation of lifestyle factors underlying the global gradient of the increasing trends of allergic diseases.

KEY WORDS

children, gut microbiota, hygiene hypothesis, rural and urban, South East Asia

INTRODUCTION

The hypothesis of 'microbial deprivation syndrome' suggests that modernization and urbanization over the last few decades have reduced human exposure to certain microbes.¹ This reduction of microbial exposure may serve as one of the root-causes contributing to the rising trends of allergic diseases in the developed world.

South East Asia is a region of unbalanced urban development. As a consequence, the prevalence of allergic diseases was found to be significantly higher in metropolitan areas such as Singapore or Bangkok, as

compared with their rural neighbors.²⁻⁴ These neighboring areas of contrasting allergy prevalence (rural *vs* urban) provide excellent research models for the identification of possible etiological factors underlying such gradient of disease prevalence.

Previous studies have demonstrated that allergic and healthy infants may differ in the counts of lactic acid bacteria (LAB), coliforms, enterococci and staphylococci.⁵⁻⁷ This study aimed to compare the counts of these bacterial groups in the fecal samples obtained from young children living in rural Thailand and urban Singapore. Since rural lifestyle may be associated with lower prevalence of allergy, and their

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Received 18 May 2007. Accepted for publication 14 August 2007.

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gut microbiota is believed to be one of the factors rendering them less prone to allergic manifestations, certain bacterial isolates from rural Thai children were identified. Furthermore, we attempted to identify lifestyle factor(s) that might potentially lead to such difference(s) in microbiota composition.

METHODS

STUDY DESIGN

The study groups comprised 73 young children (age 3.0 ± 0.5) from rural Thailand and 69 age-matched children from urban Singapore. Subjects from both centers were randomly selected from their respective population-based birth cohort studies. All the Thai children were recruited from southern Thailand, more specifically the Theppa district of Songkhla province. On the other hand, Singapore children were recruited from the National University Hospital. Parents of these children were administered a modified ISAAC⁸ questionnaire in English (Singapore) or Thai language (Thailand), accompanied with detailed explanation by either a doctor or a healthcare worker. Informed consent was obtained from all parents of subjects. The study was approved by the National University Hospital Institutional Review Board.

SAMPLE COLLECTION AND BACTERIOLOGICAL CULTURE

Fecal samples were collected into sterile plastic vials by parents, stored at -20°C and delivered to the laboratory within 20 hours. The samples were all kept cool on a dry-ice pack during transport and upon arrival to the laboratory immediately diluted with saline to make up a 0.1 g/ml concentration. After homogenizing, bacteriological enumeration was performed using the drop-plate technique by spotting 25 μl of sample in duplicate per dilution on the same plate. The bacterial counts and identification of the various genus were based on growth, color and number of colonies on selective media, Gram staining and cell morphology.⁹ Selective media used were de Man-Rogosa-Sharpe agar (MRS) (Oxoid) for LAB; Slanetz and Bartley agar (S&B) (Oxoid) for *Enterococcus spp*; McConkey purple agar (Oxoid) for *Coliforms* and Staphylococcus Medium no. 110 (Oxoid) for staphylococcus *spp*. The samples were serially diluted (10^{-1} – 10^{-8}) in saline in a bio-safety cabinet. The MRS agar plates were incubated at 37°C , 5% CO_2 for 48 hours, McConkey purple agar plates at 35°C aerobically, overnight, S&B agar plates at 35°C for 2 hours and then at 44°C for 44 hours and Staphylococcus no. 110 agar at 35°C aerobically for 48 hours.

An internal control comparison study was also conducted to compare bacterial counts by selective culture performed by lab staff from the two centers (Thai & Singapore). In addition, to check whether the experimental outcomes varied according to the facilities from the two centers, two Singapore staff

members also carried out a synchronized culture, one at the Singapore and the other at the Thai center, on the same fecal samples preserved in 30% glycerol. These counts were not significantly different from each other (data not shown).

ISOLATION AND IDENTIFICATION OF COLIFORM SPECIES

For study on the prevalence of coliform species in Thai children, at least one coliform colony on McConkey purple agar was picked from each Thai subject. Depending on their colony appearances on the McConkey purple plates, more colonies were picked from a subject if more than one type of colony morphology were observed. These isolates were confirmed with Gram negative staining and sub-cultured for species identification based on the following biochemical tests: triple sugar iron (TSI), lysine, citrate, urea, indole as well as motility.

TSI agar was used to identify the biochemical ability of the coliforms to ferment glucose, lactose or glucose based on color change. Its iron salts would also react with any H_2S produced by the growing organism to form a black precipitate. The lysine test was employed to determine any activity of deaminase and decarboxylase. The citrate test was used to determine the ability of a bacterium to utilize citrate as its only source of carbon. Bacteria can break the conjugate base salt of citrate into organic acids and carbon dioxide. The carbon dioxide can combine with the sodium from the conjugate base salt to form a basic compound, sodium carbonate. A pH indicator in the medium could detect the presence of this compound by turning blue (a positive test). Furthermore, the urea test was performed to detect ureanase and the indole test for tryptophanase. Bacterial motility was also determined.

STATISTICS

Aerobic counts were expressed in log CFU/g of wet fecal samples. The detection limit was 3.6. Since the bacterial counts were not normally distributed, the Mann-Whitney *U* test was employed for analysis. All counts were expressed as median (lower quartile, upper quartile). Univariate analysis was performed to determine the underlying environmental factors that may account for the differences in microbial counts (number of siblings, ever used antibiotics, ever ingested yogurt drink, drinking water source, rhinitis without flu, rash resembling eczema) in the Singapore group or Thailand group. Specifically, spearman's rho was calculated to assess the linear relationship between number of siblings and bacterial counts. Mann-Whitney *U* tests were performed for other factors. In addition, a multi regression analysis was performed on these environmental factors. All statistical analysis was done using the computer software SPSS. Significance was set at a *p* value < 0.05 .

Based on a study by Sepp *et al.*¹⁰ who reported a higher prevalence of *Lactobacillus* at 12 months of age in Estonian infants (63%) compared with Swedish infants (38%), we estimated a sample size of 49 subjects per group as adequate to detect a difference of 25% for a 1-sided test of 5% and a power of 90%.

RESULTS

DEMOGRAPHIC & CLINICAL CHARACTERISTICS

Singapore is a metropolitan city which focuses its economy mainly on manufacturing and financial business services, and has one of the world's highest per capita gross domestic product (GDP) of USD 26,892 (estimated in the year 2005). In contrast, Theppa is a rural village located in Songkhla province, Southern Thailand and its main economic reliance is on agricultural production. More than 90% of Singaporeans live in high rise concrete buildings, in contrast to most Theppa families who live in wooden huts with concrete flooring. Most of the families in Theppa keep farm animals for livelihood. The socioeconomic status of Singapore is somewhat higher than Theppa.

Results obtained from questionnaires are summarized in Table 1. Compared with Singapore, Thai families had more children per household (4 *vs* 1, $p < 0.0005$), and their children consumed less antibiotics (40.6% *vs* 82.0%, $p < 0.0005$) but more yogurt drinks (81.2% *vs* 54.2%, $p = 0.001$). They had higher prevalence of rhinitis without flu (42.0% *vs* 13.8%, $p < 0.0005$).

It is well known that treated tap water is freely available in Singapore. Thus, we presumed that all Singapore children drank tap water. In contrast, only 17.4% of the Thai families in Theppa had access to tap water, causing them to seek for other water sources, such as well water (56.5%) or other surface water sources (26.1%), for instance from ponds or lakes (Table 1).

BACTERIAL COUNTS

Thai children harbored significantly higher counts of LAB [7.1 (6.4, 8.3) *vs* 6.0 (5.3, 7.0) logCFU/g, $p < 0.001$], coliforms [8.9 (7.3, 10.2) *vs* 6.9 (5.7, 7.7) logCFU/g, $p < 0.001$] as well as staphylococci [5.3 (4.8, 6.3) *vs* 4.3 (3.6, 5.0) logCFU/g, $p < 0.001$] than Singapore children. However, counts of enterococci were comparable between the two populations (Thai 5.2 (3.9, 6.0) log CFU/g *vs* Singapore 5.4 (3.6, 6.4) log CFU/g, $p = 0.76$) (Fig. 1).

ASSOCIATION OF ENVIRONMENTAL FACTORS WITH MICROBIAL COUNTS

In general, the environmental factors that we included for analysis could not sufficiently explain the huge differences in microbial counts observed between the two groups of children. Among all parameters analyzed, Thai children who never had con-

Table 1 Demographic and clinical characteristics of Thai ($n = 73$) & Singapore ($n = 69$) children, significant differences are indicated

	Thai ($n = 73$)	Singapore ($n = 69$)
Average number of children per household (range) **	4.09 (2–9)	1.92 (1–4)
Prevalence of (%)		
Non-feverish wheeze	66	52
Rhinitis without flu **	42	13.8
Rash resembling eczema	18.8	28.3
Family history of atopic diseases (%)		
i) None	66	52
ii) 1 member	27.5	31.7
iii) 2 members	2.9	12.7
iv) 3 members	2	0
Antibiotics used (%) **	40.6	82
Paracetamol used (%)	56.5	64.1
Consumption of yogurt drinks (%) **	81.2	54.2
Water source (%)		
i) Tap/treated water	17.4	100 (presumed)
ii) Well water	56.5	—
iii) Surface water	26.1	—

(** $p < 0.05$).

sumed yogurt drinks showed higher counts of coliforms compared to those who had (10.12 *vs* 8.75 log CFU/g, $p = 0.046$). However, this difference was not observed in Singapore children (Tables 2A, 2B).

A multiple regression model taking into account several possible confounders was performed for every bacterial species studied (Table 3). Strong associations between counts of LAB and coliforms with geographical origins were observed. However, the association between staphylococcal counts and geographical origins was not detected in this model due to strong collinearity among geographical origins and number of siblings. When number of siblings was excluded from the analysis, staphylococcal counts were significantly different between children from the two countries ($p = 0.031$, $b = 1.322$, 95% CI -0.127 to 2.518). On the other hand, no association was found between enterococcal counts with any of the factors analyzed, including geographical origins. In addition, staphylococcal counts were probably associated with every numerical increase in sibling number ($p = 0.061$). For every 1 sibling increase, the staphylococcal counts increased by 0.2 log CFU/g (95% CI - 0.01 to 0.42) (Table 3).

SPECIES VARIETY OF COLIFORMS IN THAI CHILDREN

Out of the 74 coliform colonies isolated, the majority were identified as *Escherichia coli* ($n = 65$) or *Kleb-*

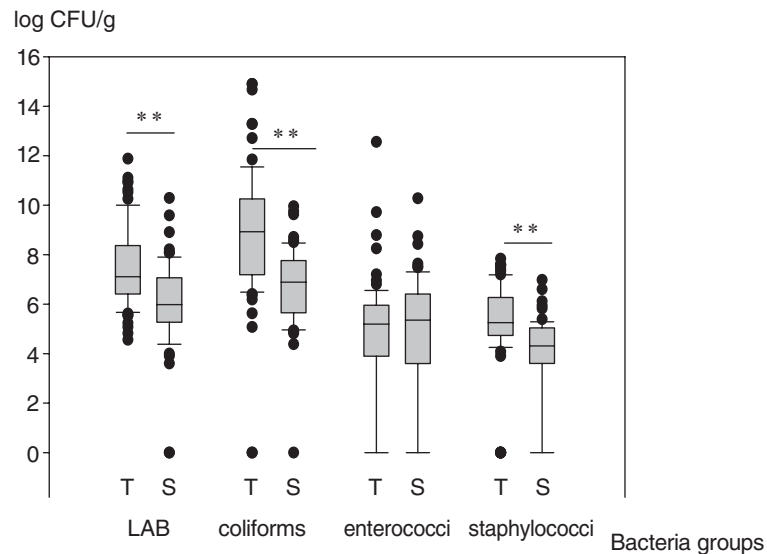


Fig. 1 Bacterial counts in rural Thai [T] ($n = 73$) and urban Singapore [S] ($n = 69$) children as determined by selective bacteriological culture. The 10th, 25th, 50th, 75th and 90th percentiles are displayed and significant differences are indicated (** $p < 0.001$).

Table 2 [A] Univariate analysis of the association of certain possible underlying confounders with the counts of 4 bacteria groups studied in Singapore children.

Singapore children	LAB		Coliforms		Enterococcus		Staphylococcus	
	Spearman's rho	p -value	rho	p -value	rho	p -value	rho	p -value
No. siblings	− 0.12	0.36	− 0.03	0.82	0.06	0.67	0.00	0.99
Only consumed tap water	Median	p -value	Median	p -value	Median	p -value	Median	p -value
Yes	6.09		6.95		5.37		4.30	
No	None		None		None		None	
Ever drank yogurt drinks	Median	p -value	Median	p -value	Median	p -value	Median	p -value
Yes	6.30	0.90	7.05	0.61	5.35	0.68	4.52	0.13
No	5.89		6.69		5.73		3.90	
Rhinitis without flu	Median	p -value	Median	p -value	Median	p -value	Median	p -value
Yes	5.92	0.60	7.63	0.31	4.92	0.30	4.76	0.49
No	6.19		6.81		5.72		4.17	
Rash resembling eczema	Median	p -value	Median	p -value	Median	p -value	Median	p -value
Yes	6.59	0.29	7.04	0.47	5.87	0.23	3.90	0.28
No	6.02		6.88		5.37		4.32	
Ever used antibiotics	Median	p -value	Median	p -value	Median	p -value	Median	p -value
Yes	6.02	0.19	6.69	0.36	5.37	0.48	4.26	0.87
No	6.95		7.53		5.19		4.26	

Table 2 [B] Univariate analysis of the association of certain possible underlying confounders with the counts of 4 bacteria groups studied in Thai children, significant difference is indicated

Thai children	LAB		Coliforms		Enterococcus		Staphylococcus	
No. siblings	Spearman's rho	p-value	rho	p-value	rho	p-value	rho	p-value
	0.15	0.23	0.04	0.73	0.09	0.45	0.15	0.21
Only consumed tap water	Median	p-value	Median	p-value	Median	p-value	Median	p-value
Yes	7.90	0.21	10.05	0.91	4.30	0.42	5.19	0.58
No	7.09		8.90		5.21		5.26	
Ever drank yogurt drinks	Median	p-value	Median	p-value	Median	p-value	Median	p-value
Yes	7.05	0.50	8.75	0.046 *	5.07	0.91	5.26	0.91
No	7.70		10.12		5.58		5.07	
Rhinitis without flu	Median	p-value	Median	p-value	Median	p-value	Median	p-value
Yes	7.15	0.44	8.72	0.15	5.11	0.63	5.21	0.54
No	7.15		9.35		5.09		5.55	
Rash resembling eczema	Median	p-value	Median	p-value	Median	p-value	Median	p-value
Yes	7.46	0.62	8.72	0.77	5.58	0.72	5.52	0.59
No	7.09		8.94		5.07		5.24	
Ever used antibiotics	Median	p-value	Median	p-value	Median	p-value	Median	p-value
Yes	7.17	0.60	8.90	0.76	5.17	0.95	5.17	0.79
No	7.07		8.98		4.98		5.52	

(* $p < 0.05$).**Table 3** Multi regression analysis of the association of certain possible underlying confounders with the counts of 4 bacteria groups studied, B (regression coefficient) and p values of all factors are shown and significant differences are indicated

Bacteria groups	LAB		Coliforms		Enterococcus		Staphylococcus	
Factors	B	p-value	B	p-value	B	p-value	B	p-value
Geographical origins	1.795	0.027 *	2.517	0.026 *	- 1.067	0.405	0.452	0.550
No. siblings	0.047	0.679	0.013	0.933	0.125	0.495	0.203	0.061
Only consumed tap water	- 0.673	0.246	- 0.149	0.854	0.710	0.442	- 0.047	0.932
Ever drank yogurt drinks	- 0.309	0.409	- 0.480	0.358	0.104	0.861	0.030	0.932
Rhinitis without flu	- 0.398	0.301	- 0.492	0.359	- 0.417	0.497	0.195	0.589
Rash resembling eczema	0.283	0.487	0.238	0.675	1.025	0.117	- 0.563	0.143
Ever used antibiotics	- 0.428	0.227	- 0.268	0.586	- 0.311	0.581	- 0.078	0.814

(* $p < 0.05$).

siella pneumoniae ($n = 7$). Other species, such as *Citrobacter freundii* ($n = 1$) and *Enterobacter cloacae* ($n = 1$), were also rarely found.

DISCUSSION

This study compared four groups of the gut microbiota from two distinct populations of children living

in South East Asia. The counts of LAB, coliforms and staphylococci were found to be significantly higher in rural Thai children than in those children living in urban Singapore. However, there was no difference between the two distinct populations in their enterococcal counts. To our knowledge, such an epidemiological survey of gut microbiota composition in children

has not been conducted in this region of the world. It has been previously suggested that there may be a protective association between certain gut bacterial counts against atopic diseases.⁵⁻⁷ Lee *et al.* recently performed a similar survey of endotoxin levels in the same populations of rural Theppa *vs* urban Singapore, and found higher endotoxin levels in rural compared with urban homes.¹¹ Our study further strengthens the claim that higher levels of certain 'microbial factors' can be found in rural environments in South East Asia. This may explain the region's escalating prevalence of allergic diseases in urban areas with increased affluence.

We also tried to identify certain environmental factors in the households that may affect the counts of these aerobic microbes studied. Among all possible factors studied, we failed to identify a single factor that may account for the huge differences in these bacterial counts between countries. It is possible that there are other factors occurring before these children were recruited, such as breastfeeding, mode of delivery, birth place hygiene etc. that may potentially affect the eventual gut community during childhood. We, however, speculate that no single factor could account for such differences. It is more likely that the entire environment in which the child was born and raised was accountable for such differences. Germ free work suggested that the types of bacteria that could colonize an organism are genetically determined. Their relative proportions, on the other hand, may still vary depending on the immediate environment.¹²

Previous clinical surveys claimed that allergic diseases in infants were more prevalent in the more developed regions of South East Asia.²⁻⁴ Our data indicate a higher incidence of 'rhinitis without flu' in rural Thai compared with urban Singapore children. The cause of this symptom in early life is heterogeneous, and allergic rhinitis is unlikely the major cause in this young age group. Although unsubstantiated in this study, infective rhinitis is a more likely etiological factor. The exact mechanisms of pediatric nonallergic rhinitis are still unclear, although several upper airway diseases are currently linked with laryngopharyngeal reflux (LPR) events.¹³ Furthermore, according to the rule of the atopic march, skin rash at this age may be more predictive of subsequent allergies, rather than rhinitis.¹⁴ The symptom of rashes resembling eczema was more prevalent in the Singapore children. (28.8% *vs* 18.8%, $p > 0.05$)

Striking differences in staphylococcal counts was observed between the two populations. This could possibly be explained by the number of siblings in the household. Older siblings may very likely contaminate their younger siblings with staphylococcal bacteria via oral-skin contact. Other practices, such as consumption of yogurt drinks, tap water and antibiotics, were not shown to have any major impact on any

of the bacterial counts studied here. Intriguingly, there was no difference in enterococcal counts between the two study cohorts. Since other bacterial groups were generally higher in the Thai children, the proportion of enterococci as a fraction of the total microbiota may be lower in the Thai children. Our previous study reported that higher enterococcal counts may be associated with the manifestation of eczema.⁹ Our observation here again highlights the possible role of enterococci in childhood allergy and future investigation is warranted. In that previous study, we have also confirmed that S&B-derived enterococci were part of the MRS-derived LAB.⁹ As higher LAB was found in Thai children, but not enterococci, the true difference between the two groups should lie in other lactic acid producing bacteria, such as lactobacilli. In another excellent comparison between regions of differential socioeconomic development, Sepp *et al.* previously reported that Estonian infants harbor higher prevalence of lactobacilli than Swedish infants.¹⁰ As an extension of this study, we also used MRS-V agars (MRS supplemented with 250 ug/ml vancomycin) to screen for 69 MRS isolates and found 5 highly vancomycin-resistant presumptive lactobacillus isolates. PCR-RFLP as well as partial sequencing of their 16S rRNA genes revealed that these Thai isolates were highly similar (>98% homology), and very closely related to the species *Lactobacillus sakei* (>98% homology). These *L. sakei* isolates from rural children may also offer exciting research prospects for *in vitro* studies regarding their influence towards immune cells.

We also found that *E. coli* was the most prevalent coliform found in the fecal samples of rural Thai children. As coliform isolates were not picked from the Singapore children, a comparison was not possible. However, as total coliform counts were higher in rural Thai children than in urban Singapore children, it can be inferred that counts of *E. coli* may very likely be higher in the rural population. Nonetheless, a protective association towards allergies could not be concluded due to the equal or higher prevalence of symptoms observed in our rural population. Nevertheless, other studies have suggested that *E. coli* was less frequently found in cesarean section-delivered infants than in vaginally delivered infants. The former more frequently carried other enterobacteria, such as Klebsiella and Enterobacter.¹⁵ However, as birth data were not collected in this study, we could not evaluate the possible associations between routes of delivery, coliforms and allergy. Previously, cesarean born subjects were found to be 2 times more likely to be sensitized to food allergens.¹⁶ Furthermore, a clinical trial which intentionally supplemented oral *E. coli* after birth was found to decrease the incidence of allergies in later life.¹⁷ These studies collectively suggest that *E. coli* as early colonizer of the infant gut might confer a protective effect against allergic diseases.

Taken together, higher levels of certain gram positive and gram negative microbes were found in young children living in rural Thai compared with their urban counterparts in Singapore. Although no single factor was identified, this difference may be explained by the contrasting environmental factors and lifestyle practices between the two regions, possibly number of siblings, source of drinking water and antibiotic use. This difference between rural and urban children was not unexpected. However, in order to further elucidate this phenomenon, the atopic prevalence of the rural population in the Theppa district has to be carefully repeated in another larger, well-planned cohort. This present investigation may thus serve as an initial study paving the way for a larger cohort study in the future.

ACKNOWLEDGEMENTS

The study was supported by the Biomedical Research Council (00/01/56/62/002) and National Medical Research Council (NMRC0971/2005), Republic of Singapore. We are grateful to Mr. Neo Keng Hwee for technical assistance and Dr. Shen Liang for statistical analysis. The voluntary participation of all subjects in the study is sincerely appreciated.

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