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# Recovery of *Lactobacillus casei* strain Shirota (LcS) from faeces of healthy Singapore adults after intake of fermented milk

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**RESEARCH ARTICLE**

## Abstract

To validate survival of *Lactobacillus casei* strain Shirota (LcS) during passage through the gastrointestinal tract of healthy Singaporean young adults, 21 participants (18-25 years old) were asked to consume a 100 ml of fermented milk drink containing $1.0\times10^8$ cfu/ml of LcS daily for 14 days, and to maintain their dietary habit and life style. During and at the end of the ingestion period, both culture method (identity confirmed by ELISA) and 16s rRNA sequencing results revealed that viable LcS (7.27 and 7.64 $\log_{10}$ cfu/g of faeces at the ingestion period Day 7 and Day 14, respectively) and *Lactobacillus* could be recovered from the faeces of all the subjects. The viable LcS count from male and female were comparable for each time point. Before consumption (baseline) and 14 days after cessation of consumption of the fermented milk, LcS was not detected in most of the subjects. In this study condition, the composition of the major gut microbiota (>0.1% in relative abundance of genus) and characteristics of defaecation such as stool consistency and frequency of defecation did not change throughout the study before and after ingestion of LcS. LcS was able to survive passage through the gastrointestinal tract of Singapore adults without sustainable colonisation, but the effect of LcS on microbiota modulation, stool consistency and frequency was not observed under this study condition.

**Keywords:** probiotics, *Lactobacillus casei* strain Shirota, health

## 1. Introduction

The microbiota in human host refers to the colonisation of microorganisms, such as fungi, bacteria, and archaea on the various habitats within the human host. The large abundance of microbes within the body establishes its basis for microbial symbiosis where these microbes play a role in the modulation of human health and physiology (Turnbaugh *et al.*, 2007). The gut microbiota makes up more than 50% of the total microbes that colonise the entire human body (Rodríguez *et al.*, 2015), and it is no doubt that the large abundance of gut microbiota plays an important role in the modulation of human health. For example, the presence of *Lactobacillus* in the gut serves to protect against pathogenic infections (Shu and Gill, 2002). Moreover, changes in the gut microbiota composition could also affect the health status of the host. Imbalance microbiome in the gut has been implicated in the pathogenesis of diseases, such as allergic disorders and irritable bowel disease (DeGruttola *et al.*, 2016). As such, the knowledge in gut microbiota composition and their relative abundance could potentially have an impact on human health paves the way for future studies on the possible use of gut microbiota to promote health.

In recent decades, probiotics have been widely available in the consumer market and are very often marketed as a functional food. Probiotics are foods or supplements that contain live microbes (Ziemer and Gibson, 1998) and adequate consumption of these probiotics have been

suggested to bring about beneficial health effect to the host (Kechagia *et al.*, 2013). Commonly used probiotics are lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* with the various strains (Hemarajata and Versalovic, 2013). The main health benefits of probiotics are an enhancement of immunity against intestinal infections, modulation of human intestinal microbiota, prevention of diarrhoea disease, colon cancer, hypercholesterolemia, and gastrointestinal tract disease, and improvement in lactose utilisation, and stabilisation of the gut mucosal barrier (Kailasapathy and James, 2000). The probiotic efficacy relies on their ability to survive passage in the digestive system. The ability of *Lactobacillus* and *Bifidobacterium* to survive in the gastrointestinal tract varies between species and strains considerably (Guergoletto *et al.*, 2010). With the possibility of different probiotics exerting different effects on the host, it is thus important that the study utilises a single strain of probiotic, where the effect on the gut microbiota could be certain that it results from the specific probiotic used.

The *Lactobacillus casei* strain Shirota (LcS) is an exclusively registered probiotic strain that is used in the fermented milk products of Yakult Honsha Co., Ltd. (Tokyo, Japan). Numerous studies have been conducted to investigate the beneficial effect of LcS. The introduction of LcS in the gut has been suggested in studies to prevent bladder cancer recurrence (Aso and Akazan, 1992). Studies have also suggested that the bacteria are able to suppress food-induced allergies and anaphylaxis (Shida *et al.*, 2002). Consumption of the probiotic could also potentially be used as a health-promoting tactic to combat obesity. A pilot study has shown that this strain of probiotic is able to effectively improve lipid metabolism of the host thereby promoting weight loss (Wang *et al.*, 2017). With the numerous health benefits that accompany the consumption of LcS, it is thus important to investigate if its consumption leads to similar effects in subjects with varying baseline gut microbiota profile or genetic background. The survivability in the gastrointestinal tract is one of the most important features of probiotics, and that of was assessed in different regions and countries (Japan (Yuki *et al.*, 1999), Thailand (Tiengrim *et al.*, 2012), China (Wang *et al.*, 2015), UK (Sakai *et al.*, 2010), Vietnam (Mai *et al.*, 2017), and Indonesia (Utami *et al.*, 2015). The survival of LcS through gut might be associated with factors such as type and composition of food consumed, lifestyle, environment, and race. For this reason, we investigate the recovery of viable LcS in Singapore adults who had different lifestyle, food habit, and the living environment from other countries.

## 2. Materials and methods

Twenty-seven healthy Chinese Singaporean adults of 18-25 years old were recruited. The subjects who are going to be 18 years old were also included in the study. Six subjects were rejected because of having fermented dairy products except for study products during the study period or unwillingness of the subjects to complete the study or difficulties in sample storage or absence during the study. The duration of the study for each subject was 42 days (Figure 1). This includes 14 days baseline period, 14 days ingestion period and 14 days follow up period. At the start of the recruitment, the subjects were instructed to abstain from the consumption of all probiotic food/beverages and fermented food, such as kimchi, tempeh, and cheese among many others. During the ingestion period, each subject consumed one bottle of study product per day (Yakult drink, containing 10 billion LcS, after lunch), and instructed to maintain their dietary habit and lifestyle, to remove these potential confounding factors. During the study, daily food intake, concomitant medications, and bowel movement were recorded. Faecal samples were collected before, during ingestion and post ingestion at specific time points. The stool characteristic was recorded and determined based on the Bristol Stool Form Scale (Lewis and Heaton, 1997). The number of viable LcS was investigated using the culture method and confirmed by Enzyme-linked Immunosorbent Assay (ELISA). The monoclonal antibody for LcS was a gift from Yakult Central Institute, Japan. The gut microbiome profile was also analysed using 16S rRNA sequencing.

Faecal DNA was extracted using phenol-chloroform and ethanol DNA precipitation after washing the samples. Then,

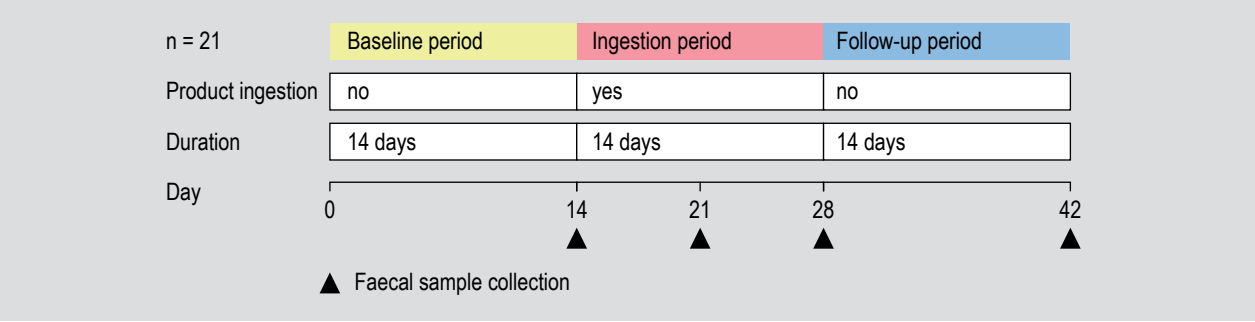


**Figure 1. Schematic diagram of study design. 14 days last for ingestion and follow-up periods, respectively. Four time points for faecal samples collection at baseline, ingestion day 7, ingestion day 14 and end of follow-up. n = number of subjects.**

the concentration of dsDNA was accurately quantified by the PicoGreen fluorescent based method. The extracted DNA was amplified at V3-V4 region of the 16S rRNA gene followed by the addition of indices to the purified amplicons for individual sample barcoding purpose. The DNA library was checked for its quantity and quality by each sample. The libraries were adjusted for their concentration and then pooled together. The pooled library was quantified again by qPCR to adjust the concentration and was loaded into the MiSeq sequencing system (Illumina, New York, NY, USA) after denaturation. After the run was completed, the demultiplexed reads were further analysed using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatic pipeline (Caporaso *et al.*, 2010; Kuczynski *et al.*, 2012) and Greengene bacterial database (gg 13_8). The appropriate graphs and statistical analysis were done by GraphPad Prism 7 (GraphPad, Inc., San Diego, CA, USA) software and Canoco5 (Microcomputer Power, Ithaca, NY, USA) software and R 3.5.2 software (RStudio, Inc., Boston, MA, USA). For statistical analysis, a non-parametric test of Friedman test and Nemenyi post-hoc pairwise comparison test were performed to find the significant differences of stool consistency, the frequency of defecation and the relative abundance of the bacterial genera across the time points. Permutational multivariate analysis of variance (PERMANOVA) test was used to compare the dissimilarities of the bacterial communities in Principle coordinate analysis (PCoA).

## 3. Results

The biodata of the subjects is summarised in Table 1. The LcS concentration was enumerated by culture method and its identity confirmed by ELISA method. The results are summarised in Table 2.

LcS was found in faecal samples of six participants after the baseline period at an average of 5.29 $\log_{10}$ cfu/g of faeces. During the ingestion period (Ingestion Day 7 and Day 14), LcS was detected at an average of 7.27 $\log_{10}$ cfu/g of faeces and 7.64 $\log_{10}$ cfu/g of faeces in all the subjects (n=21) respectively. LcS was only detected in the faeces of five volunteers on the 42$^{th}$ day (at the end of follow-up period) at an average of 6.97 $\log_{10}$ cfu/g of faeces.

**Table 1. Biodata of subjects (mean ± standard deviation).**

| | |
|---|---|
| Age (years) | 18.8±1.0 |
| Height (cm) | 164.4±0.1 |
| Weight (kg) | 60.3±11.0 |
| Body Mass Index (kg/m$^2$) | 22.1±3.0 |
| Gender (n) | |
| Female | 14 |
| Male | 7 |

Among the male and female subjects, LcS was found in faecal samples of one out of seven and five out of 14 participants after the baseline period respectively. During the ingestion Day 7, LcS was detected at an average of 7.26 and 7.28 $\log_{10}$ cfu/g of faeces for males and females, respectively. On ingestion Day 14, LcS counts for males and females were 7.66 and 7.62 $\log_{10}$ cfu/g of faeces, respectively. LcS was only detected in the faeces of one male and four female volunteers at the end of follow-up period.

The stool consistency scale was not statistically significant by Friedman test (Figure 2A). However, the frequency of defecation (Table 3) was statistically significant by Friedman test at a *P*-value of 0.021, but not statistically significant between each time point by Nemenyi post-hoc pairwise comparison test (Figure 2B).

Gut bacterial communities from any two time points were not statistically significant as performed by permutational multivariate analysis of variance (PERMANOVA) (Supplementary Table S1). The overall bacterial profile of both genders as well as each gender was shown in Figure 3. In the analysis of both genders, except for *Lactobacillus*, there were no significant difference between each time point using both the Friedman test and Nemenyi post-hoc pairwise comparison test (Supplementary Tables S2, S5 and S8). In the analysis of male and female, statistical difference was detected in various genus including *Lactobacillus* (Supplementary Tables S3, S4, S6, S7, S9 and S10).

As shown in Figure 4, Nemenyi post-hoc pairwise comparison test was performed for comparison of the relative abundance of *Lactobacillus*. Statistical differences could be seen between baseline and during the ingestion period and between during and post-ingestion period in both gender and each gender (Figure 4 and Supplementary Tables S8-S10).

## 4. Discussion

During the ingestion period, the LcS was detected at a relatively high abundance in all the subject's stools. This result indicates that the LcS in the tested fermented milk is able to survive passage through the gastrointestinal tract of Singapore adults and this confirms previous similar reports on the recovery of LcS in other countries (Mai *et al.*, 2017; Sakai *et al.*, 2010; Tienrim *et al.*, 2012; Utami *et al.*, 2015; Wang *et al.*, 2015; Yuki *et al.*, 1999). Recovery of LcS from one subject decreased to 2.85 $\log_{10}$ cfu/g faeces in the second week of ingestion, which reason is unknown.

In this study, the LcS was not detected in most of the subjects (16/21) in 14 days after consumption of the fermented milk. However, it was only detected in five subjects after consumption was stopped. This shows variation in LcS persistence during the wash-out period. The persistent

**Table 2. Enumeration of faecal LcS, based on culture and ELISA method.[1]**

| Subject IDs | Baseline | Ingestion | | Follow-up |
|---|---|---|---|---|
| | | Day 7 | Day 14 | |
| S1 (female) | ND, 0.00* | 5.57, 371,535.23* | 6.53, 3,388,441.56* | ND, 0.00* |
| S2 (female) | 3.04, 1,096.48* | 7.00, 10,000,000* | 2.85, 707.95* | ND, 0.00* |
| S3 (female) | ND, 0.00* | 7.92, 83,176,377.11* | 7.98, 95,499,258.60* | ND, 0.00* |
| S4 (female) | ND, 0.00* | 6.26, 1,819,700.86* | 7.37, 23,442,288.15* | ND, 0.00* |
| S5 (female) | ND, 0.00* | 6.54, 3,467,368.50* | 7.01, 10,232,929.92* | ND, 0.00* |
| S6 (female) | ND, 0.00* | 6.88, 7,585,775.75* | 6.34, 2,187,761.62* | ND, 0.00* |
| S7 (female) | ND, 0.00* | 5.64, 436,515.83* | 7.58, 38,018,939.63* | ND, 0.00* |
| S8 (male) | ND, 0.00* | 6.93, 8,511,380.38* | 7.50, 31,622,776.60* | ND, 0.00* |
| S9 (male) | ND, 0.00* | 6.71, 5,128,613.84* | 7.06, 11,481,536.21* | ND, 0.00* |
| S10 (male) | ND, 0.00* | 7.57, 37,153,522.91* | 7.44, 27,542,287.03* | ND, 0.00* |
| S11 (female) | ND, 0.00* | 6.32, 2,089,296.13* | 6.96, 9,120,108.39* | ND, 0.00* |
| S12 (female) | 5.11, 128,824.96* | 7.00, 10,000,000* | 6.43, 2,691,534.80* | ND, 0.00* |
| S13 (female) | 5.03, 107,151.93* | 7.07, 11,748,975.55* | 8.51, 323,593,656.93* | 8.29, 194,984,459.98* |
| S14 (female) | 2.58, 380.19* | 6.52, 3,311,311.21* | 7.80, 63,095,734.45* | 4.34, 21,877.62* |
| S15 (female) | 3.19, 1,548.82* | 7.49, 30,902,954.33* | 6.00, 1,000,000* | 5.45, 281,838.29* |
| S16 (female) | ND, 0.00* | 7.32, 20,892,961.31* | 6.58, 3,801,893.96* | 5.82, 660,693.45* |
| S17 (female) | ND, 0.00* | 7.91, 81,283,051.62* | 7.13, 13,489,628.83* | ND, 0.00* |
| S18 (male) | 6.59, 3,890,451.45* | 6.65, 4,466,835.92* | 7.95, 89,125,093.81* | ND, 0.00* |
| S19 (male) | ND, 0.00* | 7.00, 10,000,000* | 6.07, 1,174,897.55* | 3.30, 1995.26* |
| S20 (male) | ND, 0.00* | 7.49, 30,902,954.33* | 7.99, 97,723,722.10* | ND, 0.00* |
| S21 (male) | ND, 0.00* | 7.50, 31,622,776.60* | 7.79, 61659500.19* | ND, 0.00* |
| Average | 5.29 (5.74, 4.23) | 7.27 (7.26, 7.28) | 7.64 (7.66, 7.62) | 6.97 (2.45, 7.15) |
| SD | 5.93 (6.17, 4.63) | 7.38 (7.15, 7.45) | 7.86 (7.58, 7.93) | 7.63 (2.88, 7.72) |
| Maximum | 6.59 (6.59, 5.11) | 7.92 (7.57, 7.92) | 8.51 (7.99, 8.51) | 8.29 (3.3, 8.29) |
| Minimum | ND (ND, ND) | 5.57 (6.65, 5.57) | 2.85 (6.07, 2.85) | ND (ND, ND) |
| Detection rate | 6/21 (1/7, 5/14) | 21/21 (7/7, 14/14) | 21/21 (7/7, 14/14) | 5/21 (1/7, 4/14) |

[1] The values are presented in $\log_{10}$ cfu/g and cfu/g* of faeces. Detection rate is described as the detected/total number of samples. The average and standard deviation (SD) are calculated based on the data of all the subjects for each time point. The values of average, SD, maximum, minimum and detection rate for males/females are presented in parenthesis. ND = not detected.

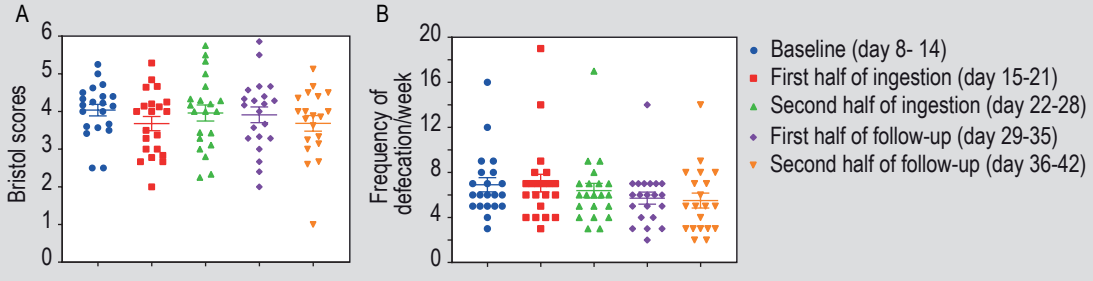


**Figure 2. Scatter plots showing Bristol stool consistency scale (A) and frequency of defaecation per week (B). Mean values and standard error of the mean (SEM) are shown.**

level of LcS could be influenced by individual dietary habit and lifestyle of Singaporeans. Some literature reports have shown a similar observation (Mai *et al.*, 2017; Utami *et al.*, 2015). As shown in Table 2, there is a large difference among individuals in the pattern of LcS recovery during the study period although the factor causing individual variation is not clear.

**Table 3. Summary of effect of *Lactobacillus casei* strain Shirota on the individual data of average Bristol stool consistency scores and frequency of defecation per week.**

| Subject ID | Baseline (day 8-14) | | First half of ingestion (day 15-21) | | Second half of ingestion (day 22-28) | | First half of follow-up (day 29-35) | | Second half of follow-up (day 36-42) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Scores | Frequency of defecation | Scores | Frequency of defecation | Scores | Frequency of defecation | Scores | Frequency of defecation | Scores | Frequency of defecation |
| S1 | 4.14 | 7 | 2.78 | 9 | 3.44 | 9 | 3.57 | 7 | 3.63 | 8 |
| S2 | 4.71 | 7 | 4.67 | 6 | 4.17 | 6 | 2.67 | 3 | 4.40 | 5 |
| S3 | 4.00 | 5 | 4.14 | 7 | 4.00 | 5 | 4.17 | 6 | 4.00 | 5 |
| S4 | 3.42 | 12 | 2.67 | 3 | 4.33 | 3 | 3.67 | 3 | 3.80 | 5 |
| S5 | 4.22 | 9 | 4.13 | 8 | 3.29 | 7 | 4.67 | 6 | 4.50 | 2 |
| S6 | 5.00 | 5 | 4.00 | 6 | 4.20 | 5 | 4.67 | 6 | 4.50 | 2 |
| S7 | 4.17 | 6 | 4.25 | 4 | 5.50 | 6 | 5.50 | 2 | 4.67 | 3 |
| S8 | 3.50 | 6 | 2.00 | 4 | 2.25 | 4 | 2.00 | 4 | 3.33 | 3 |
| S9 | 5.25 | 8 | 4.20 | 5 | 4.00 | 4 | 4.00 | 6 | 4.00 | 6 |
| S10 | 4.63 | 16 | 4.84 | 19 | 4.29 | 17 | 4.57 | 14 | 4.36 | 14 |
| S11 | 2.50 | 8 | 3.00 | 7 | 5.00 | 6 | 5.86 | 7 | 3.25 | 8 |
| S12 | 3.67 | 6 | 4.64 | 14 | 5.75 | 8 | 4.29 | 7 | 5.13 | 8 |
| S13 | 2.50 | 6 | 2.83 | 6 | 3.00 | 4 | 3.00 | 4 | 2.67 | 3 |
| S14 | 4.40 | 5 | 2.67 | 6 | 2.80 | 5 | 2.40 | 5 | 1.00 | 3 |
| S15 | 4.40 | 5 | 3.00 | 4 | 4.67 | 3 | 4.33 | 3 | - | - |
| S16 | 4.00 | 3 | 3.50 | 4 | 5.33 | 6 | 4.40 | 5 | 3.00 | 4 |
| S17 | 3.60 | 5 | 4.00 | 7 | 2.33 | 6 | 4.20 | 5 | 4.00 | 3 |
| S18 | 4.25 | 4 | 5.29 | 7 | 3.11 | 9 | 3.33 | 6 | 2.60 | 5 |
| S19 | 3.57 | 7 | 3.29 | 7 | 3.43 | 7 | 3.29 | 7 | 3.86 | 7 |
| S20 | 4.33 | 9 | 4.00 | 7 | 4.00 | 7 | 4.29 | 7 | 3.89 | 9 |
| S21 | 4.50 | 6 | 3.38 | 8 | 4.29 | 7 | 3.29 | 7 | 3.14 | 7 |
| Average | 4.04 | 6.90 | 3.68 | 7.05 | 3.96 | 6.38 | 3.91 | 5.71 | 3.69 | 5.50 |
| SD[1] | 0.70 | 2.88 | 0.86 | 3.60 | 0.98 | 2.97 | 0.96 | 2.47 | 0.92 | 2.96 |
| Maximum | 5.25 | 16 | 5.29 | 19 | 5.75 | 17 | 5.86 | 14 | 5.13 | 14 |
| Minimum | 2.50 | 3 | 2.00 | 3 | 2.25 | 3 | 2.00 | 2 | 1.00 | 2 |

[1] SD = standard deviation.

In general, average stool consistency was in a normal range during the study period, but the average frequency of defecation seems to be reduced gradually throughout the ingestion period till the end of the study. However, the individual changes of defecation frequency might have varied as shown in Table 3 because of seasonal differences in subjects daily habits including food intake.

In our study, LcS administration did not alter the relative abundance of major gut microbiota at the genus level in the analysis of whole subjects (Figure 3 and Supplementary Tables S2, S5 and S8). Similarly, the overall diversity of gut microbiota did not change in LcS administration in a past study (Stadlbauer *et al.*, 2015). Changes in the abundance of major gut microbiota may depend on the host factors: i.e. age, race, lifestyle, the health of human gut, overall health status, dietary habit and so on, and the type of host gut microbiome and foreign agent (the introduced bacteria) factors: i.e. the dose of LcS consumption, duration of feeding, time of feeding and adhesion property of LcS (Evivie *et al.*, 2017; Ouwehand *et al.*, 2001; Sanders, 2011). In the aspect of the microbial composition, most of *Lactobacillus* spp. are found in the small intestine (Reuter, 2001; Sandine, 1978). This enumeration of *Lactobacillus* based on its abundance in faeces might have under-represent changes in the small intestine. On the other hand, in the analysis of male and female, statistical differences between each time point were detected not only in *Lactobacillus* but also some genus but, there is not enough information to consider their biological and physiological significance.

However, we could see changes in the relative abundance of *Lactobacillus* among gut microbiota after LcS consumption. This result was supported by the data from the recovery

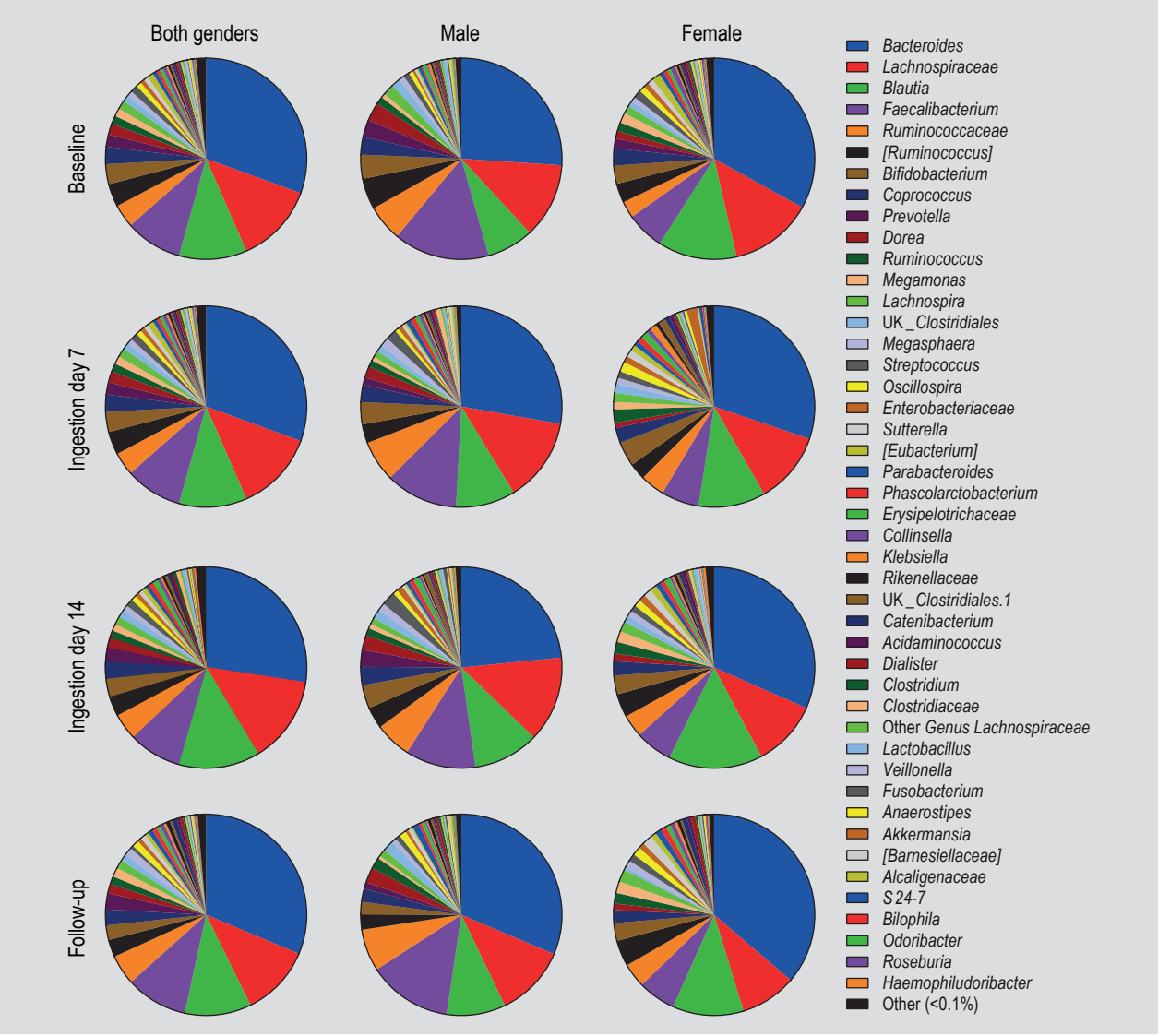


**Figure 3. The composition of faecal microbiota in all the subjects at the genus level across the time points. Both genders, female and male plots are separated into 3 columns. The relative abundance of the genus >0.1% is shown in the percentage for each time point. UK = unknown.**

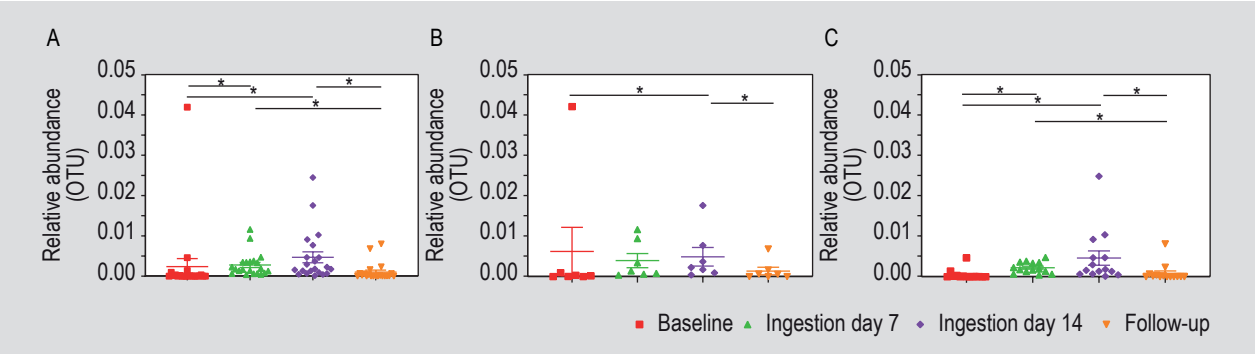


**Figure 4. Comparison of the relative abundance of *Lactobacillus* across the time points for (A) both genders (B) male (C) female. The scatter plot shows the relative abundance of *Lactobacillus* at different time points. Mean and standard error of the mean (SEM) are shown. Asterisk represents significantly different at *P*<0.05 between the two time points.**

of LcS during the ingestion period detected by ELISA. Both ELISA and 16S rRNA could not detect LcS and *Lactobacillus* respectively in most of the subjects in the post-ingestion period. The method employed to analyse the faecal microbiota in this study did not allow identification of *Lactobacillus* species. LcS may have affected the commensal lactobacilli counts during its ingestion, but it was not clear in this study.

## 5. Conclusions

The study demonstrated that LcS can survive during passage through the gastrointestinal tract of Singapore adults and this confirms previous similar reports on the recovery of LcS in other countries. The study also showed that LcS is not able to colonise and persist in the intestinal tract of Singaporeans. Under this study condition, no interference by LcS on the major microbiota composition was observed and any probiotic effects of LcS on defecation due to modulation of gut microbiota profile was not observed in Singaporeans.

## Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2018.0173.

**Table S1.** Results of permutational multivariate analysis of variance (PERMANOVA) of the gut microbial communities using Bray-Curtis distance.

**Table S2.** Results of Friedman rank sum test of major genus (>0.1% in relative abundance) for both genders.

**Table S3.** Results of Friedman rank sum test of major genus (>0.1% in relative abundance) for the female subjects.

**Table S4.** Results of Friedman rank sum test of major genus (>0.1% in relative abundance) for the male subjects.

**Table S5.** Mean and standard deviation of relative abundance of genera of both genders.

**Table S6.** Mean and standard deviation of relative abundance of genera of female subjects.

**Table S7.** Mean and standard deviation of relative abundance of genera of female subjects.

**Table S8.** Results of Nemenyi PostHoc pairwise comparison test of major genus (>0.1% in relative abundance) for both genders.

**Table S9.** Results of Nemenyi PostHoc pairwise comparison test of major genus (>0.1% in relative abundance) for the female subjects.

**Table S10.** Results of Nemenyi PostHoc pairwise comparison test of major genus (>0.1% in relative abundance) for the male subjects.

## Acknowledgements

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