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# *Lactobacillus paracasei* subsp. *paracasei* LC01 Positively Modulates Intestinal Microflora in Healthy Young Adults[§]

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***Lactobacillus paracasei* subsp. *paracasei* LC01 (LC01) can tolerate intestinal stresses and has antioxidant activity. To evaluate the effect of the bacterium on human intestinal microflora, a randomized, double-blind, placebo-controlled human trial was carried out. Fifty-two healthy adult volunteers were randomized equally to two groups. One group consumed 12% (wt/vol) skimmed milk supplemented with $10^{10}$ CFU of LC01 each day for the 4-week treatment period, and then consumed placebo in the next treatment period, separated by a 2-week washout. The other group followed the reverse order. Group-specific real-time PCR and biochemical analyses was used to determine the intestinal bacterial composition of fecal samples collected at the end of every period, and the concentration of short-chain fatty acids and ammonia. A significant inhibition in fecal *Escherichia coli* and increase in *Lactobacillus*, *Bifidobacterium*, and *Roseburia intestinalis* were observed after consumption of LC01. Acetic acid and butyric acid were significantly higher in the probiotic stage and fecal ammonia was significantly lower. The results indicated a modulation effect of LC01 on the intestinal microflora of young adults, suggesting a beneficial effect on bowel health. LC01 may have potential value as a probiotic.**

***Keywords*:** human trial, *Lactobacillus paracasei* subsp. *paracasei* LC01, intestinal microflora, short-chain fatty acid, ammonia

## Introduction

Probiotics are live microorganisms that confer a health benefit on the host when orally administered in adequate amounts (FAO/WHO, 2001). Their ability to improve the health condition of the gastrointestinal tract is especially relevant. The most frequently used probiotic microorganisms are *Bifidobacterium* and *Lactobacillus* species, a dose-related fecal consistency was observed in healthy young adults who consumed a mixture of *Bifidobacterium anialis* subsp. *lactis* BB-12 and *Lactobacillus paracasei* subsp. *paracasei* CRL-341 (Larsen *et al.*, 2006). As the beneficial effect of probiotics is strain-ependent (Bosch *et al.*, 2012), it is necessary to screen and evaluate individual strains in order to obtain satisfying properties. *Lactobacillus paracasei* subsp. *paracasei* LC01 avidly adheres to gastrointestinal mucus (Salminen *et al.*, 1996), resists gastric stress (Fernandez de Palencia *et al.*, 2008), has antioxidant activity (Kim *et al.*, 2005) and displays immunity enhancing activities (Fernandez de Palencia *et al.*, 2008). Thus, LC01 has potential value as a new probiotic.

One of the key beneficial functions of probiotics is to modulate the human intestinal microflora (Isolauri *et al.*, 2008). Gut microflora are the important determinant of human health, including digestive and metabolic efficiencies, development of the host immune system and resistance against intestinal pathogens (Hebuterne, 2003; O'Toole and Claesson, 2010). The use of probiotics as dietary supplements has proven to be efficient in raising the ratio of indigenous beneficial bacteria to harmful bacteria (Costabile *et al.*, 2010; Verdenelli *et al.*, 2011). Furthermore, the modulation of the gut microflora can influence some important physiological parameters of the gut, including short-chain fatty acids (SCFAs) and ammonia (Sakata *et al.*, 1999).

SCFAs production arises from carbohydrate and lipid fermentation by bacterial metabolism. SCFAs are one of the most important final products influencing gastrointestinal health (Matur and Eraslan, 2012). Probiotics reportedly increase the production of SCFAs both *in vitro* (Sakata *et al.*, 1999) and *in vivo* (Riezzo *et al.*, 2012), which accelerates intestinal peristaltic motion (Salminen and Salminen, 1997). The three major SCFAs are acetate, propionate, and butyrate. Among them, butyric acid is the main energy source for intestinal epithelial cells (Bekkali *et al.*, 2007), maintenance of gut integrity and prevention of several gastrointestinal diseases (Perez Chaia and Oliver, 2003).

Urea and other proteinous materials can be converted to ammonia by microflora in the large intestine (Egan *et al.*, 1986; Huang and Chau, 2012). Ammonia is considered a potential cytotoxin and carcinogen (Cummings and Bingham, 1987). Higher levels of fecal ammonia might be caused by insufficient microbial assimilation, and excess ammonia is harmful for epithelium cells and tissues (Visek, 1984), which may lead to gastrointestinal disease (Sato and Nakajima, 2005). Thus, lower colonic and fecal ammonia is beneficial

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for intestinal health.

Presently, an oral administration study assessed the effect of strain LC01 on human fecal bacteria composition. Concentrations of fecal SCFAs and ammonia, which are under the influence of microbial change, were also determined.

## Materials and Methods

### Subjects

Fifty-two volunteers [31 females and 21 males, age 24±4 years, body mass index (BMI) 23±3 kg/m$^2$] were recruited from China Agricultural University, according to their willingness and exclusion criteria. Subject information, including lifestyle, current diet, and medical history, was obtained by completion of a questionnaire before the formal trial.

The main exclusion criteria were BMI outside the range 19–29 kg/m$^2$, diagnosis with or treatment for gastrointestinal symptoms, including chronic constipation and diarrhea, and diagnosis of other serious diseases, including diabetes mellitus and cardiovascular disease. Subjects had not taken antibiotics, probiotics or prebiotics within the four week period leading up to the study. The study was approved by the Ethics Committee of China Agricultural University. Volunteers provided written informed consent before participation.

Four participants who withdrew for personal reasons were not included in the final analysis; 48 participants were available for data analysis. There were no adverse events during the study period.

### Study product

*Lactobacillus paracasei* subsp. *paracasei* LC01 strain was provided by C. Hansen A/S (Hørsholm, Denmark). The study product was based on skimmed milk (12% w/v; Mengniu Dairy Ltd., China) which was enriched with *Lactobacillus paracasei* subsp. *paracasei* LC01 strain at the level of $2 \times 10^8$ CFU/ml. Daily beverages were stored at 4°C. Probiotic-free milk was used as the control. The participants and providers could not distinguish between the two products.

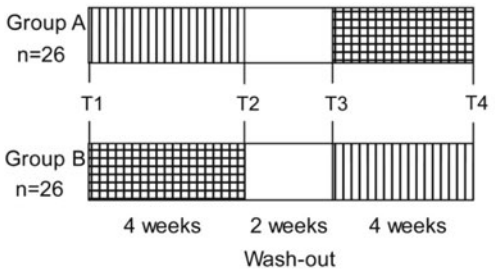


**Fig. 1. Design of the randomized, double-blind, placebo-controlled, crossover human trial.** All subjects took the placebo in the first run-in week. During the treatment period, group A followed the order of Control (▥) to Probiotic (▦), while group B followed reverse order. One product was given over the first 4-week period, followed by a 2-week wash-out period, and then volunteers received the second product during the next 4-week period. Fecal samples were collected at T0, T1, T2, T3, and T4.

### Study design and sample collection

This study had a randomized, double-blind, and placebo-controlled trial design (Fig. 1). Subjects were randomly divided into group A and group B. After the run-in period, the groups received 50 ml/day of probiotic (group A) or placebo (group B) for a period of four weeks. After a 2-week wash-out period, group A subjects received the placebo and group B subjects received the probiotic. Fecal samples were collected from each subject at the end of every period. Each participant was instructed to collect two fecal samples in separate 50 ml sterilized tubes (one tube contained RNAlater® to stabilize and protect DNA) and deliver the samples to the lab within 4 h. Samples in RNAlater® were stored at 4°C for microflora analysis. The other sample from each participant was stored at -80°C until determination of ammonia content and SCFAs analysis. Participants maintained their usual lifestyles and habitual diets throughout the study pe-

**Table 1. Primers, product size, and annealing temperature**

| Target groups or organism | Amplicon size (bp) | Annealing temperature (°C) | Primer sequence (5′→3′) | Reference |
|---|---|---|---|---|
| All bacteria | 466 | 58 | F: TCCTACGGGAGGCAGCAGT<br>R: GGACTACCAGGGTATCTAATCCTGTT | Nadkarni *et al.* (2002) |
| *Bacteroides–Prevotella* group | 418 | 59 | F: GAAGGTCCCCCACATTG<br>R: CAATCGGAGTTCTTCGTG | Bartosch *et al.* (2004) |
| *Bifidobacterium* genus | 437 | 60 | F: GGGTGGTAATGCCGGATG<br>R: TAAGCCATGGACTTTCACACC | Bartosch *et al.* (2004) |
| *Enterococcus* genus | 144 | 61 | F: CCCTTATTGTTAGTTGCCATCATT<br>R: ACTCGTTGTACTTCCCATTGT | Rinttila *et al.* (2004) |
| *Escherichia coli* subgroup | 340 | 61 | F: GTTAATACCTTTGCTCATTGA<br>R: ACCAGGGTATCTAATCCTGTT | Malinen *et al.* (2003) |
| *Lactobacillus* group | 341 | 58 | F: AGCAGTAGGGAATCTTCCA<br>R: CACCGCTACACATGGAG | Heilig *et al.* (2002) |
| *Roseburia intestinalis* | 146 | 56 | F: GCATGACCTGGTGTGAA<br>R: TTGGGCCGTGTCTCAG | Makivuokko *et al.* (2010) |
| *Fusobacterium prausnitzii*[2] | 158 | 61 | F: CCCTTCAGTGCCGCAGT<br>R: GTCGCAGGATGTCAAGA | Rinttila *et al.* (2004) |
| *Eubacterium* group | 429 | 55 | F: CCCTTCAGTGCCGCAGT<br>R: GTCGCAGGATGTCAAGA | Rinttila *et al.* (2004) |

riod, except for the consumption of other probiotics or any antibiotics. The cross-over design permitted the evaluation of the subjective outcome, as the between-subject variations was reduced effectively, along with a lower number of subjects, as compared with a parallel study (Woods *et al.*, 1989).

### DNA extraction and fecal microflora analysis

Bacterial genomic DNA was extracted from fecal samples by the glass bead-phenol-chloroform method (Via and Falkinham, 1995) within two weeks after collection. DNA was dissolved in Tris-EDTA to a final volume of 200 µl. The integrity and concentration of the extracted DNA were qualitatively determined by 1% agarose gel electrophoresis.

Real-time quantitative PCR was carried out to determine the dominant fecal bacteria. The V3 variable region of 16S rDNA was amplified using commercial primers (Invitrogen, China), which are listed in Table 1. The PCR reaction mixture (adjusted to 50 µl with sterile deionized water) contained 1 µl template DNA, 0.5 µmol/L (final concentration) each primer, and 5 µl of 2 × Ultra SYBR Mixture (CWBIO, China). Reactions were run in an ABI PRISM 7000 Sequence Detection System device (Applied Biosystems, USA) as follows: 10 min at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at annealing temperature, 30 sec at 72°C, and 5 sec at 78°C for the signal detection. After PCR amplification, the dissociation curve program was run to provide values for specific product testing. Quantification was performed by comparison of $C_t$ values between unknown DNA samples and standard curves generated from known copies of amplified products, as copy numbers of DNA amplicon per gram of feces, after correction for dilution.

### Ammonia analysis

Fecal ammonia content was determined using a previously described indophenol blue procedure (Brinkworth *et al.*, 2009). Briefly, ammonia was oxidized by sodium hypochlorite and a blue end-product formed in the presence of sodium nitroprusside. The ammonia concentration was measured at 630 nm using a UV-2102 spectrophotometer (UNICO, China), and the standard curve was based on an appropriate reference solution, as µmol/g fecal sample.

### SCFAs analysis

Frozen fecal samples were prepared as previously described (Goossens *et al.*, 2003) with slight modification. Briefly, fecal samples were homogenized in 1 ml diethyl ether containing 1 mmol/L heptanoic acid as the internal standard and 50 µmol/L HCl. Free SCFAs were extracted after centrifugation at 5000×*g* for 10 min. Two microliters of the diethyl ether extract was injected for gas chromatography measurement.

A GC-14C gas chromatograph (SHIMADZU, Japan) containing a WCOT fused silica (25 m × 0.32 mm internal diameter) chromatographic column coated with FFAP-CB (df 0.5) was used. The conditions were: oven temperature, initially held at 50°C for 1 min, raised to 140°C at a rate of 10°C/min, then raised to 240°C at 30°C/min after 1 min; injector temperature, 230°C; detector temperature, 230°C; gas flow rate, 20 ml/min. The carrier gas was nitrogen, 260 kpa. The injection type was split in a 1:50 ratio. Concentrations of SCFAs (acetic acid, propionic acid, and butyric acid) were determined based on standard curves of reference solutions, as the number of micromoles per gram of feces after correction for dilution.

### Statistical analysis

Distribution normality of data was examined by the Kolmogorov-Smirnow test before hypothesis testing. Results are the Mean±SD. The data of bacterial counts were shown to be normally distributed, and were analyzed by repeated-measures analysis of variance with Bonferroni's adjustment (T1 of the two groups were considered as the Baseline stage, T2 of group A and T4 of group B as Control, T4 of group A and T2 of group B as Probiotic) relevant to all subjects. When a difference was found, multiple comparisons were performed (LSD Fisher test) to determine the groups between which there was a significant difference (Valerio *et al.*, 2011). To further investigate how reversed order affect bacteria populations for each group, the differences between

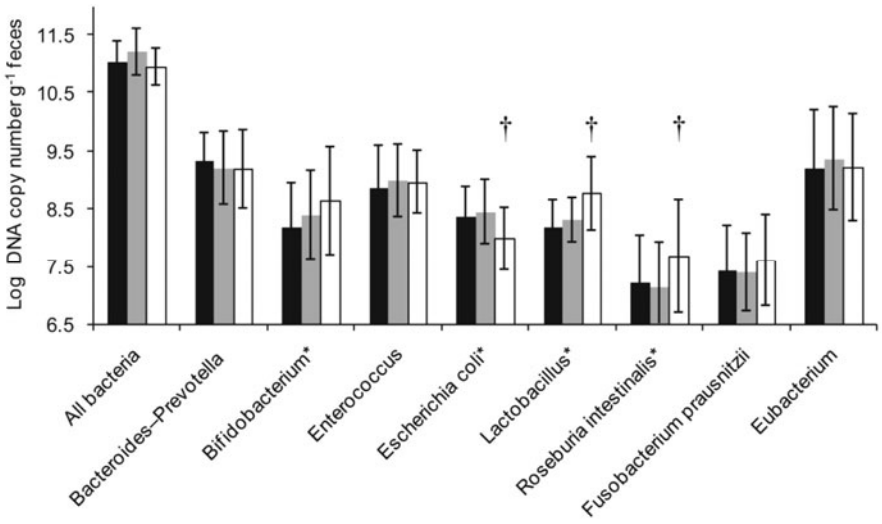


**Fig. 2. Bacterial populations in fecal samples after administration of LC01 and placebo, relevant to all subjects (n=48):** Baseline, black bar; Control, dark-grey bar; Probiotic, white. Values (Log copy numbers/g of feces) were reported as Mean±SD. The repeated-measures ANOVA with Bonferroni's adjustment was used to compare among three stages, and **P*<0.05 were considered significant. †Values within the same bacteria which are significantly different from each other (*P*<0.05) based on LSD Fisher post hoc test.

pre- and post-placebo treatment, pre- and post-wash out treatment, pre- and post-probiotic treatment were analyzed by the paired Student *t*-test (Valerio *et al.*, 2011). SCFAs and ammonia data, did not comply with the normal distribution, thus the Friedman repeated measures analysis of variance relevant to all subjects were used to investigate the effects of the two dietary treatments on SCFAs and ammonia, followed by Dunn's test multiple comparisons to determine the groups between which there was a significant difference (Riezzo *et al.*, 2012). To further investigate the influence of reversing order, the non-parametric Wilcoxon test was performed (Valerio *et al.*, 2011). Analysis was performed with SPSS Statistics 17.0 software (SPSS, USA). Statistical significance was set at $P<0.05$.

## Results

### Microflora analysis

Effects of probiotic intervention on the fecal microbiota of all subjects are shown in Fig. 2. The number of *Lactobacillus* and *Roseburia intestinalis* in the probiotic stage were significantly higher than the baseline ($P<0.001$, $P=0.016$) and control ($P=0.016$, $P=0.01$), while the bacterial count of *Escherichia coli* was significantly lower than the baseline ($P<0.001$) and control ($P<0.001$). Numbers of *Bifidobacterium* in the probiotic stage increased significantly compared to the baseline ($P=0.002$). No significant changes were found in other dominant microflora groups, indicating a maintenance of a balanced indigenous microecology of healthy subjects.

Effects of the timing of consumption of the probiotic beverage on fecal microbiota are summarized in Supplementary data Table S1. Similar results were evident for the four bacterial genera, except for marginal differences in *Bifidobacterium*, where the level of *Bifidobacterium* increased in both groups, but was significant only in subjects of group A (T4 to T3), who received the probiotic after placebo. This may be explained by the high base number of *Bifidobacterium* in group B. The washout period (T2 to T3) caused a significant decrease in *Lactobacillus* ($P=0.04$), a general reduction in *Roseburia intestinalis* ($P=0.076$), as well as a remarkable increase in counts of *E. coli* ($P=0.002$). At the end of washout, they all restored to almost the same levels as determined at T1. Of note, after the 2-week washout, the group B data indicated a carryover effect of the probiotics only for *Bifidobacterium*, where the bacterial population in the T3 period was slightly higher than that of the T2 period ($P>0.05$).

### Ammonia analysis

Table 2 displays the ammonia data of all subjects, and significant differences among three stages were observed ($P=0.029$). The administration of LC01 caused a significant decrease of the values for fecal ammonia (versus baseline $P=0.006$). The probiotic beverage had the same significant effect on fecal ammonia in group A and B during probiotic-treatment periods (Supplementary data Table S2). In group B, there was no significant change during the wash-out period ($P=0.456$), but the values significantly increased during the following control period ($P=0.016$). These data indicated a carryover effect of the probiotics, which might last longer than 2 weeks, reflecting their persistence in the gut.

**Table 2. Concentrations of ammonia, acetic acid, propionic acid, and butyric acid in fecal samples after the administration of LC01 and placebo, relevant to all subjects**

| | Baseline | Control | Probiotic | *P* |
|---|---|---|---|---|
| Ammonia | 28.36 ± 16.48 | 29.49 ± 17.94 | 20.14 ± 10.82† | 0.029* |
| Acetic acid | 66.92 ± 33.76 | 67.27 ± 30.72 | 91.91 ± 43.32† | 0.013* |
| Propionic acid | 8.47 ± 4.74 | 8.96 ± 3.96 | 8.75 ± 3.96 | 0.712 |
| Butyric acid | 5.18 ± 2.40 | 5.46 ± 1.92 | 6.93 ± 3.72† | 0.031* |

Data (μmol/g feces) were reported as Mean±SD. Statistical evaluations were performed using the Friedman one way repeated measures analysis of variance, *$P<0.05$ were considered to be significantly different among three stages. †Mean values which differ from each other significantly ($P<0.05$) based on Dunn's all-pairwise multiple comparison post-test.

### SCFAs analysis

Table 2 summarizes the SCFAs values of all subjects. Compared with both baseline and control stages, acetic acid and butyric acid were increased significantly ($P<0.05$) in the probiotic stage, while the value of propionic acid did not show significant change among the three periods ($P>0.05$). Separate analysis of the group data (Supplementary data Table S2) revealed a similar upward trend of acetic acid and butyric acid. It is noteworthy that acetic acid and butyric acid in group B both decreased significantly from period T2 to T3 ($P=0.009$ and 0.023, respectively), suggesting a wash-out of the probiotics.

## Discussion

The LC01 intervention caused significant increases in *Bifidobacterium*, *Lactobacillus*, *Roseburia intestinalis*, and a decrease in *Escherichia coli*. Similar results have been previously obtained when several other *Lactobacillus paracasei* strains were used (Valerio *et al.*, 2011; Verdenelli *et al.*, 2011). Microbial counts shifted towards lower values of potentially harmful bacteria (such as *E. coli*) and higher values of lactobacilli and bifidobacteria, which are considered to be beneficial for the health of the colon (Gibson *et al.*, 2004).

Fecal ammonia is partly derived from urea, which is produced from amino acid degradation and the tricarboxylic acid cycle, in which bacteria possessing urease activity play a key role (Huang and Chau, 2012). As shown in our data, the ingestion of LC01 could decrease the daily fecal ammonia output. The decreased ammonia might reflect the reduced survival of deleterious microorganisms in the intestinal tract. Additionally, bacterial breakdown of proteinous materials in the large intestine is another main source of ammonia (Sakata *et al.*, 1999). Thus, the decreased ammonia should indicate decreased protein degradation or increased protein synthesis in which ammonia was used as substrate. This might be a result of an increased utilization of carbohydrates (and increased production of SCFAs) indicating a more balanced energy supply.

In SCFAs analysis, both acetic acid and butyric acid were increased significantly by the probiotic treatment. The high

level of butryric acid did not exactly match previous results (Riezzo *et al.*, 2012), in which *Lactobacillus paracasei* produced a slight increase of propionic acid only. The difference of the test strains might be the possible reason. SCFAs are effective in maintaining the balance of water and electrolytes (Rabbani *et al.*, 1999), and in providing resistance to pathogenic microorganisms, inflammation and tumor growth (Chander *et al.*, 2006; Worthley *et al.*, 2011). The present data suggested that the ingestion of LC01 could increase the daily fecal output of acetic acid and butyric acid, which may be helpful to improve intestinal health.

It is worth noting that the increase of SCFAs corresponded to the decrease of fecal ammonia after ingestion of LC01. As described above, this may be explained by the utilization of energy sources for the intestinal microorganisms. In fact, intestinal SCFAs production is a major promoting force for the microflora utilization of $NH_4^+$ due to pH change (Brinkworth *et al.*, 2009). Meanwhile, the generation of ammonia lessens. Moreover, the process of ammonia detoxification and SCFAs increase in the colon has been interpreted as an interplay of the microbiota to establish appropriate growth conditions (Wutzke *et al.*, 2010).

In conclusion, human consumption of *Lactobacillus paracasei* subsp. *paracasei* LC01 exerted positive effects on the intestinal microflora of young adults. These benefits included a marked inhibition of *E. coli*, increases of *Lactobacillus*, *Bifidobacterium*, and *Roseburia intestinalis*, stimulated production of SCFAs, including propionic acid and butyric acid, and a significantly decreased level of ammonia. The changes are indicative of improved bowel health and a decreased risk of colonic disease. The findings suggest the potential value of LC01 as a probiotic food supplement.

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