Survival of the Probiotic *Lacticaseibacillus paracasei* strain Shirota (LcS) in the Gastrointestinal Tract of Generally Healthy Adults

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1. List of abbreviations

AE	adverse event
BMI	body mass index
C	Celsius
CFU	colony forming unit
d	day(s)
DNA	deoxyribonucleic acid
g	gram(s)
GI	gastrointestinal
h	hour(s)
ITT	intent-to-treat
kg	kilogram
LcS	*Lacticaseibacillus paracasei* strain Shirota
LOD	limit of detection
mg	milligram
mL	milliliter
mm Hg	millimeter mercury
PCR	polymerase chain reaction
PP	per protocol
RNA	ribonucleic acid
SD	standard deviation

2. Introduction

Probiotics are being used with increasing frequency in medicine and by the general population given the increasing evidence on the beneficial effects on human health. The probiotic efficacy relies on their ability to survive in the digestive system and then to proliferate in the gut. Factors related to survivability in the GI tract include the probiotic strain, as well as other intrinsic and extrinsic factors, and the ability to survive in the GI tract varies considerably among lactobacilli species. Ingested bacteria are exposed to adverse conditions starting as soon as they reach the stomach, with survival influenced by the time required to leave the stomach. The gastric emptying rate, therefore, is an important feature for the survival of bacteria. Factors such as type and composition of food consumed, lifestyle age, environment and race are known to influence the gastric emptying rate and, thus, influence the survival of the probiotic bacteria. The small intestine, especially its proximal part, contains hydrolytic enzymes and bile salts known to have a lethal effect on microorganisms. Thus, ability to pass easily through this compartment of the GI tract may also substantially affect the survival of ingested bacteria.

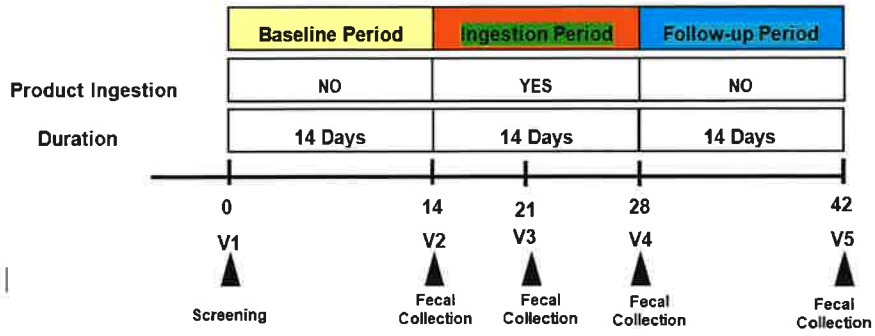
The aim of this study is to investigate the survival of LcS in the intestine of generally healthy adults after intake of fermented milk (Yakult). Similar studies have been conducted by other investigators. For example, clinical studies conducted in Japan, Vietnam, China, and the United Kingdom have reported on the survival of LcS in the intestine in healthy adults. The study described in this proposal is intended to investigate whether the survival of LcS is the same or not in generally healthy adults in the U.S., who may have different ethnicities (e.g., genetic factors), as well as lifestyles, food habits, and environmental influences compared to these previous studies

3. Study objectives

The objective of this study is to investigate the survival of LcS in the human GI tract after consumption of fermented milk in generally healthy adults, 18 to 40 years of age (inclusive), with a body mass index (BMI) $\geq$18.5 and $\leq$29.9 kg/m^2.

4. Trial design and visit structure

This is a single-arm, open label study with one screening visit (Visit 1; Day 0), one baseline visit (Visit 2; Day 14), two intervention visits (Visit 3 and 4; Days 21 and 28), and one post-intervention follow-up visit (Visit 5; Day 42).



Eligible subjects will be enrolled and start a 14-d run-in period (baseline) with the instruction to avoid fermented dairy and non-dairy products. Participants will be instructed to collect a stool sample sometime after 7pm the day before and prior to the next visit (Visit 2; Day 14). Visit 2 (Day 14) is the end of the run-in and start of the ingestion period. The first serving of the study product will be consumed during the clinic visit after participants have consumed breakfast (at home) and obtained/dropped off their stool samples. Subjects will be instructed to consume 1 bottle of study product within 30 minutes of breakfast every day. Additional stool samples will be collected at visit 3 (Day 21), Visit 4 (Day 28), and Visit 5 (Day 42). Pertinent data collection points are presented below in the abbreviated flow chart.

Abbreviated Flow Chart

	Screening/ Start Run-in	**End Run-in/ Start Study Product**	**Interim Study Visit**	**End Product/ Start Follow-up**	**End Follow-up**
Visit	**1**	**2**	**3**	**4**	**5**
Days	**0**	**14**	**21**	**28**	**42**
Clinic Visit	X	X		X	X
Study Instructions & Query	X	X	X	X	X
Review Daily Diary	X	X	X	X	X
Dispense Fecal Collection Kit	X	X	X	X	
Fecal Collection Kit Returned		X	X	X	X
Compliance			X	X	
Adverse Events		X	X	X	X

The daily dairy will capture the date and time of daily product consumption, the occurrence of any of adverse events (AEs), concomitant medication use, and compliance to diet restrictions (no fermented foods or probiotic supplements).

5. Definition of endpoint measures

5.1. Primary endpoint

Change in LcS number (CFU/g feces) from baseline (Visit 2; Day 14) to the end of the ingestion period (Visit 4; Day 28). Bacterial count will be conducted at Institute for Food Safety and Health (IFSH; Bedford Park, IL) using the culture method (FOM-LLV) and PCR approaches, and reported as CFU/g feces.

5.2. Secondary endpoint(s)

- Change in LcS number (CFU/g feces) from baseline (Visit 2; Day 14) to during the ingestion period (Visit 3; Day 21).
- Change in LcS from the end of the ingestion period (Visit 4; Day 28) and to the end of follow-up (Visit 5; Day 42)

5.3. Safety outcome endpoint(s)

- Overall incidence of product-emergent AEs
- Number, type, and duration of Serious Adverse Events (SAEs)

6. Definitions of analysis sets

6.1. Intent-to-treat (ITT) population

The intent-to-treat (ITT) population includes all subjects who were enrolled and serves as the primary analysis population

6.2. Per protocol (PP) population

The per protocol (PP) population is a subset of the ITT population in which subjects will be excluded for, but not limited to, the following reasons:

- Missing consumption of product for two or more days during study product ingestion period
- Not consuming the study product the day before stool collection
- Missing appointments
- Use of prohibited drugs or any products thought to alter the primary outcome variable during the study
- Not adhering to instructions as outlined in the protocol

All decisions regarding the exclusions from the PP population will be documented following the data review meeting which will occur prior to database lock.

7. Determination of sample size

Sample size was determined based on previous studies, which indicate enrolling a sample size of N=25 will provide sufficient data for a significant effect. No subjects will be replaced in the event of early terminations.

8. Statistical methods

Unadjusted descriptive statistics will be provided per measured time point. Continuous variables will be summarized with mean, standard deviation, median, and range. Categorical variables will be summarized with counts and percentages. All tests of significance are two-sided and considered significant at the 0.05 level. SAS (version 9.4 or higher) or R (version 4.2.0 or higher) will be used for statistical analyses. All analyses will be conducted for the ITT and PP populations.

8.1. Demographic and anthropometric measures

Subject demographic and anthropometric measures of enrolled subjects will include:

- Collected at screening visit 1
    - Age (years)
    - Sex (Male, Female)

- Ethnicity (Hispanic/Latino, Not Hispanic/Latino)
- Race (White, Black/African American, Asian, Native Hawaiian/Other Pacific Islander, American Indian/Alaskan Native, Other). Note "more than one race" will be used if more than one race is selected
- Weight (kg)
- Height (cm)
- BMI (kg/m^2)
- Heart rate (bpm)
- Systolic blood pressure (mmHg)
- Diastolic blood pressure (mmHg)

8.2. Outcome analyses

For samples where LcS is not detected, counts will be set to the limit of detection to allow for statistical analysis[1]. Descriptive plots will be provided for the distribution of LcS at each time point (i.e. boxplots) as well as line graphs for each subject. LcS number over time will be analyzed with a repeated measures model with an unstructured covariance structure where the LcS number at each time point will be included in the response vector. The model derived absolute change in LcS from baseline (day 14) to each of days 21, day 28, and 42 will be estimated along with the corresponding 95% confidence interval. In the event model assumptions are violated (i.e. constant variance and/or normality of residuals), a log transformation followed by a rank transformation will be considered. Note, if a rank transformation is used, contrast statements will be used to test the change from baseline but an estimate will not be calculated.

It is possible that the baseline LcS number for all subjects will be non-detectable which would likely result in the model assumption failure. If there are no detectable LcS levels at baseline and the repeated measure model assumptions are violated after exploring possible data transformations, then a model will be fit that only considers the post-consumption time points where the subject is on active product (day 21 and day 28). The estimated mean and 95% CI at day 21 and day 28 would then reflect an estimated increase from baseline. The proportion of subjects that return to baseline (day 14) by day 42 will be estimated along with a 95% CI. Additionally, in the subset of subjects that have elevated LcS while on study product measured at day 21 or day 28, as compared to baseline (day 14), the proportion of subjects that return to baseline by day 42 will be estimated along with a 95% CI.

Product emergent-AEs and SAE will be described. The proportion of subjects with at least 1 possibly, probably, or definitely related AE will be estimated along with the corresponding 95% CI.

9. Handling of missing data

For outcomes measured at multiple time points post baseline, statistical models that account for unbalanced data will be used. Specifically, missing data will be handled by maximum likelihood estimated of mixed models which assumes data is missing at random.

10. Deviations from statistical plan and other issues

During the analysis and reporting process, any deviations from the statistical analysis designed for this protocol will be described and justified.

11. Changes from protocol

N/A

12. References

1. Cox AJ, Makino H, Cripps AW, West NP. Recovery of Lactobacillus casei strain Shirota (LcS) from faeces with 14 days of fermented milk supplementation in healthy Australian adults. Asia Pac J Clin Nutr. 2019;28(4):734-739. doi: 10.6133/apjcn.201912_28(4).0009. PMID: 31826370.