

Original Article

Recovery of *Lactobacillus casei* strain Shirota (LcS) from faeces with 14 days of fermented milk supplementation in healthy Australian adults

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Background and Objectives: A key measure for classifying bacteria as a probiotic is the ability to survive gastric transport and be recoverable in faeces. The aim of this study was to determine whether *Lactobacillus casei* strain Shirota (LcS) could be recovered in the faeces of healthy young Australian adults following ingestion of a fermented milk drink. **Methods and Study Design:** A cohort of 25 healthy individuals (male/female: 14/11; age: 29.3±6.6 years; BMI: 25.3±2.7 kg/m², mean±SD) ingested one 65 ml bottle of fermented milk containing 6.5×10⁹ LcS live cells daily for 14 days. Participants provided a faecal sample at day 0, day 7 (mid-supplementation), day 14 (end of supplementation) and 14 days after cessation of the supplement (day 28) for assessment of the number of viable LcS via microbial culture on selective media with confirmation using a colony-direct polymerase chain reaction and species-specific primers. **Results:** The supplement was well tolerated by participants. No LcS colonies were recovered from participants prior to ingestion of the fermented milk drink. All participants had recoverable LcS colonies at day 7 and day 14, with a mean recovery of 6.5±1.1 and 6.4±1.1 log₁₀ CFU/g of faeces (mean±SD) at each time point respectively. LcS was detectable in only one sample at 14 days following the cessation of supplementation. **Conclusions:** Live LcS is recoverable in faeces from healthy Australian adults following daily ingestion of a fermented milk drink.

Key Words: *Lactobacillus casei* strain Shirota, probiotics, Yakult, faecal

INTRODUCTION

Probiotics, defined in 2001 by the World Health Organisation^{1,2} as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host, continue to be of strong interest as a supplement to maintain good health. Initial evidence for the use of probiotics was for gut health, in particular gastrointestinal infections and antibiotic-associated diarrhoea,³ but has extended to illnesses associated with the respiratory and urogenital tracts and skin, metabolic and immune regulation and, more recently, mental health.⁴ Understanding the mechanisms by which probiotics may exert these positive health effects necessitates consideration of the survivability of probiotic bacteria within the gastrointestinal tract (GIT).

The GIT provides a niche environment for a rich microbial community. The intestinal microbiome has been the subject of extensive analysis, particularly its composition and stability within and between individuals.⁵ Containing more than 10¹³ individual bacteria belonging to hundreds of unique species, the microbiome plays a key role in the ontogeny of the immune system and the regulation of mucosal homeostasis, particularly in the gut. Animal and *in-vitro* studies have shown that the microb-

iota interacts with the mucosal epithelium and underlying immune cells, produces metabolic substrates (such as short chain fatty acids from non-digestible starches for energy), vitamins and enzymes, and are able to modulate the secretion of neuropeptides within the GIT.⁶ Given evidence of its extensive interaction with its host, the microbiome has been classified as an organ in its own right⁷ and the potential to modulate the microbiome to influence health outcomes continues to receive attention.

The increasing demand for probiotics both as supplements and in fortified foods has led to a growing focus by regulators and the scientific community on the selection of bacterial strains to be defined as potential probiotic bacteria. A number of key criteria have been identified, in particular resistance to intestinal acid and bile, survival of

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gastric transport, and to be non-toxic.⁸ Considerable evidence suggests *Lactobacillus casei* strain Shirota (LcS), contained in a commercially available fermented milk drink, meets these criteria.⁹⁻¹¹ Importantly, LcS is recoverable as live bacteria through bacterial culture rather than through molecular techniques that, while more sensitive, do not provide important information on survival of the bacteria in the digestive tract. The viability of probiotics may alter their effects at the mucosa, with live strains having a greater effect on the composition of the intestinal microbiota and on immune responses in comparison to dead cells.¹² Accounting for the viability of ingested strains may be as important as considering strain specificity when considering the potential benefits of probiotic supplementation.

Given that the effects of probiotics are strain specific and may vary between different population groups it is necessary to confirm previous findings that LcS is recoverable in various population cohorts.^{11,13-15} The aim of this study is to examine whether LcS is recoverable in faeces after ingestion of a fermented milk drink containing live LcS in healthy Australian adults.

METHODS

Design

A single-arm open-label trial was conducted over 42 days to assess the effects of supplementation with a fermented probiotic milk drink on the faecal recovery of LcS. The study consisted of three phases: (i) a 14 day pre-test period (day -14 to 0) in which subjects ceased consuming probiotic supplements or foods that contained probiotics or prebiotics, (ii) a 14 day ingestion period (day 0 to day 14) with faecal sampling at day 0, day 7 and day 14, and (iii) 14 day follow up period (day 15 to 28) at the end of which participants provided another faecal sample (Figure 1).

The Griffith University Human Research Ethics Committee provided ethics approval for the project (AHS/37/14/HREC). The study was prospectively registered with the Australian New Zealand Clinical Trials Registry (ANZCTR; ACTRN12618001607268).

Participants

Participants were recruited from the local community of the Gold Coast, Australia. Interested individuals were initially screened against the inclusion criteria including aged 18-40 years, body mass index (BMI) <30 kg/m², and reported history of daily bowel movements. Individuals were excluded if they had a history of gastrointestinal illness/symptoms (diarrhoea, constipation, abdominal pain, irritable bowel syndrome, inflammatory bowel dis-

ease, Coeliac Disease), were lactose intolerant, or had used antibiotic, antidiarrheal or laxative medications or probiotic supplements in the last 30 days. All participants were fully informed of the study procedures and provided written informed consent prior to participation.

Participants attended the Griffith University Clinical Trials Unit for an initial screening assessment prior to commencing the study at which time height and weight were recorded and a questionnaire regarding their bowel habits, including the Bristol Stool Scale¹⁶, was completed.

Supplementation

The probiotic supplement used was a commercially available fermented milk drink containing 6.5 billion LcS per 65 mL (8.0 log₁₀ cfu/mL) (Yakult, Yakult Australia, Melbourne, Australia). Participants were asked to consume one 65 mL bottle daily following breakfast and to keep the supplements stored in a fridge. Participants were asked to record their consumption on a daily checklist to allow assessment of compliance. Participants were also required to refrain from consuming non-study probiotics in supplement form or in fermented foods during the trial.

Sample collection

Faecal samples were collected at day 0 (end baseline period), day 7 (mid-point of supplement period), day 14 (end of supplementation) and again 14 days after supplementation (day 28) (Figure 1). Participants were instructed to defecate on to water resistant paper placed in a toilet bowl and to immediately collect a portion of faecal material into a container with a specially designed scoop. The sample had to be free from urine or water contamination and was stored in a cold container (10 °C) until transport to the laboratory (within 12 hours of collection).

Microbial culture and identification

The culture and enumeration of LcS in faeces was undertaken according to the method of Tiengrim¹¹ with modifications. Briefly, faecal samples were homogenised in nine volumes (weight:volume) of transport medium. A 10-fold dilution series of each faecal suspension was then prepared in phosphate buffered saline (PBS) to 10⁻⁶. A volume of 100 uL of each diluted sample was spread over lactitol-LBS vancomycin (LLV) plus fosfomycin medium (LLV-FOM) plate and incubated aerobically at 37°C for 96 hours. Plates that had 30-300 large, white, dome shaped colonies were counted for LcS enumeration. A positive control prepared from the same batch of the fermented milk drink consumed by participants (and diluted at 10⁻⁵, 10⁻⁶ and 10⁻⁷) was also cultured at each time point. The limit of detection was considered to be 2.0 log₁₀ cfu/g

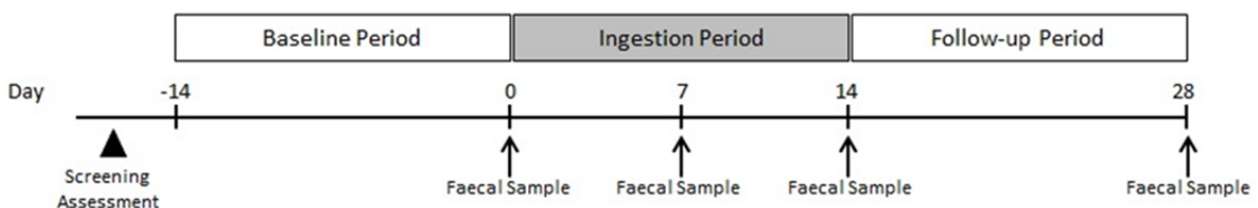


Figure 1. The study was design as single-arm open-label trial consisting of a 14 day baseline period, 14 day ingestion period and 14 day follow-up period with faecal samples collected prior to, during and following the ingestion phase.

faeces.

Confirmation of colonies as LcS was determined using a colony-direct polymerase chain reaction (PCR) method¹⁷ and species specific primers.¹⁸ Briefly, for each sample, eight LcS-like colonies were selected at random from a single plate (at the dilution containing 30-300 colonies; Figure 2A). Colonies were suspended directly in PCR reaction mix containing PCR Buffer (Takara Bio Inc, Shiga, Japan), 1.5 mM MgCl (Takara Bio Inc), 200 uM dNTPs (Takara Bio Inc), 0.75 U Taq Polymerase (Takara Bio Inc), 0.26 uM forward primer (5'-CTCAAAGCCGTGACGGTC-3'), 0.26 uM reverse primer

(5'-CACTAGGATTATTAGCACCACGT-3') and nuclease free water to a final reaction volume of 25 uL. Reactions underwent thermal cycling consisting of 95 °C for 5 min; 35 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 40 s; and finally 1 cycle of 72 °C for 180 s. PCR products were resolved on a 1.5% agarose gel to confirm product size (~500 bp) in relation to the positive control (Figure 2B).

The number of LcS per gram of faecal sample was calculated using the following formula:

$$\text{LcS (cfu/g)} = \frac{[(\text{number of LcS-like colonies}) \times (\text{LcS+}) \times (\text{dilution of faecal sample})]}{[(\text{inoculation volume of faecal sample (i.e 0.1 mL)}) \times 100]}$$

Statistical analysis

The pre-specified primary analysis involved a within group repeated measures comparison of the change in LcS recovery between day 0 (end of pre-test), day 7 (mid-point of supplementation), day 14 (end of supplementa-

tion) and 14 days after supplementation (day 28). Sample size was based on previous studies that reported a significant increase in the faecal recovery of LcS with a similar protocol.¹¹ Recovery data were not normally distributed and were log (log₁₀) transformed prior to analysis to reduce non-uniformity of error. For samples where LcS was not detected, counts were set to the limit of detection to allow for statistical analysis. Change in LcS counts over the four time-points were assessed initially using a one-way analysis of variance (ANOVA). Differences between time-points (day 7 and day 14 compared to day 0) were evaluated using a paired-sample t-test. Differences between genders at a given time point (day 7 and day 14) were evaluated using a Student's t-test. Significance was set at $p=0.05$ and data is presented as mean \pm standard deviation (SD).

RESULTS

A total of 25 participants were recruited to the study, with data from all participants included in the final analysis (Figure 3). In general the cohort consisted of young adults (14 male, 11 female; age 29.3 \pm 6.6 years; BMI: 25.3 \pm 2.7 kg/m²) with a reported history of daily bowel movements and a Bristol Stool score of 3.8 \pm 0.7.

The supplement was generally well tolerated; one participant reported mild gastrointestinal symptoms (bloating) exclusively in the first 72 hours of supplementation, another participant reported bloating periodically during the baseline, supplementation and follow-up phases. Assessment of compliance using a daily checklist indicated 100% compliance for all participants. All faecal samples were collected as scheduled, with the exception being

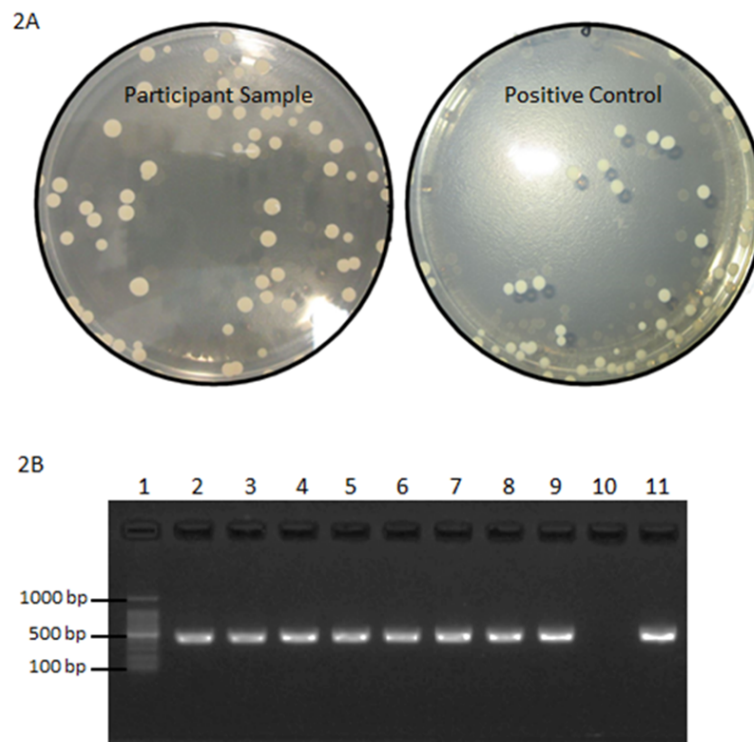


Figure 2. A) Example image of colony density for counting for a participant sample relative to the positive control. Large white dome shaped colonies were selected as LcS. (B) Example image of resolution of PCR products from colony-specific PCR for a participant sample relative to positive control. PCR products were resolved on a 1.5% agarose gel. Lane 1: molecular weight marker (100 base pair DNA ladder, TaKaRa Bio Inc, Japan); Lanes 2-9 individual colonies from a single participant sample; Lane 10: not used; Lane 11: positive control.

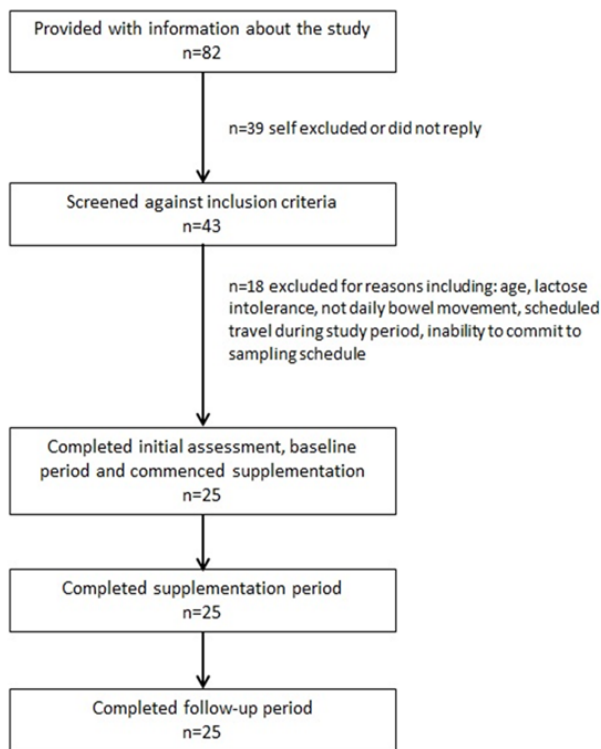


Figure 3. CONSORT diagram illustrating participant recruitment and retention in the study

samples from two participants at day 28 which were collected early (day 25) due to unanticipated travel.

LcS Recovery

No individuals had detectable LcS prior to the start of supplementation. LcS counts changed significantly over the four time-points ($p=5.56 \times 10^{-44}$). Compared to day 0, the mean recovery of LcS at day 7 was $6.48 \log_{10}$ cfu/g of faeces ($p=2.54 \times 10^{-16}$) and $6.43 \log_{10}$ cfu/g faeces ($p=9.03 \times 10^{-17}$) at day 14. Only one sample had detectable LcS at day 28 ($4.8 \log_{10}$ cfu/g).

No differences in recovery were noted between males and females at either day 7 ($6.54 \log_{10}$ v $6.41 \log_{10}$ cfu/g; $p=0.80$) or day 14 ($6.36 \log_{10}$ v $6.53 \log_{10}$ cfu/g; $p=0.70$). However, variation in the recovery of live LcS between individuals was noted (Table 1). In addition, the pattern of change differed between individuals; 16 individuals showed consistent recovery (<1 log fold change) between day 7 and day 14, five individuals showed a marked decrease (>1 log fold decrease; average -1.69 ± 0.40 log fold) in recovery between day 7 and day 14, and four individuals showed a marked increase in recovery (>1 log fold increase; average: 1.56 ± 0.43 log fold) between day 7 and day 14. There were no discernible differences in key attributes (age, gender, BMI) between individuals with different patterns of change in LcS recovery.

DISCUSSION

In unravelling the mechanisms via which probiotic bacteria are able to exert positive health effects, improved understanding of probiotic viability through the GIT is an important consideration. This study investigated the faecal recovery of LcS from a fermented milk drink in healthy Australian adults. The probiotic beverage was

well tolerated and LcS recovery averaged approximately $6.5 \log_{10}$ CFU/g faeces after 7 days of supplementation and was largely unchanged after a further 7 days of supplementation. These findings extend the growing evidence that LcS in a fermented milk drink is both safe and able to survive gastric transport to be recoverable in faeces.

Maintaining the viability of probiotic microorganisms and delivering them to the GIT is considered essential for the health promoting benefits of these supplements. Survival through the acidic environment of the GIT is strain specific and dependent on the matrix containing the microorganism.¹⁹ Findings from the current study are consistent with existing reports of the survival of LcS through the intestine. This includes several studies involving otherwise healthy adults,^{20,21} as well as in critically ill children²² and the elderly.²³ Recovery of live LcS was not dissimilar to other studies ($6.6 \log_{10}$)²⁴ involving healthy adults consuming a similar dose and as expected, was lower than studies involving consumption of higher doses.^{11,18} LcS was detected in only one sample at day 28, however, it was not possible draw direct comparison regarding the persistence of LcS following the cessation of supplement between this and earlier studies due to variation in the dose consumed, duration of the ingestion period and length of follow up. It is noted that studies using higher doses have reported persistence of LcS in a proportion (12-30%) of samples at 7 days following the cessation of supplementation.^{14,15} Collectively these findings, in conjunction with our own, suggests that for most individuals persistence of LcS following the cessation of supplementation at the 6.5 billion daily dose employed in the current study is less than 14 days and possibly less than 7 days.

One interesting aspect of the current study was the obvious individual responses both in regard to numbers of recovery and pattern of change (either increase, decrease or consistent recovery) between day 7 and day 14 samples. Despite consumption of a consistent dose by all participants and reported high rates of compliance (100%), minimum and maximum numbers of recovery differed by over four log fold. Reasons for this disparity are not clear, but might be related to differences in dietary composition, GIT acidity, gut motility or other aspects of gut physiology, although all participants were required to have a history of daily bowel movements for inclusion in the study. Such large variation between individuals means that prescription of an optimum LcS dose may not be a one-size-fits-all proposition. Indeed, not all studies report linear increases in faecal recovery of ingested probiotic species with increases in dose.²⁵⁻²⁷ In addition, lower doses of a probiotic supplement have been reported to elicit greater immunomodulation than higher doses²⁸ which is promising from the perspective of minimising the already low risk of any possible adverse events, while achieving positive effects for the host. Future studies may choose to also assess various aspects of gut physiology to better understand individualised patterns of recovery.

The potential for positive effects of LcS have been reported by others previously. Survival of the probiotic bacteria through the GIT to allow for an interaction between the ingested bacteria and the gut epithelial and underlying

Table 1. Recoverable *Lactobacillus casei* strain Shirota (LcS) expressed as colony forming units per gram faeces (cfu/g) for individuals at each of the four sampling points

Participant #	Gender	Recovery of live LcS – Log ₁₀ cfu/g faeces (cfu/g)			
		Day 0	Day 7	Day 14	Day 28
1	F	ND	3.8 (6.1×10 ³)	5.8 (5.7×10 ⁵)	ND
2	F	ND	6.7 (4.9×10 ⁶)	6.6 (4.3×10 ⁶)	ND
3	F	ND	7.1 (1.2×10 ⁷)	6.8 (6.8×10 ⁶)	ND
4	F	ND	8.1 (1.2×10 ⁸)	8.1 (1.3×10 ⁸)	ND
5	F	ND	5.3 (2.0×10 ⁵)	7.2 (1.5×10 ⁷)	ND
6	F	ND	7.0 (9.1×10 ⁶)	8.1 (1.1×10 ⁸)	ND
7	F	ND	8.0 (1.0×10 ⁸)	6.0 (9.2×10 ⁵)	ND
8	F	ND	7.6 (4.2×10 ⁷)	6.5 (3.4×10 ⁶)	4.8 (6.5×10 ⁴)
9	F	ND	5.2 (1.4×10 ⁵)	5.6 (4.0×10 ⁵)	ND
10	F	ND	5.1 (1.3×10 ⁵)	5.1 (1.2×10 ⁵)	ND
11	F	ND	6.8 (6.0×10 ⁶)	6.1 (1.2×10 ⁶)	ND
12	M	ND	6.5 (3.0×10 ⁶)	7.8 (6.1×10 ⁷)	ND
13	M	ND	6.9 (7.1×10 ⁶)	7.2 (1.7×10 ⁷)	ND
14	M	ND	8.1 (1.2×10 ⁸)	6.1 (1.3×10 ⁶)	ND
15	M	ND	6.2 (1.5×10 ⁶)	6.9 (8.2×10 ⁶)	ND
16	M	ND	7.7 (5.0×10 ⁷)	7.7 (4.8×10 ⁷)	ND
17	M	ND	6.0 (1.1×10 ⁶)	5.9 (7.0×10 ⁵)	ND
18	M	ND	7.0 (1.1×10 ⁷)	5.2 (1.4×10 ⁵)	ND
19	M	ND	4.2 (1.5×10 ⁴)	3.4 (2.5×10 ³)	ND
20	M	ND	7.0 (9.6×10 ⁶)	5.5 (3.3×10 ⁵)	ND
21	M	ND	6.0 (1.1×10 ⁶)	6.5 (3.1×10 ⁶)	ND
22	M	ND	5.6 (3.6×10 ⁵)	6.0 (9.9×10 ⁵)	ND
23	M	ND	7.1 (1.4×10 ⁷)	7.7 (4.7×10 ⁷)	ND
24	M	ND	6.6 (4.1×10 ⁶)	6.7 (5.2×10 ⁶)	ND
25	M	ND	6.7 (5.4×10 ⁶)	6.5 (3.4×10 ⁶)	ND

ND: not detected.

mucosal immune system is proposed to underpin any beneficial effects. Supplementation with LcS has been associated with a reduction in URTI in endurance athletes,¹⁰ the incidence of hard and lumpy stools in healthy adults²⁹ and the duration of gastroenteritis in the elderly.²³ These effects may be mediated through a variety of mechanisms initiated by microbial-host interaction in the colon, such as the induction of cytokines and immune cell activity,^{30,31} although consistent findings regarding changes in immune parameters are not yet established.³² The recovery of viable LcS in faecal samples in this study provides further support for possible interaction between ingested LcS and the host mucosal immune system.

Daily supplementation for 14 days with an LcS-containing fermented milk drink, led to the recovery of viable LcS in the faeces of healthy Australian adults. This indicates that the consumed LcS was able to withstand the harsh chemical environment of the upper GIT and reach the colon. These results are consistent with research conducted in Asia and Europe involving LcS-containing fermented milk drinks and provide a platform for further evaluation of the possible beneficial clinical effects that may be achieved in various settings using this probiotic strain.

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AUTHOR DISCLOSURES

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