

# *Lactobacillus casei* Strain Shirota Supplied as Beverage Regulates Gut Microbiota in High-Fat Diet Fed Hamster

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## Article Information

Received date: May 18, 2017

Accepted date: May 26, 2017

Published date: May 31, 2017

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## Abstract

The probiotic strain *Lactobacillus casei* strain Shirota (LcS), has been consumed in the form of beverages in many countries. This paper studies the gut microbiota regulation effect of the beverage, instead of the strain itself. The evaluation is conducted using beverage with  $10^8$  cfu mL<sup>-1</sup> LcS and 0.2 g mL<sup>-1</sup> sugar on high-fat-diet hamster model. High-throughput sequencing of gut microbiota showed that some of the non-dominant populations were regulated positively by LcS beverage gavage, against the changes induced by high-fat diet, such as *Erysipelotrichales*, *Lactobacillales* and *Burkholderiales* at Class level, *Erysipelotrichaceae*, *Lactobacillaceae* and *Rikenellaceae* at Family level, *Coprococcus*, *Allobaculum*, *Lactobacillus* and *Akkermansia* at Genus level, and *Akkermansia muciniphila* and *Lactobacillus reuteri* at species level. There was no significant difference in core microbiome, dominant populations or  $\alpha$ -diversity between control and treated groups. In conclusion, the LcS beverage can regulate gut microbiota composition in high-fat diet hamster model by changing non-dominant populations.

## Introduction

*Lactobacillus casei* strain Shirota (LcS) is a well-known probiotic [1]. In China, LcS is usually consumed within high-sugar drinks (about 20% sugar in it) [2], while there are opposite effects of LcS and high-sugar drinks on health.

Previous studies usually had researches on LcS or high-sugar drinks separately. Refined carbohydrates are attracting attention due to their adverse health effects. Besides weight gain, hyperglycemia, dental caries, studies have shown that there was lower bacterial diversity and richness in high-sugar diet mice (10% sugar and 90% basic food) [3]. While LcS has beneficial effects on host health via intestinal flora regulation [4]. The diversity and population of gut microbiota is important. The composition of gut microbiota is closely related to immunity, emotion [5], and many chronic diseases including cancer, inflammation, insulin resistance, dyslipidemia and diabetes [6-10]. Chatelier has shown that bacterial richness is different between obese and non-obese individuals [11]. The gut microbiota composition or gut bacterial richness is not immutable. Energy-restricted diet could increase the abundance of most gene clusters, especially low gene count, which is always characterized by more marked overall adiposity, insulin resistance, dyslipidaemia and inflammatory phenotype [12]. In addition to dietary intervention, probiotics can modify gut microbiota more effectively by producing chemicals [13,14]. Human experiments have demonstrated the ability of LcS to modulate gut flora [4], making LcS intake an effective way to maintain the healthy gut micro-ecology.

Consequently, because the contrary effects of LcS and sugar on gut microbiota modulation, the health effects of LcS beverage with sugar are confused. In this pilot study, we try to illustrate the co-effect of LcS and sugar on gut microbiota composition. High-fat-diet hamster model, whose metabolic pathways were similar to human being's [15], was used to evaluate the regulation effects of LcS beverage with high-sugar on the gut microbiota.

## Materials and Methods

### Animal experiments

SPF Syrian golden hamsters, male and 8 weeks old, were purchased from Charles River Laboratory Animal Technology Co., and raised in cages separately in SPF animal lab of Chinese CDC at 20-26°C with a 12 hours light-12 hours dark cycle, based on Walter's methods [16]. Random

**Table 1:** Beverages used in treated groups.

Treated groups	Dosages and reagents /100 g body weight
T1	0.5 mL beverage and 1.5 mL sterilized distilled water
T2	1.0 mL beverage and 1.0 mL sterilized distilled water
T3	2.0 mL beverage

allocation was taken to evenly divide 40 hamsters into 2 control groups (C1, C2) and 3 treated groups (T1, T2, T3), 8 hamsters in every group. Group C1 was fed with basic feed, while group C2, T1, T2, T3 were fed with High-Fat diet (HF-diet). The feed was purchased from Animal Center of Academy of Military Medical Science. The fat-fed was consists of 80% basic feed, 10% yolk and 10% fat.

Treated groups were given different doses of beverages (Table 1) by gavage once a day for 6 weeks, and control groups were given 2 mL sterilized distilled water per 100 g body weight. All hamsters were allowed freely access to chow and tap water. There was no significant difference between initial body weights. Significant body weight difference between control and treated groups at the end of the experiment indicated that the HF-diet models were successfully established. The body weight was recorded once a week in order to adjust the gavage volume. At the end of the experiment, fecal samples were collected.

All procedures were conducted according to the Laboratory Animal Administration Rules for animal care and approved by ethics committee (Details are given in the supplementary materials).

### Beverages with LcS

The commercial beverage Yakult was used for gavage in different dosages. There are 108 cfu mL<sup>-1</sup> LcS and 0.2 g mL<sup>-1</sup> sugar in it. 3 different dosages were used in 3 treated groups (Table 1). The dosages used in this study were determined by the general consumption of 100 mL 60 kg<sup>-1</sup> body weight. The low, medium and high doses were 3, 6 and 12 times of human body dose.

### DNA extraction and PCR amplification

The DNA of the fecal samples was extracted with reagents prepared by BGI. The V4 region of the 16S rRNA gene was amplified by PCR using universal primers below. The information content of V4 sequences was sufficient to identify phylogenetic affinity in a reference [17].

515F (5'-GTGCCAGCMGCCGCGGTAA-3'), 806R (5'-GGACTACHVGGGTWTCTAAT-3').

### High-throughput sequencing and bioinformatics analysis

Sequencing and analysis is accomplished by BGI Tech. The qualified DNA is used to construct libraries. For PCR product, the

**Table 2:**  $\alpha$ -diversity comparison results among groups (mean  $\pm$  SD).

Indices	C1	C2	T1	T2	T3	p-value*
Chao	465.7 $\pm$ 11.9	496.7 $\pm$ 17.0	499.2 $\pm$ 30.3	506.8 $\pm$ 30.6	486.2 $\pm$ 21.9	0.11062
Ace	458.3 $\pm$ 23.2	481.0 $\pm$ 17.5	489.0 $\pm$ 35.1	503.8 $\pm$ 29.2	483.6 $\pm$ 22.3	0.21721
Shannon	4.1 $\pm$ 0.4	4.2 $\pm$ 0.3	4.1 $\pm$ 0.4	4.1 $\pm$ 0.5	4.0 $\pm$ 0.4	0.99501
Simpson	0.05 $\pm$ 0.03	0.04 $\pm$ 0.02	0.05 $\pm$ 0.03	0.06 $\pm$ 0.05	0.06 $\pm$ 0.04	0.64139

\*Statistical significance was set at p-value < 0.05.

jagged ends of DNA fragment are converted into blunt ends by using T4 DNA polymerase, klenow fragment and T4 polynucleotide kinase, then they are added an 'A' base to each 3' end to make it easier to add adapters. After all that, fragments too short are removed by Ampure beads. For genomics DNA, we use fusion primer with dual index and adapters for PCR, fragments too short are removed by Ampure beads too. In both cases, only the qualified library can be used for sequencing [18]. The bioinformatics analysis is carried on with sequencing data.

The raw data is filtered to eliminate the adapter pollution and low quality to obtain clean reads [19]. Paired-end reads with overlap are merged to tags [20] and generated with Illumina HiSeq platform. Any reads with sequencing adapters, N-base, poly-base, low-quality etc are filtered out with default parameters. Tags are clustered to Operational Taxonomic Units (OTUs) with a 97% threshold by using UPARSE [21]. For further bioinformatics analysis, Venn diagram,  $\alpha$ -diversity, Principal Component Analysis (PCA) and taxonomic classification are applied. Venn diagram is drawn by Venn diagram [22] of software R (v3.1.1) according to unique OTUs amount.  $\alpha$ -diversity is applied for analyzing complexity of species diversity for a sample through several indices, including Chao1, Ace, Shannon [23,24] and Simpson. The indices are calculated by Mothur (v1.31.2) [25]. The complexity of sample is proportional with the first 3 values, while with a negative correlation with Simpson value. PCA [26] of OTU is applied with the relative abundance value of each OTU in every group, which was calculated by the OTU abundance information. OTU representative sequences are taxonomically classified using Ribosomal Database Project (RDP) Classifier (v.2.2) trained on the Green genes database, using 0.8 confidence values as cut-off [27-29].

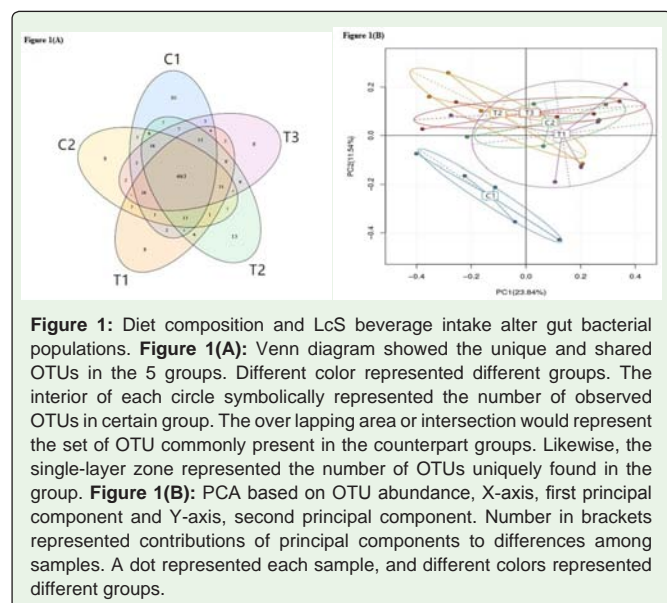
### Statistical analysis

Gathered data were analyzed using Microsoft Excel-2010 and SPSS 16.0. Measured values were expressed as the Mean  $\pm$  SD, data among groups were compared by one-way ANOVA, and statistical significance was set at P < 0.05.

## Results and Discussion

### OTU analysis

We got 607 OTUs in group C1, 615 OTUs in C2, 614 OTUs in T1, 638 OTUs in T2 and 631 OTUs in T3. Number of OTUs per sample primarily represented the degree of sample diversity. Diversity was measured by number of OTUs and  $\alpha$ -diversity. Mean and standard deviation ( $\pm$ SD) were calculated based on the indices of all samples. If p-value is less than 0.05, there is significant difference in  $\alpha$ -diversity among the groups. Results in table 2 show no significant difference in  $\alpha$ -diversity among groups. Namely, HF diet or LcS beverage intake had no effect on gut microbiota diversity in hamster model. In Kristensen's systematic review of the potential evidence for an effect of probiotic supplementation on the composition of human fecal microbiota, it was found that there was not enough evidence for an



impact of probiotics on fecal microbiota  $\alpha$ -diversity. Health benefits of probiotic supplementation would rely on other ways [30] (Table 2).

The Venn diagram, drawn by Venn Diagram of software R (v3.1.1), which can visually display the number of unique OTUs in multi-groups, shows the unique and shared OTUs in the 5 groups (Figure 1A). As shown in Figure 1A, a core microbiome, accounting for the vast majority of gut flora, is shared by all groups, and a small number of OTUs are unique to certain group. It indicates that, the core microbiome is relatively stable in the case of dietary intervention in hamster. The results shown in Venn diagram, with a core microbiota shared by all the groups, is in keeping with the research done by Falony [31], which identifies a global human core microbiota. In order to display the differences among the groups, PCA is used to construct 2-D graph to summarize factors mainly responsible for this difference, similarity is high if two samples are closely located (Figure 1B). As shown in Figure 1B, OTU composition changes a lot in different samples, which suggests gut flora composition variation. Further analysis on taxonomic annotation will find out the existence reasons of core microbiome and flora composition variation hereinafter.

### Taxonomic profiling of gut microbiota

The taxonomic composition distribution in different groups was analyzed by UPARSE [27] on OTUs at 97% threshold implemented in R [29]. All OTUs, which could be aligned to reference sequence, were compared between groups at every taxonomic level (Figure 2). The taxonomic profiling of each group were determined at *Phylum*, *Order*, *Class*, *Family*, *Genus*, and *Species* level separately. We found that less than 5% of them could be determined to species level, about 30% to genus level, 88-90% to family level, and 98-99% to the rest levels. As shown in Fig. 2A, there were more *Bacteroidetes* than *Firmicutes* in all the groups, while the proportion of *Bacteroidetes* was increased and *Firmicutes* was decreased in HF diet groups. This result is not so consistent with known results in human or mice [32,33], in which the *Firmicutes* to *Bacteroidetes* ratio is increased in obesity. It

is deduced to be species difference. But seen from another angle, it is consistent with known results that the proportions of *Bacteroidetes* and *Firmicutes* are closer in basic-food group with low-body weight.

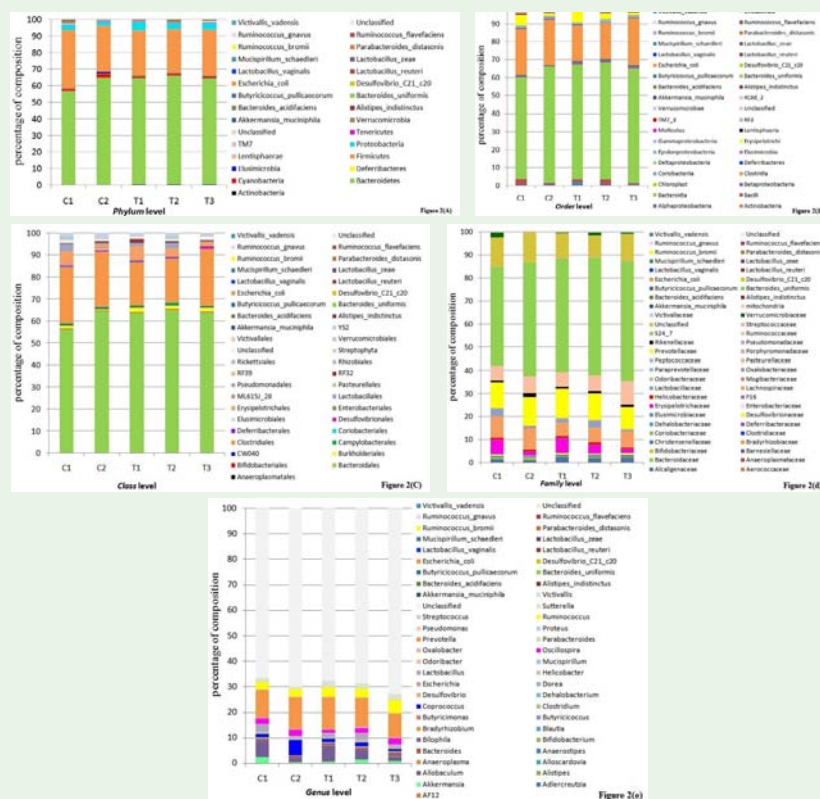
HF diet changed the flora composition at all taxonomic levels as shown in group C2 (Figure 2). Compared with C2, the dominant populations were affected quite little by LcS beverage intake in T1, T2, T3, such as *Firmicutes* and *Bacteroidetes* at Phylum level, *Bacteroidia* and *Clostridia* at Order level, *Bacteroidales* and *Clostridiales* at Class level, S24\_7 (a new Family under *Bacteroidales*) and *Prevotellaceae* at Family level, *Prevotella* and *Ruminococcus* at Genus level. This result was in accordance with the Venn diagram analysis above, and was a rational explanation to the stable core microbiome. While for the non-dominant populations, the amounts of some of the bacteria in HF-diet hamsters were adjusted closer to that of basic feed group, such as *Erysipelotrichi* and *Verrucomicrobiae* at Order level, *Erysipelotrichales*, *Lactobacillales* and *Burkholderiales* at Class level, *Erysipelotrichaceae*, *Lactobacillaceae* and *Rikenellaceae* at Family level, *Coprococcus*, *Allobaculum*, *Lactobacillus* and *Akkermansia* at Genus level, and *Akkermansia muciniphila* and *Lactobacillus reuteri* at species level (Figure 3). It showed that LcS beverage intake had the positive regulation effects in the treated groups in the beneficial of the host, and explained the gut flora composition variation shown in PCA analysis (Figure 1B).

Among the non-dominant populations above, compared with group C2, some of them were increased in treated groups, such as *Erysipelotrichi*, which was reported to be less in human adults with type 2 diabetes than in the control [10], *Verrucomicrobiae*, which accounted for a higher proportion in High Gene Count (HGC) individuals [11], *Allobaculum*, which had a protective effect in gut [34], *Akkermansia* which had a negative relationship with glucose intolerance [35], *Akkermansia muciniphila* which could reduce body weight and relieve the symptoms of Type-2 diabetes in obese mice [36] and *Lactobacillus reuteri*, which was recognized as probiotics generally [37]. While some of them were decreased, for example, *Rikenellaceae*, which could be increased by HF diet [38]. Namely, according to the existing research result, both of the alterations caused by LcS beverage were beneficial to host health.

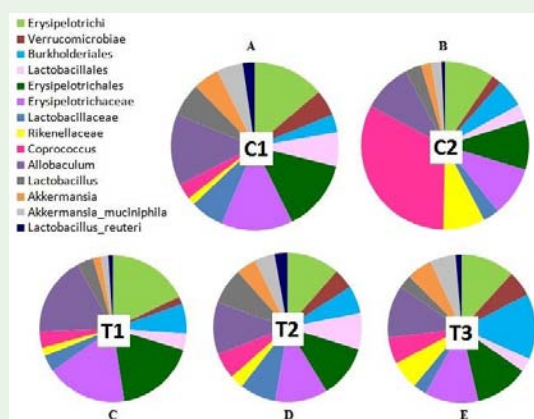
There are many hypotheses about how probiotics influenced the gut microbiota, including promoting homeostasis of the gut microbiota, impacting the function of colonizing microbes, and so on [39-41]. While the potential mechanism for probiotic functionality is often assumed to their ability to impact the human microbiota. Just like the results in this paper, LcS beverage has distinctly adjustment action on the non-dominant microflora. In the treated groups, the composition the non-dominant microflora is sufficiently close to the control group with basic feed (Figure 3), and it suggests that some of the adverse changes induced by HF diet are restrained by LcS beverage effectively.

We have not found a significant dose-dependent relationship between LcS beverage and gut microbiota regulation effect in this study. In treated groups, all reagent concentration had modulation effect on gut flora composition. Maybe it was because the quantitative variation of probiotics intake was too tiny to lead to dose-dependent response, for the huge number of gut microbiota [42]. Further study is needed to illuminate the mechanism of the modulation effects.





**Figure 2:** Taxonomic profiling at different level (A, Phylum level, B, Order level, C, Class level, D, Family level, E, Genus level, F, Species level ) The taxonomic profiling of each group were determined at Phylum, Order, Class, Family, Genus, Species level separately, and we found that less than 5% of them could be determined to species level, about 30% to genus level, 88-90% to family level, and 98-99% to the rest. The dominant populations were affected quite little by LcS beverage intake in T1, T2, T3, compared with C2. This result was in accordance with the Venn diagram analysis above, and a rational explanation to the stable core microbiome.



**Figure 3:** Non-dominant populations affected by HF diet and regulated by LcS beverages. The percentages of the non-dominant bacteria at Order, Class, Family, Genus, Species levels were drawn in one pie graph for each group as shown in Figures 3A-3E, in order to show changes of non-dominant bacteria at different levels clearly. The same color represented the same bacteria in all the 5 pie graphs, and the sectorial are a represented the percentage of counterpart bacteria. So we could intuitively understand the variation caused by HF diet and LcS beverage intake. Compared with group C2, treated groups gut flora composition was more similar to group C1, which demonstrated the regulation effect of LcS beverage intake on gut microbiota, which was good for host health. This result could explain the gut flora composition variation shown in PCA analysis.

## Conclusions

The LcS beverage, in spite of sugar in it, has the regulation function on the non-dominant microflora composition in hamster gut, which has been changed adversely by HF diet. Particularly, it could increase the bacteria beneficial to the host, which was reduced by HF diet, on behalf of the host health.

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