Short Communication

Recovery of Lactobacillus casei strain Shirota (LcS) from the intestine of healthy Vietnamese adults after intake of fermented milk

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To demonstrate the gastrointestinal survival of *Lactobacillus casei* strain Shirota (LcS) in healthy Vietnamese adults, a fermented milk drink containing LcS was administered daily for 14 days. Twenty-six healthy Vietnamese adults took part in the study. Each participant consumed 65 mL of a fermented milk drink containing LcS daily for 14 days. The drink contained a dose of 10^8 CFU/mL LcS. Fecal samples were collected before, during and after consuming the fermented milk drink. LcS was confirmed by culture and ELISA. After 7 and 14 days of ingesting fermented milk drink, LcS was recovered from fecal samples at average of 5.0×10^7 CFU/g feces (n=26) and 5.4×10^7 CFU/g feces (n=26), respectively. LcS persisted in 8 voluteers until day 42 (after 14 days stopping fermented milk drink) at 0.0033×10^7 CFU/g feces (n=8). We confirmed survival of LcS after passage through the gastrointestinal tract of Vietnamese adults.

Key Words: *Lactobacillus casei* Shirota, fermented milk drink, Vietnamese adults, probiotic, survival

INTRODUCTION

Probiotics exert their beneficial effects through interactions with the gut. The exact mechanisms are still not known. Several mechanisms have been proposed, including lowering intestinal pH, decreasing colonization and invasion by pathogenic bacteria, and modifying the host immune response.[1-3] A number of health effects are associated with probiotic use. The main health benefits are enhancement of immunity against intestinal infections; immune enhancement;[4-6] prevention of diarrheal disease;[7,8] prevention of colon cancer;[1] prevention of hypercholesterolemia;[1-3] improvement in lactose utilization;[2] prevention of upper gastrointestinal tract disease; and stabilization of the gut mucosal barrier.[2]

Probiotics have been consumed by humans in one form or another for over 100 years, with a generally good safety record. No known or potential risks are expected from the use of probiotics. No pathogenic or virulent properties have been found for lactobacilli, bifidobacteria or lactococci.[9-12]

Lactobacillus and *Bifidobacterium* are lactic acid bacteria that are able to produce lactic and acetic acid, which may lower intestinal pH and suppress the growth of various pathogenic bacteria. These probiotic bacteria may also produce various substances such as hydrogen peroxide, organic acids, bacteriocins and biosurfactants, which are toxic to pathogenic bacteria. *Lactobacillus* species strain GG is known to secrete a low molecular weight compound that inhibits a broad spectrum of Gram-positive, gram-negative and anaerobic bacteria. The non-pathogenic yeast *Saccaromyces boulardii* produces a protease that decreases the toxicity of *Clostridium difficile* toxin A and B.[1,2]

In vitro, LcS is resistant to gastric acid, stomach bile, and artificial gastric juice.[13] In addition, clinical trials in Japanese humans demonstrated the resistance of LcS to gastric passage. The recent studies by Touhy et al at the University of Reading in the UK and Dr Tiengrim at Siriraj Hospital in Thailand also demonstrated the recovery of LcS in the gastrointestinal tract of healthy volunteers.[14,15]

LcS was reported to be effective for improving of diarrhea, intestinal microbiota and the gut environment.[16,17] The effectiveness of LcS in preventing and treating constipation has been reported in several studies.[18] Koebnick conducted a trial on 70 patients with chronic constipation using LcS (6.5 billion per day) for 4 weeks. After 2 weeks, 89% of the experimental group improved significantly in measures of constipation compared with the control group. The frequency of bowel movements increased 6 times/week, the state of distention and bloating signifi-

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cantly improved, and there were no reported side effects.[18] Sgouras et al showed *in vitro* and in human clinical trials that LcS inhibits the bacterium *Helicobacter pylori* that causes stomach ulcers.[19] In addition, LcS also showed a significant effect on enhancing immunity to fight infections and cancer, especially enhanced NK cell activity, cells that destroy viruses.[20]

Probiotics are being used with increasing frequency as a treatment for several medical conditions. Many studies have supported the beneficial effects of probiotics for human health. Probiotic efficacy relies on their ability to survive in the digestive system and their ability to proliferate in the gut. The viability of probiotics depends on the strain used and both intrinsic and extrinsic factors.[21,22]

The ability of *Lactobacillus* and *Bifidobacterium* to survive in the gastrointestinal tract varies considerably between species.[23] Ingested bacteria are exposed to adverse conditions starting as soon as they reach the stomach. Survival in the stomach depends on the time required by the bacteria to leave the stomach. Thus, the gastric emptying rate is an important feature for the survival of bacteria. The small intestine, particularly its proximal part, contains hydrolytic enzymes and bile salts that are known to have lethal effects on microorganisms. Thus, the passage through this compartment may also significantly affect the survival of ingested bacteria.[24,25] Factors such as the type and composition of food consumed, lifestyle, age, environment and race influence the gastric emptying rate and thus also influence the survival of probiotic bacteria.

The aim of this study was to investigate the recovery of LcS from the intestine of healthy Vietnamese adults after intake of a fermented milk drink. A similar study has been conducted by other investigators. This study investigated the recovery of LcS in adults who have different lifestyles, food habits, environments and races compared with subjects from other studies.

There is no consensus regarding the minimum number of microorganisms that must be ingested to obtain a beneficial effect. Probiotic dosing varies depending on the product and specific indication. Typically, a probiotic should contain several billion microorganisms (1-20 billion colony-forming units).[7] In this study, retail fermented milk product containing over 6.5 billion LcS per 65 mL (10^8 CFU/mL) was used. A study conducted by Yuki *et al* showed that the consumption of 125 mL of fermented milk product (10^8 CFU/ mL) for 3 days had a mean recovery of 10^7 CFU per gram feces.[22]

MATERIALS & METHODS

Volunteer recruitment

Twenty-six healthy volunteers from the students and staff of Hanoi University of Science and Technology and the National Institute of Nutrition, Vietnam, between the ages of 18 and 35 were recruited for this study. The volunteers were not pregnant, were free from underlying gastrointestinal complaints (constipation, diarrhea, abdominal pain, irritable bowel syndrome), were not taking any medications with gastrointestinal activity, had not taken antibiotics for 30 days prior to starting the study, and did not consume probiotic or prebiotic foods during the study period. The present study were approved by the Ethical Committee in National Institute of Nutrition, Ministry of Health Vietnam (No 457/QĐ-VDD) and carried out in accordance the code of ethics of the World Medical Association (Declaration of Helsinki).

Study design and implementation

The study consisted of a 14-day baseline period, a 14-day ingestion period, and a 14-day follow-up period. At the screening visit, demographic data, eligibility criteria, medical history and concomitant medications were recorded. Those data, except medical history, were obtained through a questionnaire. The investigator filled in the questionnaire during the screening visit.

During the baseline period, subjects continued their normal diet with the exclusion of fermented dairy products. The subjects were requested to fill in a subject diary on a daily basis that consisted of questions on study product intake (only for ingestion period), other food intake (if any), number of bowel movements, change in stool consistency, any medications received, and any symptoms of discomfort (e.g., diarrhea, constipation, vomiting, gassing, sensation of illness). Fecal samples were collected at visit 1 (on the morning of day 14), when the subject visited the study team; the sample was marked as "sample day 14". The diaries were also collected at visit 1.

Visit 1 was the end of the baseline period and the start of the ingestion period. On day 1 of the ingestion period (day 14 of the study), the study team gave each subject a diary and one bottle of study product that subjects should drink after having lunch. For the next 13 consecutive days (the ingestion period, from day 14 until day 27), the study team visited the subjects every day after lunchtime. The study team gave the subjects one bottle of study product, which the subjects drank immediately. Subjects were not allowed to consume any other fermented dairy milk. The subjects were requested to fill in the subject diary on a daily basis. Upon completion of the ingestion period, a fecal sample and the subject diary were collected at visits 2 (on the morning of day 21) and 3 (on the morning of day 28). The samples were marked as "sample day 21" and "sample day 28".

Subjects who successfully completed the ingestion period entered the follow-up period. During the follow-up period, subjects continued their normal diets with the exclusion of fermented dairy products. The subjects were requested to fill in the subject diary on a daily basis. A fecal sample and the subject diary were collected at visit 4 (on the morning of day 42) during a visit of the subject to the study team; the sample was marked as "sample day 42".

Product handing

Fermented milk containing 6.5 billion LcS per 65 mL (10^8 CFU/mL) is produced by Yakult company in Vietnam. The nutrition composition of the tested fermented milk consist energy of 57.7 kcal, protein of 0.8 g, fat of <0.1 g and carbonhydrate of 12.4 g. The tested fermented milks were for oral use only. Subjects drank 1 bottle per day of the tested fermented milk after having lunch for 14 consecutive days (day 14 to day 27). During the ingestion period, the study team visited the subjects every day to give them the tested fermented milk (one bottle each day).

The subjects drank it after having lunch under supervision of study team.

The tested fermented milks were ready to use, and it is suggested that the product be consumed cold. The tested fermented milk was supplied by the investigator/study team. Each pack was labeled in accordance with applicable laws and regulations. Each pack contained a label and an instruction sheet in the local language.

The tested fermented milks were stored in a refrigerator (<10°C) and protected from direct sunlight. The tested fermented milks were used before their expiration date.

Assessment of the recovery of LcS

Stool collection

A fecal sample was collected in a special container at home by each subject. Materials and instructions for fecal sample collection were provided to the subjects by the study team during the baseline visit, ingestion visit and follow-up visit. Fecal samples were collected into sterile container with scoop built into the inside of the lid. Prior to the stool collection schedule, subjects were provided with the sample kit collection (sterile condition). Fecal samples were collected by the subjects and immediately transported to the site/laboratory in a cold storage container. Once fecal samples were received from subjects, they were immediately used for LcS analysis.

Enumeration of LcS from fecal samples

The LcS levels at the end of the baseline period (day 14), one week after ingestion (day 21), the at the end of ingestion period (day 28), and at the end of follow-up period (day 42) were measured by the Laboratory at the Institute of Microbiology and Biotechnology using a culture method (FOM-LLV agar [14]) and an ELISA method.

The fecal samples were homogenized in 9 volumes PBS by weight. A 10-fold dilution series of each fecal suspension was made using PBS. Then, each diluted fecal suspension was inoculated onto FOM-LLV agar plates and smeared on the surface of the plates. The plates were incubated aerobically at 37°C for 96 hours. Using the ELISA method, large, white, dome-shaped colonies were identified as LcS or not.[14]

Assessment the improvement of gut health

The stool consistency during the baseline, ingestion and follow-up period was measured by the subjects using a 7-point scale (Bristol Stool Form Scale) and recorded in the subject diary. The subjects were requested to analyze their stool consistency immediately after defecation using pictures provided by the study team during the screening visit. The subjects were trained to use the pictures in the Bristol Stool Form Scale by the study team.[26]

The number of bowel movements (number of movements per day) in the baseline, ingestion and follow-up periods were measured by subjects and recorded in the subject diary.

Assessment of safety and tolerability of the tested fermented milk

Safety parameters in this study were defined as the overall incidence of adverse events between study entry (day 0 at baseline period) until the end of the study (day 42 at follow-up period). The number, type and duration of serious adverse events, incidence and severity of diarrhea and constipation and discomfort (vomiting, gassing, changes in defecation frequency) were checked.

Study compliance

Compliance was based on the tested fermented milk consumed, intake of other fermented dairy milks and concomitant medications and was reported at each scheduled visit for all subjects. The study team reviewed the study diaries together with the subjects to check compliance. Compliance was reinforced at each study visit.

Statistical analysis

This study was a preliminary study to investigate the recovery of LcS from the intestines of healthy adults after the intake of LcS in fermented milk. The 26 subjects were considered adequate for the study.

Baseline data were described and summarized either as the means and standard deviations or medians and interquartile ranges, as appropriate for continuous data, or as numbers and percentages for categorical data. Parameters were evaluated using t-tests or non-parametric tests in SPSS 16.0.

RESULTS

Subject's characteristics at screening

Twenty-six subjects participated in the study, including 3 males and 23 females (88.5% female). The average age was 22.2±4.1 (ages 18-35), and 88.5% of subjects were students at Hanoi University of Science and Technology; the remainder were office staff (Table 1).

Tolerability and safety of the tested fermented milk

Over the 42 days of the study, subjects did not have any changes in body weight and BMI. The average body weight was 50.1 kg, and the average BMI was 20.7 kg/m^2 (data not shown). There were no differences in the body weight of subjects between the visits. No subjects ingested any yogurt products, fermented products or prebiotic products, except for the 14-day ingestion period. All sub-

Table 1. Characteristics of subjects at screening

Parameters	Value
Sex[†]	
Men	3 (11.5)
Women	23 (88.5)
Age[‡]	22.2±4.1
Body weight (kg)[‡]	49.9±6.6
Height (cm)[‡]	157±6.6
BMI (kg/m)[‡]	21.4±1.6
Systolic blood pressure (mmHg)[‡]	97.9±8.8
Diastolic blood pressure (mmHg)[‡]	59.8±7.4
Job[†]	
Student	23 (88.5)
Office staff	3 (11.5)
Chronic diseases[†]	
Yes	0
No	26 (100)

[†]The values are expressed as the number (or percentage) of subjects.
[‡]The values are expressed as the mean±standard deviation.

Table 2. The percentage of subjects with illness or taking medication during the study

Study period	Illness (cough, headache and flu)	Taking medication	Digestion related signs (diarrhea, flatulence)
Baseline period (0-13 days)	4 (15.4)	6 (23.1)	0
Ingestion period 1 (14-21 days)	4 (15.4)	2 (7.7)	0
Ingestion period 2 (22-28 days)	2 (7.7)	1 (3.8)	0
Follow-up period (29-42 days)	0	2 (7.7)	0

The values are expressed as the number (or percentage) of subjects.

Table 3. The percentage of subjects with constipation and the number of bowel movements during the study

Study periods	Constipation‡	Bowel movements‡
Baseline period (0-13 days)	2 (7.7)	13.6±3.9
Ingestion period 1 (14-21 days)	1 (3.8)	12.7±2.9
Ingestion period 2 (22-28 days)	0	12.2±2.7
Follow-up period (29-42 days)	0	11.1±3.5

‡The values are expressed as the number (or percentage) of subjects
‡The values are expressed as the mean±standard deviation

jects drank all of the required study products (one 65 mL bottle of fermented milk/day after lunch).

Table 2 shows that during the 42-day study, some subjects felt sick or developed a cough, headache and flu (4/26 subjects in the baseline period and ingestion period 1; 2/26 subjects in ingestion period 2 and no subjects in the follow-up period).

For medications, there were 6 people (23.1%) who used cough, cold and allergy medications due to the change of weather in the baseline period. During the ingestion period, 2/26 subjects took panadol, flu and weather allergy medications. No subjects took any antibacterial drugs during the 42-day study. Diarrhea, flatulence, bloating and vomiting did not occur. No side effects including allergy problems were reported during the 42-day trial.

The effects of study product on constipation, stool consistency and frequency of defecation

In the first 14 days, there were 2 subjects with constipation, accounting for 7.7%. After one week of using the tested fermented milk, the number of constipated subjects dropped to one, and after 2 weeks, constipation was not observed in any of the subjects. This effect was maintained until the end of the follow-up period (Table 3).

Table 3 also shows the number of bowel movements. Although the average number of bowel movement in each period was not changed, bowel frequency showed improvement tendency, with 24/26 subjects defecating every morning (one time/day) after 14 days of drinking the tested fermented milk (data not shown).

In the baseline period, 9 subjects had hard stools (including constipation), accounting for 34.5% (7.6% with constipation). After one week of using the tested fermented milk, this number decreased to only 5 subjects with hard stools (19.2%). The number of hard stools declined statistically significantly after 2 weeks of ingestion (7.7%, 0% constipation). However, after not using the tested fermented milk, the number subjects with hard stools had a trend towards increasing, with 5 subjects having hard stools (19.2%), but none with constipation (Table 4).

After one week of drinking the tested fermented milk, the number subjects with soft blobs increased to 19.2%, which was higher than the baseline period. After 2 weeks of ingestion, the percentage of subjects with normal sausage stools increased to 84.6%, higher than in other periods.

Survival of LcS upon gastrointestinal transit

In the baseline period, LcS was not detected in 19 subjects but was detected in 7 subjects at concentrations ranging from 1.40×10^3 to 2.37×10^5 CFU/g feces. From the result of previous studies, it was not likely that the colonies detected at the end of baseline period could be false positive.[14,27] These 7 subjects may have consumed the tested fermented milk before, and LcS was thus maintained for prolonged periods of time, although these subjects had been "washed out" during the 14 days before the ingestion period.

After 1 and 2 weeks of ingestion, LcS was recovered from all 26 fecal samples at an average of 5.0×10^7 CFU/g feces and 5.4×10^7 CFU/g feces, respectively (mean±SD, n=26). These CFU counts were significantly higher compared with the baseline period and the follow-up period ($p<0.0001$). LcS was recovered at very high levels (2.29×10^8 CFU/g feces) from some fecal samples (data not shown).

After 2 weeks of not using the tested fermented milk, LcS persisted in 8 subjects at concentrations ranging from 1.80×10^3 to 7.90×10^5 CFU/g feces (Table 5).

DISCUSSION

The current study was designed as a clinical trial with evaluations before and after the intervention. The study was conducted on 26 healthy volunteers aged 18-35 years at the National Institute of Nutrition and Hanoi University of Science and Technology. The study was divided into 3 periods with 4 evaluation periods. There were 14 continuous days of using one bottle of the tested fermented milk every day (the tested fermented milk is a fermented milk drink that contains over 6.5 billion LcS per 65 mL/bottle). All subjects received training and counselling. Study supervisors and collaborators closely monitored and exactly

Table 4. Change in stool consistency

Bristol Stool Form Scale	Baseline period	Ingestion period 1	Ingestion period 2	Follow-up period
Hard lumps	1 (3.8)	0	0	0
Lumpy	1 (3.8)	1 (3.8)	0	0
Hard sausage	7 (26.9)	4 (15.4)	2 (7.7)*	5 (19.2)
Normal sausage	16 (61.5)	16 (61.5)	22 (84.6)	19 (73.1)
Soft blobs	1 (3.8)	5 (19.2)*	2 (7.7)	2 (7.7)
Mushy	0	0	0	0
Watery	0	0	0	0

The values are expressed as the number (or percentage) of subjects.
*$p<0.05$ (compared with baseline period).

Table 5. The number of the subjects with LcS detected in feces and the average LcS level by study period

Study period	Number of subjects	LcS ($\times 10^7$ CFU/g feces)†
Before drinking the tested fermented milk (V1)	7/26	0.0014±0.0049
After one week of using the tested fermented milk (V2)	26/26	5.0±5.6*
After 2 weeks of using the tested fermented milk (V3)	26/26	5.4±7.2*
After 2 weeks of not using the tested fermented milk (V4)	8/26	0.0033±0.0154

†The values are expressed as the mean ± standard deviation of LcS counts.
*$p<0.0001$ (compared with V1 and V4).

recorded all data. The results of current study show that LcS was recovered and that drinking the tested fermented milk improved stool consistency in Vietnamese adults.

Different species of *Lactobacillus* and *Bifidobacterium* may exist in the digestive tract. The existence of bacteria in the stomach depends on factors such as the pH, time, and species of bacteria. Bacteria can pass through the stomach and live in the intestine.[23-25]

In the present study, all subjects drank the tested fermented milk containing over 6.5 billion of LcS/65 mL (10^8 CFU/mL). After one week of ingestion, LcS levels increased from an average of 0.0014×10^7 to 5.0×10^7 CFU/g feces. After 2 weeks, the number of LcS in feces increased to 5.4×10^7 CFU/g faces. This result indicates that the LcS in the tested fermented milk is able to survive passage through the gastrointestinal tract of Vietnamese adults. The ability of LcS to survive passage through the human gastrointestinal tract was reported by Yuki et al in a study that showed consuming 10^{10} LcS/125 mL fermented milk product for 3 days led to an average of 10^7 CFU/g feces of LcS.[22] This present study confirms previous reports on the survivability of LcS present in a retail product.[14,15,23-25]

In the present study, after 14 days of stopping the tested fermented milk ingestion, LcS persisted in 8 subjects. It could be the case that LcS can adapt to colonize the gastrointestinal tract of Vietnamese subjects and can be maintained for prolonged periods. Factors such as food ingredients, age, environment and race may affect the rate of gastric emptying and can thus affect the survival and colonization ability of probiotic bacteria. *In vitro,* LcS has been shown to be resistant to artificial gastric acid and bile juices.[13] In addition, clinical trials in humans proved the resistance of LcS to digestive juices.[14,15] The results of this study demonstrated that LcS in the the tested fermented milk is able to survive passage through the gastrointestinal tract of Vietnamese adults.

LcS was reported to be effective for diarrhea by improving the intestinal microbiota and the gut environment.[16,17] LcS was shown to be effective for the prevention and treatment of constipation.[18] Koebnick conducted a trial on 70 patients with chronic constipation using LcS at 6.5 billion per day for 4 weeks. The results showed that constipation improved significantly in 89% of subjects in the intervention group, the frequency of bowel movements increased to 6 times/week, distention and bloating significantly improved, and no side effects were reported.

In the present study, the percentage of subjects with constipation showed declining trend after a 2-week intake of the tested fermented milk. The result of this study showed the possibility that the tested fermented milk could improve stool consistency and bowel movement frequency. This result in the present study is consistent with previous reports that LcS improved stool consistency and defecation frequency.[16-18]

The beneficial effects of LcS may be explained by various mechanisms. Changes in the composition of the intestinal microbiota may result in changes in metabolites of bacterial fermentation and in enhanced intestinal motility, attributable to a lowering of the fecal pH value or a shortening of transit time. Some studies showed that drinking LcS modifies the composition and metabolic activity of the intestinal microbiota. These mechanisms may contribute to the beneficial effects on constipation after 2 weeks of LcS ingestion.

The effect of LcS on stool consistency in the present study could be caused directly by the presence of LcS during the 14 days of ingestion. Stool consistency is related to a number of factors, including transit time. The significant improvement in stool consistency observed may be explained by a shortened transit time. This was suggested in previous a report by Koebnick.[18] However, it is necessary to conduct further research on improving constipation.

In conclusion, we have shown that LcS survives passage through the gastrointestinal tract of Vietnamese adults.

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