

Effect of oral consumption of capsules containing *Lactobacillus paracasei* LPC-S01 on the vaginal microbiota of healthy adult women: a randomized, placebo-controlled, double-blind crossover study

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Supplementary methods

Study participants

Forty nondiseased (healthy) women were assessed for participating in the VAG-LPC 14 [“Evaluation of the effect of PREGYN capsules (containing *Lactobacillus paracasei* LPC-S01) consumption on the vaginal microbiota composition of healthy adult women: a randomized, placebo-controlled, double-blind cross-over study”] study at U.O.C. Obstetrics and Gynecology of Sacco Hospital in Milan (**Fig. 1**). Thirty-seven women (aged 34.9 ± 10.7 y; BMI: 22.5 ± 2.7 kg/m²) entered the run-in period and were randomized into groups receiving the probiotic or placebo according to the crossover design shown in Fig. 1. The eligibility criteria were good general health, age between 18 and 45 y, and a signed informed consent form. The exclusion criteria were as follows: urogenital infections in the previous 3 months (especially bacterial vaginosis); urogenital abnormalities; ascertained or planned pregnancy or breastfeeding; intake of antibiotics and/or probiotics in the month before the start of the trial; systemic or topical therapy with steroids, glucocorticoids with rapid hepatic passage such as beclometasone dipropionate or budesonide, in progress or within the month prior to enrollment; chronic intestinal inflammatory diseases; intestinal diseases of infectious, actinic, endocrine or drug origin (microscopic colitis); immunodeficiency; malignant neoplasms of any kind, or a history of previous malignancy (history of other malignant neoplasms that were surgically removed without recurrence within the five years prior to entry into the study were still eligible); any renal, hepatic, hematological, cardiovascular, pulmonary, neurological, psychiatric, immunological, gastrointestinal or endocrine disease, if clinically significant; any severe pathology that could interfere with the treatment; recent history or suspicion of alcohol or drug abuse; presence of dementia of any kind or other possible causes of progressive deterioration of the ability to understand or any psycho-physical disability that reduces the ability to take the prescribed therapy as planned; inadequate reliability or the presence of conditions that could lead to a lack of voluntary compliance with the protocol; or previous participation in this study.

Treatment and study scheme

Each volunteer was initially trained on the entire procedure to be followed, which consisted of a total of 5 visits for each volunteer, each to be performed 14 days after the end of menstruation by the subject. During the first visit (V0), the informed consent and personal data of the volunteer were collected. In addition, the volunteer received general information about dietary changes to be followed in the following 4 weeks of the run-in period. Specifically, the volunteers were asked to follow their conventional diet with the intake of probiotic fermented milks, probiotic food supplements, and prebiotic food supplements prohibited. At the end of this run-in, the volunteers were randomized into groups receiving 1 capsule per day of Pregyn® or placebo for 4 weeks. The volunteers were asked to take the capsule on an empty stomach in the morning, at least 10 min before breakfast or, in case of forgetfulness, in the evening, before going to bed and at least 2 h after the last meal. Following the first 4 weeks of treatment, the volunteers were subjected to 4 weeks of wash-out identical to the run-in period. At the end of the wash-out period, the volunteers took 1 capsule a day of Pregyn® or placebo for 4 weeks according to the crossover design.

Quantification of Gardnerella vaginalis in vaginal swab samples

Real-Time quantitative (qPCR) protocols were adopted for the quantification of the *Gardnerella vaginalis* in vaginal total DNA targeting the lactate dehydrogenase gene, with primers Gard_LdhF, 5'-GTTATTACTGCTGGTGCTCG-3' and Gard_LdhR, 5'-GCTCGCCAGCAATATAAGCG-3'. A gradient PCR was initially performed to standardize the qPCR conditions and also validation of the primer specificity was performed using DNA of several bacterial species (such as lactobacilli and bifidobacteria) commonly found in vagina. The qPCR amplifications were carried out in a final volume of 15 µl containing 7.5 µl of EvaGreen® Supermix and 0.5 µM of each primer. Total 50 ng of vaginal DNA was used in each reaction. Samples were amplified with the following program: initial hold at 95°C for 3 min, and 39 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Melting curves were analyzed to confirm the specificity of the amplification products. To generate the standard calibration curve for *Gardnerella vaginalis* quantification, 750 µl vaginal samples was added with different number of *Gardnerella* cells (starting from 10⁶ downwards

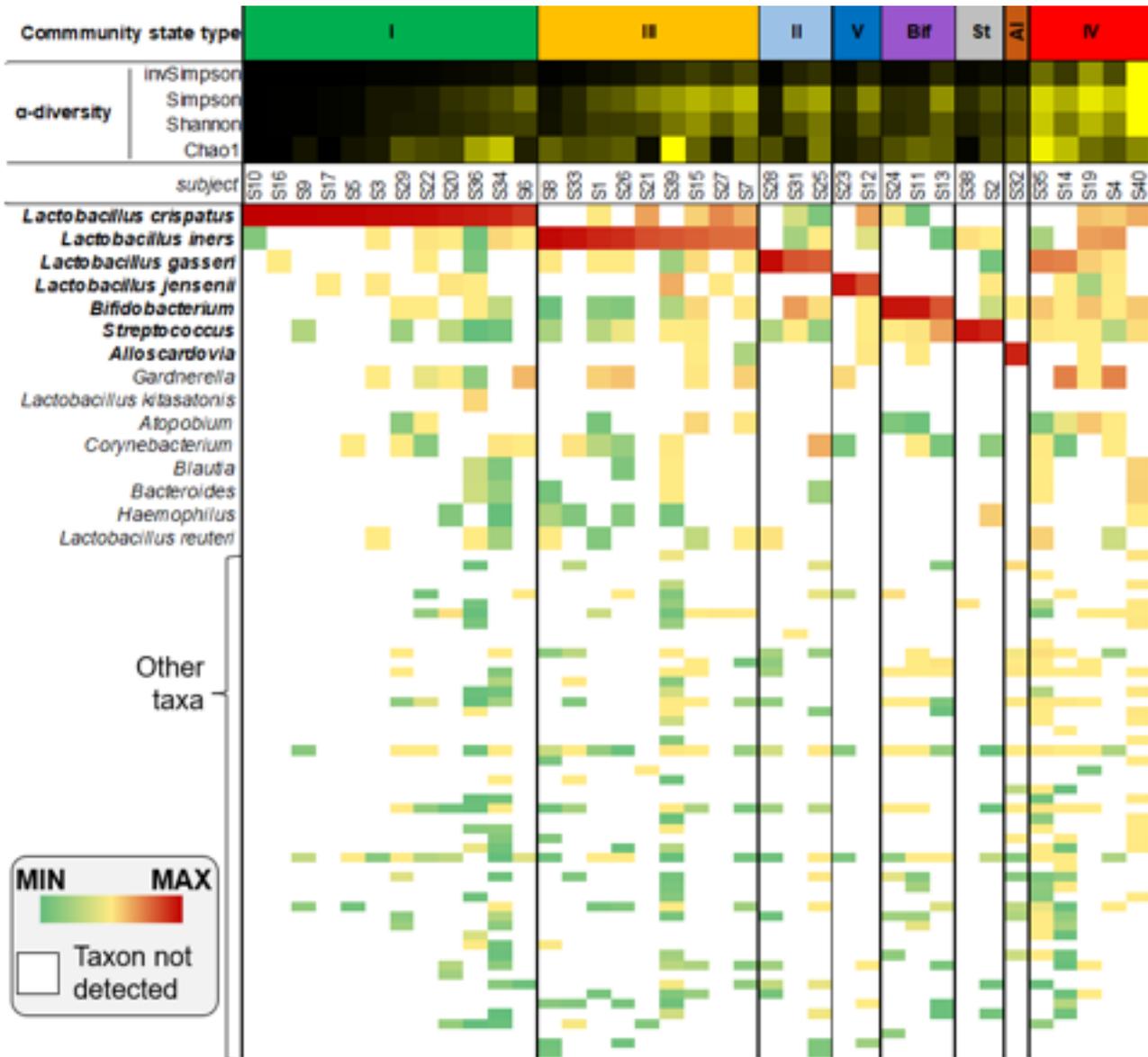
up to 1 cell and also negative control without addition of any cells), total DNA was extracted from each preparation as described in main materials and methods. For qPCR, 50 ng DNA from each preparation (samples added with different number of cells) were used and standard calibration curve was prepared. The equation of the derived standard curve was used to calculate the correlation between Ct values and the concentration of bacterial cells per swab.

Supplementary Table S1. Adverse events (AE) recorded during the trial. *More than one adverse event per subject occurred.

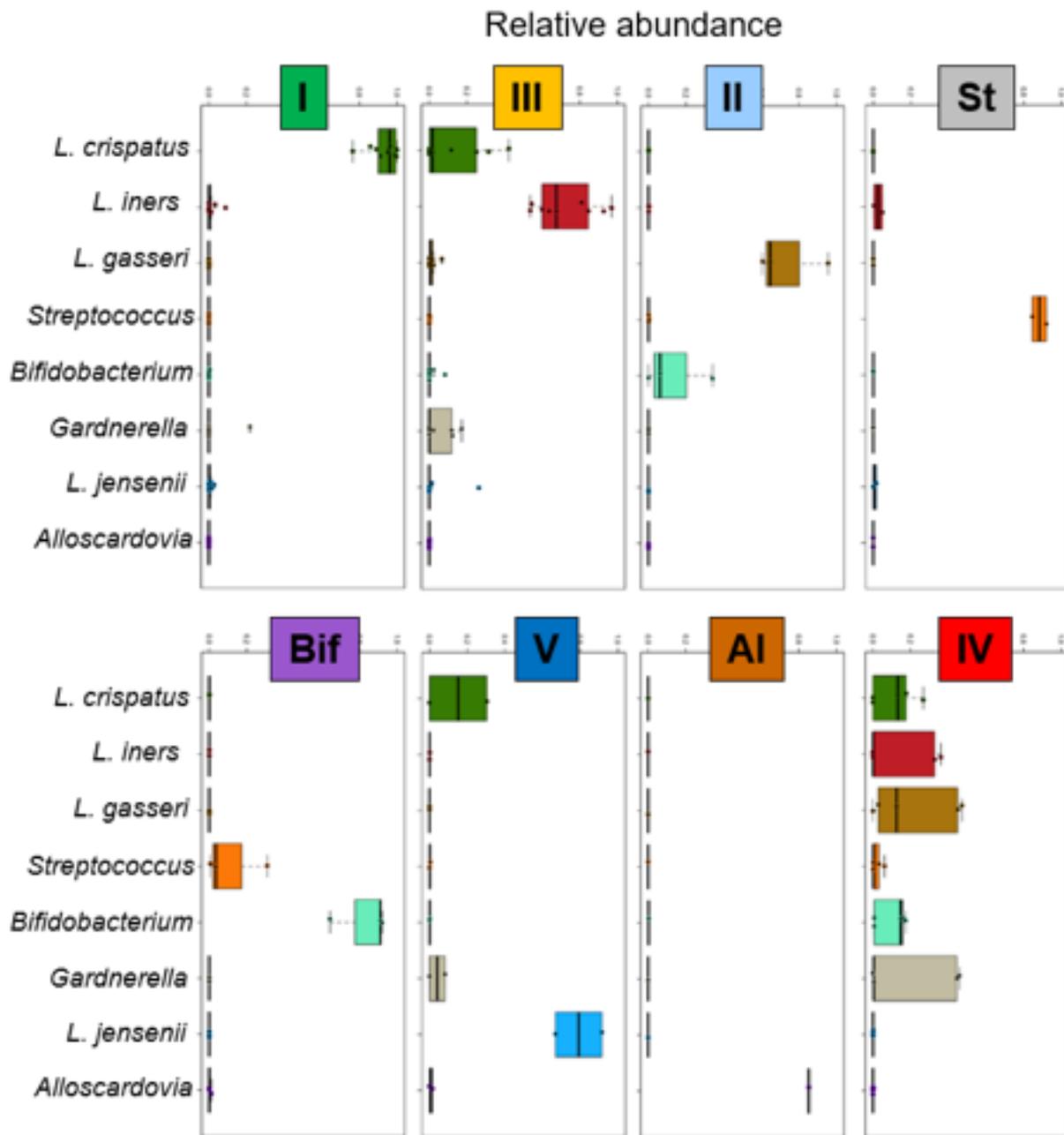
	Pregyn	Placebo
Total number of AE (N)	39	55
Number of AE per subject <i>[Mean (standard deviation)]</i>	2.17 (1.29)	3.24 (1.82)
Number of AE with suspected relatedness to the treatment* <i>[N (%)]</i>	24 (61.5%)	32 (58.2%)
Number of AE by severity level (N)		
- Mild	27	33
- Moderate	10	21
- Severe	2	1

Supplementary figure S1. Composition of the vaginal microbiota at baseline (V1) of the 37 reproductive-age women enrolled in the VAG-LPC 14 study. A, heatmap of \log_{10} -transformed relative abundances of microbial taxa found in the vaginal bacterial communities; α -diversity indices are shown as black-yellow heatmap where color indicates the value from minimum (black) to maximum (bright yellow). B, Tukey's boxplots of the bacterial taxa characterizing the identified vaginal community state types.

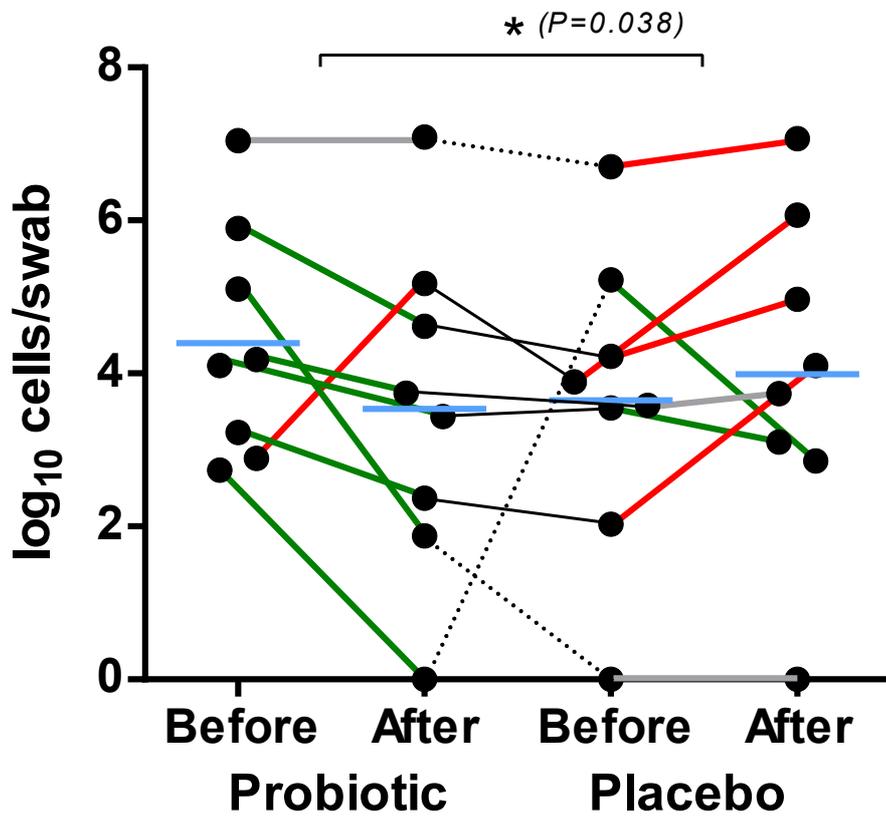
A.



B.



Supplementary figure S2. Absolute quantification by qPCR of *Gardnerella* cells in vaginal swabs. Only volunteers in whose vaginal mucosa LPC-S01 cells have been detected are shown. Lines collect samples belonging to the same volunteer. Red lines indicate increases of the cell abundance of more than 10% during an intervention; green lines indicate reductions of the cell abundance of more than 10% during an intervention. Grey lines indicate modification of the cell abundance of less than 10% during an intervention. Dotted lines indicate volunteers that were randomized to have the placebo intake phase before the probiotic intervention. Light blue lines indicate the mean. Statistics is according to repeated measure ANOVA.



Supplementary figure S3. Bacterial genes derived from KEGG Ortholog count prediction that were found to be significantly changed after the probiotic or the placebo phases. P values are according to the Wilcoxon signed-rank test. the black-yellow heatmap indicates median values; white boxes indicate a median of zero. Δ , deviation between the median abundances of the gene after and before the treatment; ∇ , positive deviation (i.e., the gene relative abundance increased after the treatment); \wedge , negative deviation (i.e., the gene relative abundance decreased after the treatment).

Probiotic

KEGG orthology	Functional gene	Role in metabolism	P value	before	after	Δ
K13051	beta aspartyl-peptidase (threonine type)_ EC.3.4.19.5	Amino acid metabolism/Alanine, aspartate and glutamate metabolism	0.010			\wedge
K03606	putative colanic acid biosynthesis UDP-glucose lipid carrier transferase	Cellular Processes/Cellular community/Biofilm formation	0.030			\wedge
K05776	molybdate transport system ATP binding protein	Environmental Information Processing/Membrane transport	0.036			\wedge
K03503	DNA polymerase V_EC.3.4.21	Genetic information processing/DNA repair and recombination proteins	0.039			\wedge
K06284	transcriptional pleiotropic regulator of transition state genes	Genetic information processing/Transcription factors	0.030			∇
K03646	colicin import membrane protein	Signaling and cellular processes/Transporters	0.029			\wedge
K03543	multidrug resistance protein A	Signaling and cellular processes/Transporters	0.041			\wedge
K10548	putative multiple sugar transport system ATP binding protein	Signaling and cellular processes/Transporters	0.047			\wedge
K10547	putative multiple sugar transport system permease protein	Signaling and cellular processes/Transporters	0.047			\wedge
K06113	arabanan endo-1,5 alpha-L-arabinosidase_EC.3.2.1.99	Unclassified	0.014			\wedge

Placebo

KEGG orthology	Functional gene	Role in metabolism	P value	before	after	Δ
K01917	glutathionylspermidine synthase_EC.6.3.1.8	Amino acid metabolism (other)/Glutathione metabolism	0.038			\wedge
K10206	LL-diaminopimelate aminotransferase_EC.2.6.1.83	Amino acid metabolism/Lysine biosynthesis	0.042			\wedge
K01850	chorismate mutase_EC.5.4.99.5	Amino acid metabolism/Phenylalanine, tyrosine and tryptophan biosynthesis	0.020			\wedge
K10907	aminotransferase_EC.2.6.1	Amino acid related enzymes	0.030			\wedge
K00812	aspartate aminotransferase_EC.2.6.1.1	Amino acid related enzymes/Biosynthesis of other secondary metabolites	0.040			\wedge
K01787	N-acylglycosamine 2-epimerase_EC.5.1.3.8	Carbohydrate metabolism/Amino sugar and nucleotide sugar metabolism	0.014			\wedge
K00176	2-oxoglutarate ferredoxin oxidoreductase subunit delta_EC.1.2.7.3	Carbohydrate metabolism/Citrate cycle (TCA cycle)	0.027			\wedge
K00177	2-oxoglutarate ferredoxin oxidoreductase subunit gamma_EC.1.2.7.3	Carbohydrate metabolism/Citrate cycle (TCA cycle)	0.024			\wedge
K01677	fumarate hydratase subunit alpha_EC.4.2.1.2	Carbohydrate metabolism/Citrate cycle (TCA cycle)	0.031			\wedge
K01678	fumarate hydratase subunit beta_EC.4.2.1.2	Carbohydrate metabolism/Citrate cycle (TCA cycle)	0.044			\wedge
K01960	pyruvate carboxylase subunit B_EC.6.4.1.1	Carbohydrate metabolism/Citrate cycle (TCA cycle)	0.046			\wedge
K00169	pyruvate ferredoxin oxidoreductase_alpha subunit_EC.1.2.7.1	Carbohydrate metabolism/Glycolysis-Gluconeogenesis; Citrate cycle (TCA cycle)	0.037			\wedge
K00172	pyruvate ferredoxin oxidoreductase_gamma subunit_EC.1.2.7.1	Carbohydrate metabolism/Glycolysis-Gluconeogenesis; Citrate cycle (TCA cycle)	0.042			\wedge
K01685	altronate hydrolase_EC.4.2.1.7	Carbohydrate metabolism/Pentose and glucuronate interconversions	0.034			\wedge
K01051	pectinesterase_EC.3.1.1.11	Carbohydrate metabolism/Pentose and glucuronate interconversions	0.043			\wedge
K14941	2-phospho L-lactate guanylyltransferase_EC.2.7.7.68	Energy metabolism/Methane metabolism	0.043			\wedge
K00336	NADH quinone oxidoreductase subunit G_EC.1.6.5.3_NADH dehydrogenase I subunit G_EC.1.6.5.3	Energy metabolism/Oxidative phosphorylation	0.040			\wedge
K02315	DNA replication protein DnaC	Genetic information processing/DNA replication proteins	0.037			\wedge
K01205	alpha N acetylglucosaminidase_EC.3.2.1.50	Glycan biosynthesis and metabolism/Glycosaminoglycan degradation	0.007			\wedge
K00324	NAD(P) transhydrogenase subunit alpha_EC.1.6.1.2	Metabolism of cofactors and vitamins/Nicotinate and nicotinamide metabolism	0.046			∇
K00325	NAD(P) transhydrogenase subunit alpha_EC.1.6.1.2	Metabolism of cofactors and vitamins/Nicotinate and nicotinamide metabolism	0.032			∇
K03150	thiamine biosynthesis ThiH	Metabolism of cofactors and vitamins/Thiamine metabolism	0.043			\wedge
K08384	stage V sporulation protein D_sporulation specific penicillin binding protein	Peptidoglycan biosynthesis and degradation proteins	0.041			\wedge
K02653	type IV pilus assembly protein PilC	Signaling and cellular processes/Secretion system	0.045			\wedge
K02856	L-rhamnose H+ transport protein	Signaling and cellular processes/Transporters	0.025			\wedge
K06409	stage V sporulation protein B	Signaling and cellular processes/Transporters	0.046			\wedge
K03518	carbon monoxide dehydrogenase small subunit_EC.1.2.99.2	Unclassified	0.047			\wedge
K10670	glycine reductase_EC.1.21.4.2	Unclassified	0.040			\wedge
K09777	hypothetical protein	Unclassified	0.042			\wedge
K00179	indolepyruvate ferredoxin oxidoreductase_alpha subunit_EC.1.2.7.8	Unclassified	0.035			\wedge
K00180	indolepyruvate ferredoxin oxidoreductase_beta subunit_EC.1.2.7.8	Unclassified	0.035			\wedge
K07033	None	Unclassified	0.049			\wedge
K07757	sugar phosphatase_EC.3.1.3.23	Unclassified	0.042			\wedge
K05919	superoxide reductase_EC.1.15.1.2	Unclassified	0.047			\wedge

MIN MAX

Supplementary figure S4. Results of strain-specific qPCR quantification of *L. paracasei* LPC-S01 cells in fecal samples. V1-4, time point of fecal sample collection, according to the study design reported in Fig. 1. Data are reported as log₁₀ cells/g of feces. n.d., not detected; N.A., samples not available. LPC-S01 cell quantifications are reported in a yellow-red heatmap from the from minimum (yellow) to maximum (red) concentration.

Subject	<i>L. paracasei</i> LPC-S01 concentration (log ₁₀ cells/g of feces)			
	V1	V2	V3	V4
S01	n.d.	8.1	n.d.	n.d.
S02	n.d.	n.d.	n.d.	6.6
S03	n.d.	5.9	n.d.	n.d.
S04	n.d.	n.d.	n.d.	6.8
S05	n.d.	n.d.	n.d.	6.8
S06	n.d.	6.6	n.d.	n.d.
S09	n.d.	6.2	n.d.	n.d.
S15	n.d.	6.7	n.d.	n.d.
S17	n.d.	6.4	n.d.	n.d.
S20	n.d.	5.6	n.d.	n.d.
S21	n.d.	6.0	n.d.	n.d.
S27	n.d.	7.1	n.d.	n.d.
S32	n.d.	6.6	n.d.	n.d.
S07	n.d.	n.d.	n.d.	n.d.
S10	n.d.	n.d.	n.d.	7.1
S16	n.d.	n.d.	n.d.	6.7
S19	n.d.	n.d.	n.d.	6.6
S22	n.d.	n.d.	n.d.	7.4
S24	N.A.			
S28	n.d.	n.d.	n.d.	n.d.
S29	n.d.	n.d.	n.d.	6.3
S31	n.d.	n.d.	n.d.	6.6
S33	n.d.	n.d.	n.d.	6.3
S35	n.d.	n.d.	n.d.	6.6