Viability of *Lactobacillus casei* Strain Shirota (LcS) from Feces of Thai Healthy Subjects Regularly Taking Milk Product Containing LcS

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Objective: *To demonstrate the recovery of Lactobacillus casei strain Shirota (LcS) from feces of Thai subjects who regularly took LcS containing milk product for 1 week and demonstrate the disappearance of LcS after stopped taking milk product.*
Material and Method: *First fecal samples were collected from 20 healthy adults at 10 days after they abstained from all lactobacillus containing milk products. Second specimens taken after the subjects ingested LcS containing milk product for 7 days and third specimens at 7 days after they stopped taking LcS containing milk product. All the fecal specimens were culture for LcS using LLV-FOS culture medium and enumeration of LcS was calculated. All stool samples were also tested for the presence of LcS by using nested PCR to confirm the presence of LcS obtained from culture method.*
Results: *Both culture and nested PCR method showed that all the stools samples obtained from subjects prior to the administration of LcS containing milk product were devoid of LcS, except for 3 specimens which showed weakly positive test for PCR. At 7 days after ingesting LcS containing milk product, all stool specimens were positive for LcS on both culture and PCR method. At 7 days after stopped taking LcS containing milk product, 1/19 specimens were positive from culture and 6/19 specimens were positive for PCR method.*
Conclusion: *LcS could survive in the gastrointestinal tract of Thai subjects and could be recovered from the feces after ingestion.*

Keywords: *Lactobacillus, L. casei, Enteric flora, Probiotics, Probiotic survival, Normal host, Healthy volunteer*

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For a probiotic to be able to exert its beneficial effects in human body, it needs to be shown that it can survive through the passage of human gastrointestinal tract and be able to be recovered from human feces after oral ingestion[1]. *Lactobacillus casei* strain Shirota (LcS) has been used in the production of fermented milk products for many decades and it is one of the most extensively studied probiotics. Several studies had shown that LcS can survive through gastrointestinal tract in Japanese and European population using selective media and monoclonal antibody testing/molecular identification[2-6]. In a double-blind prospective study carried out in Spain on other probiotics, *Lactobacillus delbrueckii* and *Streptococcus thermophilus* were found in feces in only 10 (10.52%) of 96 individuals after consumption of fresh yogurt and in 2 (2.10%) of 96 individuals after consumption of pasteurized yogurt[7]. So far, there had been no study to show the recovery of LcS after regularly consumption of milk product containing LcS in Thai population for whom the ecology of intestinal flora might be different. Thai people have a unique life style which is different from Japanese population. They probably eat less fish and seafood, consume more spices and hot condiments. They also live a life style which probably is less hygienic than Japanese in general. These factors may affect the ecology of microflora in the intestinal tract of different human populations and culture. It is interesting to demonstrate the recovery of LcS from feces in Thai population regularly taking LcS containing milk product.

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Material and Method

Subjects

The 20 healthy adult individuals who had no subjective symptom in abdomen (*e.g.*, constipation, diarrhea or abdominal pain) and could intake milk product containing LcS during the period of study were included in this project. They were 11 female and 9 male with age range of 25-57 years old.

Fecal samples

The first fecal sample was collected from each subject who had not been taken any probiotic-milk product before the intervention for 10 days. After that they ingested the 80 ml of milk product containing LcS 8 x 10^9 CFU by 3 times a day after meal for 7 consecutive days and thereafter, the 2nd fecal samples were collected from them. The last fecal samples were collected after stopping the ingestion of probiotic for 7 days. Most of the fecal specimens were collected on site and a few were transported from their homes to the laboratory in an ice box within few hours.

Culture and enumeration of LcS in feces

Each 0.5 g of fecal sample was homogenized in 4.5 ml of 0.1% yeast extract solution. The 10 fold dilution series of fecal suspension were performed in 0.1% yeast extract solution from 10^{-1} to 10^{-8}. The 0.1 ml of each fecal dilution was spread over lactitol-LBS-vancomycin (LLV) plus fosfomycin medium (LLV-FOM) plate and incubated aerobically at 35°C for 96 hours: the LLV-FOM medium was prepared by modification of LLV medium by adding fosfomycin to inhibit the growth of Gram-negative intestinal bacteria. The large, white and dome-shaped colonies formed on the LLV-FOM agar plate that had 30-300 colonies were regarded as those of LcS and were counted.

LcS confirmation by ELISA method

Ten LcS-like colonies out of the 30-300 colonies detected on the LLV-FOM agar plate were confirmed to be LcS by ELISA as described by Yuki et al[2]. The cells of LcS and *Bifidobacterium breve* YIT 4065, which were fixed with formaldehyde, were used as the positive control (PC) and negative control (NC) for ELISA, respectively. Each of LcS-like colonies, PC and NC were picked for approximately 10 µl and suspended in 100 µl of carbonate buffer containing 15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.6) in each well of 96 well microtiter plate. The blank wells that contained only carbonate buffer were also performed. The plate was incubated overnight at 4°C. After washing 3 times, the plate was blocked with 120 µl of carbonate buffer containing 1% bovine serum albumin and incubated at 37°C for 1 hour. Then the plate was washed with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) supplemented with 0.05% Triton X-100, reacted with 90 µl of appropriate dilutions of LcS monoclonal antibody solution in carbonate buffer with 1% BSA and incubated at 37°C for 1.5 hour. Next, the plate was washed, treated with 100 µl of a 1,000-fold dilution of the peroxidase-conjugated anti-mouse immunoglobulin (ICN Pharmaceuticals Inc, Tokyo, Japan) in carbonate buffer with 1% BSA. After washing, the color was developed by adding 100 µl of 0.4 mg/ml o-phenylen-ediamine in citrate buffer [0.51% citric acid, 50 mM Na_2HPO_4, 0.02% H_2O_2 (added immediately before use); pH 5.0] at 37°C for 10 min. Then the reaction was stopped by adding 50 µl of 2.5 M sulfuric acid. The absorbance was measured at 490 nm. The sample OD (S) was OD_{490} of the sample well minus OD_{490} of the blank well. The positive OD (P) was OD_{490} of the well of PC minus OD_{490} of the well of blank. The negative OD (N) was OD_{490} of the well of NC minus OD_{490} of the well of blank.

When "S" was more than 0.5, the colony was determined as LcS positive. If the value of "P" and "N" were lower than 0.5 and higher than 0.5, respectively, the test was not valid and need to be controlled and performed again.

Calculation of the number of LcS in fecal sample

The percentage of LcS positive (LcS^+) was number of LcS-positive colonies divided with number of LcS-like colonies picked up from a LLV-FOM agar plate and multiply with 100. The number of LcS in fecal sample (CFU/g) was calculated by the following formula

$$\text{LcS (CFU/g)} = \frac{\text{(number of LcS-like colonies)} \times (\text{LcS}^+) \times \text{(dilution of fecal sample)}}{\text{(inoculation volume of fecal sample, ml)} \times 100}$$

Nested PCR for detection of LcS in feces

DNA extraction from stool samples was performed by using QIAamp DNA stool mini kit (QIAGEN, Valencia, CA, USA). Two microliters of DNA template were use in each PCR tube (total volume of 20 µl). Outer primers, pLcS-57F (CTCAA AGCCGTGACGGTC) and pLcS-597R (CACTAGG ATTATTAGCACCACGT)[4] and inner primers, LcS3 (ACCTTTGCCACCAGTTGA) and LcS4 (ATTAGA GCGGGATTGACG) (the present study); were employed in nested PCR assay.

The first round amplification reaction (total volume of 20 µl) consisted of 2 µl of DNA template, 1x

PCR buffer, 2 mM $MgCl_2$ (Gibco/BRL, Gaithersberg, USA), 200 µM of each dNTP (Amresco, USA), 20 ng of each primer, pLcS-57F and pLcS-597R, 0.5 U of *Taq* DNA polymerase (Gibco/BRL, Gaithersberg, USA). PCRs were performed in a thermal cycler (Model 2400, Perkin-Elmer, USA) using the following cycling conditions: an initial denaturation at 94°C for 5 min and 30 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C. The final extension step was 7 min at 72°C.

The two microliters of 1:50 diluted product of the first round PCR were used as DNA template in the second round amplification reaction. The mixture and condition of the second round amplification reaction were the same as those of the first round reaction, except for primers (LcS3 and LcS4 instead of pLcS-57F and pLcS-597R).

The amplification products were run on 2% agarose gel and visualized following ethidium bromide staining. To confirm the authenticity of the products, some of them were randomly selected to be determined for DNA sequence.

Ethical permission

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Results

Detection of LcS in feces by culture method

The number of LcS in fecal sample is shown in Table 1. Culture method showed that all the stools samples obtained from subject prior to the administration of LcS containing milk product were devoid of LcS. At 7 days after ingesting LcS containing milk product, LcS was recovered from all stool specimens (the average bacterial counts of LcS: 8.04 x 10^7 CFU/g feces). One out of 19 specimens were LcS positive at 7 days after stopped taking LcS containing milk product.

Detection of LcS in feces by nested PCR

The detection of LcS in all fecal samples was also performed by nested PCR. The result revealed that

Table 1. *Lactobacillus casei* strain Shirota (LcS) detection in feces using culture method and nested PCR

No.	Sex	LcS ,CFU/g. feces and Nested PCR results					
		Before ingestion		After LcS-milk ingestion products		After 7 days stop ingestion	
		Culture (CFU/g)	Nested PCR	Culture (CFU/g)	Nested PCR	Culture (CFU/g)	Nested PCR
1	M	Negative	Negative	1.58 x 10^5	Positive	Negative	Positive
2	F	Negative	Negative	1.94 x 10^7	Positive	Negative	Positive
3	M	Negative	Negative	1.47 x 10^7	Positive	Negative	Negative
4	M	Negative	Negative	4.39 x 10^8	Positive	Negative	Negative
5	F	Negative	Negative	2.01 x 10^8	Positive	Negative	Negative
6	F	Negative	Negative	2.57 x 10^7	Positive	Negative	Negative
7	F	Negative	Negative	3.87 x 10^7	Positive	Negative	Negative
8	F	Negative	Negative	2.35 x 10^6	Positive	2.24 x 10^5	Positive
9	F	Negative	Negative	1.27 x 10^8	Positive	Negative	Negative
10	F	Negative	Negative	1.57 x 10^8	Positive	Negative	Negative
11	F	Negative	Negative	1.29 x 10^6	Positive	Negative	Weakly positive
12	F	Negative	Positive	1.24 x 10^8	Positive	Negative	Weakly positive
13	F	Negative	Negative	1.64 x 10^7	Positive	Negative	Negative
14	F	Negative	Positive	1.07 x 10^8	Positive	Negative	Negative
15	M	Negative	Negative	2.25 x 10^7	Positive	Negative	Negative
16	M	Negative	Negative	6.29 x 10^4	Positive	Negative	Negative
17	M	Negative	Negative	1.10 x 10^7	Positive	ND*	ND*
18	M	Negative	Positive	3.59 x 10^5	Positive	Negative	Negative
19	M	Negative	Negative	1.12 x 10^8	Positive	Negative	Negative
20	M	Negative	Negative	1.88 x 10^8	Positive	Negative	Positive

* ND = Not done, because specimen was not received

LcS could be detected in the feces of all subjects after one week of LcS-containing milk product ingestion whereas only six out of 19 subjects still carried LcS after stopping taking the milk product for one week. Interestingly, LcS could also be detected in fecal samples of three subjects before taking LcS-containing milk product Table 1.

Discussion

In the present study, the culture medium used for culturing LcS was slightly different from the medium used in earlier Japanese study as it also contained fosfomycin which was not present in the culture media of other studies because the authors' preliminary survey showed that feces from Thai subjects contained numerous gram-negative organisms which gave rise to confusing colonies among those of LcS. Hence, fosfomycin was added in the medium to inhibit growth of gram-negative bacilli and allowed the growth of LcS to be detected more easily, if present.

At the beginning of the present study, all stool samples collected from subjects who had not been taken any *Lactobacillus* containing milk products for 10 days prior to the present study, showed no LcS from any specimens sent for culture. However, for nested PCR assay, three of the specimens from the *Lactobacillus* non-exposed subjects showed weakly positive results for LcS PCR. It was postulated that some genetic residual products may be persistent in some subjects enabling the very sensitive PCR assay to detect them and giving weakly positive results, or it may be that there were cross reactions of some indigenous organisms with the tested genetic marker of LcS. Another factor which may affect the culture sensitivity is the dilution of cultured effluent which started at 1:10 dilution, making it impossible to detect bacterial concentration of less than 200 CFU per ml. Hence, the culture technique is obviously less sensitive than the nested PCR technique. Anyhow, two of these subjects did not show positive results of PCR assay after having stopped taking LcS containing product for one week, while one of these subject showed weakly positive PCR testing at one week after stopping ingesting LcS containing product.

All subjects who took LcS containing milk product, showed positive results on both culture and PCR assay at day 7 after taking LcS containing milk product. These results confirmed the positive recovery of LcS in stool of subjects who took LcS containing milk product for 7 days at the t.i.d. dose of LcS containing milk product (each bottle of milk product contained 8 x 10^9 CFU of LcS), This result was consistent with the results from Japanese and European studies[2-6], confirming that LcS can survive in human intestinal tract of Thai subjects who have different eating habits, life style, culture and perhaps, different ecology of bowel flora.

Repeated culture and PCR assay after having stopped taking LcS containing milk product for a week showed absence of LcS from feces of most subject, except for 1 in 20 subjects who showed the persistence of LcS from culture and 6 out of 19 specimens who were positive for PCR at 7 days after stopping milk product ingestion. These results reflected that PCR is probably more sensitive than culture method in detecting LcS from feces, but may not reflect the viability of the bacteria. Only the culture method is the reliable mean for proving the viability and survival of LcS in the present study. However, detection limit of culture method used was 100 CFU/g of feces. Therefore, negative result could mean either absence of LcS or presence of LcS of less than 100 CFU/g of fecal sample.

The presence of viable LcS in feces indicated that the organism could tolerate both gastric and bile salts. It has been shown that lactobacilli exhibit several health benefits[8] such as its antagonistic activity against some gastro-intestinal pathogens[8-11], contribution to eradicate *Helicobacter pylori* in patients receiving *H. pylori* treatment[12] and anti-inflammatory activity[13]. However, the LcS should be taken cautiously in patients with ulcerative bowel[14].

Conclusion

Recovery of LcS from feces was demonstrated in Thai healthy volunteers who regularly took LcS containing milk product for one week. This result confirmed the survival of LcS in the human gastrointestinal tract of a different nationality other than Japanese and European.

Potential conflicts of interest

None.

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การตรวจพบ *Lactobacillus casei strain Shirota (Lcs)* จากอุจจาระของอาสาสมัครไทยที่ดื่มนม ซึ่งมีเชื้อ *LcS*

สุรภี เทียนกริม, อมรรัตน์ ลีลาภรณ์, สถาพร มนัสสถิตย์, วิษณุ ธรรมลิขิตกุล

วัตถุประสงค์: *เพื่อแสดงให้เห็นว่าสามารถตรวจพบเชื้อ Lactobacillus casei strain Shirota (LcS) จากอุจจาระของอาสาสมัครไทยที่ดื่มนมซึ่งมีเชื้อ LcS เป็นเวลานาน 7 วัน และตรวจไม่พบเชื้อ LcS หลังจากหยุดดื่มนมซึ่งมีเชื้อ LcS เป็นเวลานาน 7 วัน*

วัสดุและวิธีการ: *เก็บอุจจาระ 3 ครั้ง ครั้งที่ 1 เก็บก่อนดื่มนมซึ่งมีเชื้อ Lactobacillus เป็นเวลา 10 วัน ครั้งที่ 2 และ 3 เก็บหลังจากดื่มนมซึ่งมีเชื้อ LcS เป็นเวลา 7 วัน และหลังจากหยุดดื่มนมซึ่งมีเชื้อ LcS นาน 7 วัน, ตามลำดับ อุจจาระที่เก็บมาทุกครั้งจะนำมาเพาะเชื้อบนอาหารเลี้ยงเชื้อพิเศษ lactitol-LBS-vancomycin (LLV) และเติม fosfomycin (LLV-FOM) เพื่อนับจำนวนเชื้อ และตรวจยืนยันว่าเป็น LcS โดยวิธี ELISA นอกจากนี้ยังได้ตรวจหาสารพันธุกรรมของเชื้อด้วยวิธี nested PCR*

ผลการศึกษา: *ก่อนดื่มนมซึ่งมีเชื้อ LcS ตรวจไม่พบเชื้อ LcS ทั้งวิธีเพาะแยกเชื้อ และ nested PCR (ซึ่งมีเพียง 3 รายที่ให้ผล nested PCR บวกอ่อนๆ) หลังจากดื่มนมซึ่งมีเชื้อ LcS 7 วัน ตรวจพบเชื้อ LcS ได้หมดทั้งสองวิธี ส่วนหลังจากหยุดดื่มนมซึ่งมีเชื้อ LcS 7 วัน ผลการเพาะแยกเชื้อ สามารถตรวจพบเชื้อ LcS 1/19 ราย ส่วนวิธี nested PCR พบ 6/19 ราย*

สรุป: *แสดงให้เห็นว่าเชื้อ LcS สามารถมีชีวิตอยู่ได้ตลอดระบบทางเดินอาหารของอาสาสมัครไทย และตรวจพบได้จากอุจจาระหลังจากดื่มนมซึ่งมีเชื้อ LcS 7 วัน*