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**L. rhamnosus** CNCM I-3690 survival, adaptation, and small bowel microbiome impact in human

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**ABSTRACT**

Fermented foods and beverages are a significant source of dietary bacteria that enter the gastrointestinal (GI) tract. However, little is known about how these microbes survive and adapt to the small intestinal environment. Colony-forming units (CFU) enumeration and viability qPCR of *Lactobacillus rhamnosus* CNCM I-3690 in the ileal effluent of 10 ileostomy subjects during 12-h post consumption of a dairy product fermented with this strain demonstrated the high level of survival of this strain during human small intestine passage. Metatranscriptome analyses revealed the *in situ* transcriptome of *L. rhamnosus* in the small intestine, which was contrasted with transcriptome data obtained from *in vitro* cultivation. These comparative analyses revealed substantial metabolic adaptations of *L. rhamnosus* during small intestine transit, including adjustments of carbohydrate metabolism, surface-protein expression, and translation machinery. The prominent presence of *L. rhamnosus* in the effluent samples did not elicit an appreciable effect on the composition of the endogenous small intestine microbiome, but significantly altered the ecosystem’s overall activity profile, particularly of pathways associated with carbohydrate metabolism. Strikingly, two of the previously recognized gut-brain metabolic modules expressed *in situ* by *L. rhamnosus* (inositol degradation and glutamate synthesis II) are among the most dominantly enriched activities in the ecosystem’s activity profile. This study establishes the survival capacity of *L. rhamnosus* in the human small intestine and highlights its functional adjustment *in situ*, which we postulate to play a role in the probiotic effects associated with this strain.

**Introduction**

The Food and Agriculture Organization (FAO) and World Health Organization (WHO) define probiotics as live organisms that, when consumed in sufficient amounts, confer a health benefit to the host.\textsuperscript{1} Most of the currently marketed probiotics belong to the bacterial families of the Bifidobacteriaceae or Lactobacillaceae,\textsuperscript{2} which are commonly applied in fermented food products. They also inhabit various environments, including the human body.\textsuperscript{3} Among the Lactobacillaceae, various probiotic strains of the species *Lacticaseibacillus rhamnosus* have been described and were shown in multiple studies to elicit beneficial effects on the host. In particular, *L. rhamnosus* was associated with relieving symptoms caused by intestinal microbiota dysbiosis, disruption of intestinal homeostasis, and amelioration of alcohol-induced liver injury.\textsuperscript{4–6} Among the *L. rhamnosus* strains that displayed several probiotic qualities, *L. rhamnosus* CNCM I-3690 was shown in preclinical studies to restore impaired intestinal barrier functions via its anti-inflammatory effect\textsuperscript{7,8} and tight junction modulation\textsuperscript{9} to protect against oxidative stress in a colitis model,\textsuperscript{10} and limit some of the immune and metabolic impairments caused by the pathobiont *Bilophila wadsworthia*.\textsuperscript{11} Additionally, administration of *L. rhamnosus* CNCM I-3690 in a murine model of diet-induced obesity was shown
to limit weight gain, improve glucose-insulin homeostasis, and reduce hepatic steatosis. Finally, in a clinical study that explored the effects of this strain on the gut-brain axis it was reported to significantly reduce subjective stress levels in healthy adults in an academic stress model. These studies underline the broad range of health benefits associated with the consumption of this candidate probiotic strain *L. rhamnosus* CNCM I-3690, warranting further research into its survival and activity during intestinal tract passage.

Passage of the human gastrointestinal tract constitutes a dynamic and challenging environment for ingested bacteria (e.g., probiotics), which may get harmed due to their exposure to gastric juice, bile salts, and host- or microbiome-derived antimicrobial or antagonistic interactions. Nevertheless, various studies have demonstrated the effective survival of probiotic Lactobacillaceae after human intestine transit, mostly employing enumeration of probiotic bacteria in stool samples but also exemplifying their low relative abundances (<1%) in feces (for a review, see). Contrary to this, studies that evaluated the survival and abundance of probiotic bacteria in the small intestine (SI) concluded that these bacteria (transiently) constitute a prominent fraction of the SI microbial ecosystem. Analogously, we have demonstrated that *L. rhamnosus* CNCM I-3690 can transiently inhabit the human SI at a relative abundance of 10–75% in some participants but substantially lower abundance in others. This high versus low colonization efficacy of the ingested *L. rhamnosus* CNCM I-3690 per participant was associated with predominance of carbon- versus amino acid-derived energy metabolism in the endogenous SI microbiome, where the latter situation was reflected by the high relative abundance of the Peptostreptococcaceae in the subject’s SI. Thereby this study provided mechanistic insight in what was previously referred to as colonization-permissive versus -resistant microbiome communities.

With the prominent temporal presence of *L. rhamnosus* CNCM I-3690 in the small intestine, it may be anticipated that they significantly participate in the overall metabolic activity of the SI microbiome. By contrast, such participation would be substantially smaller in the large intestinal microbiome, where these probiotic bacteria constitute only a small fraction (<1%) of the overall community. Importantly, the SI is responsible for most of the nutrient digestion and absorption and is commonly estimated to be responsible for approximately 85–90% of the overall caloric intake in humans. Rodent models have shown that the SI microbiota is involved in regulating a range of metabolic and physiological characteristics ranging from lipid digestion and adsorption, whole-body glucose homeostasis, bile acid metabolism and cycling, as well as contributing to the bioavailability of several important micronutrients like vitamin K and B12 and the circadian rhythm of immune homeostasis. Moreover, the health-importance of the SI microbiome has been highlighted by the association of SI microbial dysbiosis with the pathogenesis of functional gastrointestinal disorders, small intestinal bacterial overgrowth, environmental enteric dysfunction, and other pathologies. Overall, these studies support the need for a deeper understanding of the diet-microbe-host interactions in the SI region, where transient high-level presence and activity of probiotic strains may have an important impact.

The SI is a particularly harsh environment for bacterial life due to its short transit time, high concentration of digestive enzymes and bile salts, and antimicrobial peptides produced by Paneth cells. Consequently, the SI microbial community has a substantially lower density and diversity than the large intestinal community, ranging from approximately $10^4$ to $10^7$ bacteria per mL in the duodenum and distal ileum, respectively. However, in contrast to the colon, the SI microbiome remains relatively poorly characterized due to the invasive technologies required for sampling this segment of the digestive tract. Recent developments in minimally invasive sampling capsules for small intestine microbiome and metabolome analyses may solve these sampling problems in the future. As an alternative, subjects that underwent surgical colon removal (total colectomy) as a remedy for intestinal diseases like colorectal cancer or inflammatory bowel diseases (ulcerative colitis or Crohn’s) have been used to obtain samples from the small intestinal lumen. In a subgroup of
these subjects, the ileum is connected to a stoma in their abdominal wall (ileostomists), allowing non-invasive and repeated collection of effluent of the SI tract.\textsuperscript{30,35,36} Despite this history of intestinal disease, the SI of these individuals is considered to function similarly to the SI tract of a normal individual,\textsuperscript{37} in the absence of comorbidities and need for medication. The normal functioning of the small intestine in these ileostomists is reflected by the significant similarity between the microbiota in the effluent samples and samples obtained from the proximal (jejunum, ileum) regions of the SI in healthy volunteers.\textsuperscript{30} These effluent studies also revealed an individual-specific and highly dynamic ecosystem composition in the SI.\textsuperscript{15} Despite its compositional variation (between individuals and over time), the SI microbiome is commonly dominated by fast-growing facultative anaerobe bacteria that thrive in a habitat characterized by a high nutrient availability and composition fluctuation.\textsuperscript{30} This nutrient adaptability of the SI microbiome is reflected by its enrichment for genes related to acquisition (import) and utilization of simple carbohydrate substrates, rather than pathways associated with complex carbohydrates degradation that are enriched in the colonic microbiome.\textsuperscript{15,30}

Additionally, metatranscriptome analyses in ileostoma effluent samples established high-level expression of sugar phosphotransferase systems, pentose phosphate-, lactate-, acetate-, and propionate-fermentation pathways by some of the most commonly found genera, \textit{Streptococcus}, \textit{Veillonella}, \textit{Staphylococcus}, \textit{Escherichia}, \textit{Clostridium} and \textit{Bacteroides}.\textsuperscript{30,35,38} Although several studies have investigated the survival of ingested (probiotic) bacteria during SI transit,\textsuperscript{39–41} little is known about their specific activity or their impact on the overall microbiome activity profile in this intestinal region.

In the present study, 10 ileostomists consumed a dairy product fermented by \textit{L. rhamnosus} CNCM I-3690 as part of a standardized breakfast. Following consumption, ileostoma effluent was collected for 12 h to assess the effects of product consumption on the SI microbiota composition, but also to determine the population size and viability of \textit{L. rhamnosus} CNCM I-3690. In addition, ileostoma effluent metatranscriptomics was employed to identify the \textit{in situ} \textit{L. rhamnosus} CNCM I-3690 transcriptional adaptation, by comparative transcription analysis using various \textit{in vitro} cultivation conditions. Finally, the relative contribution of the \textit{L. rhamnosus} CNCM I-3690 transcriptome to the SI microbiome metatranscriptome highlighted its contribution to metabolic modules associated with carbohydrate utilization\textsuperscript{42} and those proposed to participate in modulation of the gut-brain axis.\textsuperscript{43}

**Results**

**Study design and assessment of \textit{L. rhamnosus} survival to the gastrointestinal passage**

To evaluate the ability of \textit{L. rhamnosus} to reach the upper gastrointestinal tract in a viable state and survive the passage of the small intestine, ileal effluent samples from 10 subjects were collected prior (0 H) and collectively in 4-h time intervals during an overall 12-h period (0–4, 4–8, and 8–12) after consumption of \textit{L. rhamnosus} fermented product (total bacterial intake \( \sim 10^{11} \times 10^{11} \text{ CFU} \)) as part of a standardized breakfast. After collection and homogenization, sample aliquots were prepared for downstream analyses: metataxonomics, live-dead staining followed by V-PCR and serial dilution and plating in selective media. Importantly, \textit{L. rhamnosus} was not detectable at baseline (no colonies detected on vancomycin-MRS plates, data not shown) and reached its recovery peak after 8 h of ingestion (Figure 1a). The total amount of \textit{L. rhamnosus} CNCM I-3690 recovered during the 12-h sampling period of ileal effluent varied between subjects (Figure 1, panel b, max total recovery VOL13: \( 1.2 \times 10^{11} \text{ CFU} \), min recovery VOL11: \( 3.62 \times 10^{8} \text{ CFU} \)). Approximately \( 5 \times 10^{10} \text{ CFU} \) were recovered per subject, corresponding to approximately 30% of the number of ingested bacteria. A notable degree of fluctuation was observed in the ileal effluent volumes obtained at the different time points in the different volunteers, which impacted on the estimated \textit{L. rhamnosus} concentration per volume (Figure 1, panel b) that on average was \( 2.1 \times 10^{8} \text{ CFU/ml} \) but ranged from \( 1.1 \times 10^{9} \text{ CFU/ml} \) (max; VOL03-8 H) to \( 4.4 \times 10^{4} \text{ CFU/ml} \) (min; VOL11-8 H).

We assessed relative viability of the total \textit{L. rhamnosus} population by V-PCR, able to distinguish between live and dead bacteria (see Materials
L. rhamnosus CNCM I-3690 recovery and concentration in ileal effluent. Samples were collected prior and collectively obtained during 4-h periods over a total timespan of 12 h (4, 8, and 12) after consuming L. rhamnosus CNCM I-3690 fermented product. Serial dilutions were plated in triplicate and L. rhamnosus CNCM I-3690 colonies were enumerated and identity confirmed after 4 (black dots), 8 (magenta dots), and 12 (green dots) hours, while the total amount recovered per volunteer is indicated with a blue asterisk. L. rhamnosus CNCM I-3690 recovery peaked after 8 h in most volunteers (Panel Figure 1a, b), while it was not detected before consumption (data not shown). Ileostomy effluent volumes fluctuated considerably, affecting the L. rhamnosus concentration per sample (Panel b), averaging approximately $2 \times 10^8$ CFU/mL.

Figure 2. The small intestinal environment induces microbiota composition analysis and L. rhamnosus recovery. a) L. rhamnosus CNCM I-3690 gene copies recovery in ileal effluent, assessed via qPCR using both PMAxx treated (black bars) and non-treated samples (pink-bars). Samples were collected prior and collectively obtained during 4-h periods over a total timespan of 12 h (4, 8, and 12) after consuming L. rhamnosus CNCM I-3690 fermented product and prior of it (0 H); b) overview of the microbiota composition throughout the study per volunteer at genus level, only the top 25 genera are displayed (comprising >95% of the total community). The genus formerly known as Lactobacillus is colored in red.
and Method), revealing that on average, 82% of \textit{L. rhamnosus} population was in a viable state (Figure 2a), underlining the high survival capacity of \textit{L. rhamnosus} during gastrointestinal passage. The resulting viable count estimates obtained by qPCR tended to be lower than those obtained by CFU enumerations (average accuracy of 55%), which is in agreement with previous studies that showed similar discrepancies between the two techniques.\textsuperscript{44} These analyses indicate that a relatively high proportion of ingested \textit{L. rhamnosus} CNCM I-3690 reaches the end of the SI in a viable form within 12 h from ingestion, although considerable variations in estimated survival rates and SI passage kinetics are seen between individuals (Max VOL13: 72%, Min VOL11: 0.2%, Average: 30%).

\textbf{Analysis of \textit{L. rhamnosus} fermented product consumption on SI microbial composition}

To assess the effect of \textit{L. rhamnosus} on the small intestinal microbiota, the microbial composition was evaluated via 16S rDNA sequencing. The present observations confirm our previously reported conclusions small intestinal microbiota composition and the impact of \textit{L. rhamnosus} ingestion is highly individual-specific.\textsuperscript{15} The subject ID clearly illustrated this individuality explaining approximately 50% of the total data variation at species level (Supplementary Figure S1) and substantial variability of the \textit{L. rhamnosus} relative abundance in individual effluent samples. Moreover, there is a strikingly high degree of fluctuation in the microbial community composition during the 12 h in which samples were collected (Figure 2b).

The consumption of the \textit{L. rhamnosus} CNCM I-3690 fermented product did not elicit a significant modification of the endogenous microbiota beyond the appearance of the ingested bacterial strain in variable relative abundance, nor did it significantly impact the \(\alpha\) or \(\beta\) diversity of the endogenous microbial community (data not shown). These observations underline the dynamic nature of the SI microbiome, and that the short-term impact of \textit{L. rhamnosus} fermented milk consumption on the SI microbiota is negligible, which we also observed upon prolonged consumption of the same fermented product (2 weeks, daily).\textsuperscript{15}

\textbf{Transcriptome analysis of \textit{L. rhamnosus} recovered from small intestinal effluent}

To evaluate the \textit{in situ} transcriptional activity of \textit{L. rhamnosus} CNCM I-3690, we employed previously determined metatranscriptome data.\textsuperscript{15} These datasets were mined for the \textit{L. rhamnosus} small intestinal \textit{(in situ)} transcription and compared to \textit{L. rhamnosus} CNCM I-3690 transcriptome profiles obtained for 5 \textit{in vitro} growth conditions, including MRS broth without supplementation of a carbon source (MRS-C), supplemented with glucose (MRS+G), lactose (MRS+L), or mannose (MRS+M), and milk. Principal component analysis (PCA) showed separate clustering of the \textit{in situ} samples relative to the MRS samples, which were also distinct from the samples obtained from \textit{L. rhamnosus} grown in milk (Figure 3a). Comparison of the Bray-Curtis dissimilarities within the same 'sample group' showed that the \textit{in situ} samples have a significantly higher degree of variability (Supplementary figure S2), which we interpret to reflect the individuality of the niche and ecosystem of the small intestinal tract.

To investigate the gene expression of \textit{L. rhamnosus} \textit{in situ} in comparison to its transcription profile when grown under \textit{in vitro} conditions, we performed differential expression analysis, using edgeR, identifying 1572 differentially expressed genes (FDR adjusted \(p\)-value \(\leq 0.05\)), of which 390 are highly differentially expressed (Log\(_2\) FC \(\geq 2\)). Notably, these numbers of differentially expressed genes correspond to 53 and 13% of the total \textit{L. rhamnosus} gene repertoire, respectively, which illustrates the major transcriptional adaptation of this bacterium during its transit through the intestinal tract. Finally, most of the highly differentially expressed genes, (295, \(\sim 75\%\); Figure 3b) were expressed at a higher level in the \textit{in situ} samples compared to the \textit{in vitro} samples.

\textbf{\textit{L. rhamnosus} highly modulates carbohydrate metabolism in situ}

The stringently selected differentially expressed genes (Figure 3b) were used to perform Gene Set Enrichment Analysis (GSEA), resulting in the identification of 13 significantly enriched KEGG categories among the genes that were differentially
expressed in the intestinal tract compared to the \textit{in vitro} conditions, most of which were induced in the intestinal tract (Figure 3c). Notably, 11 of the induced categories belonged to the overarching category of “Metabolism,” of which 8 were categorized under “Carbohydrate metabolism,” but which also included the “Uridine monophosphate biosynthesis” pathway, and the “Thiamine metabolism” (Supplementary Table S1).

Conversely, we found that genes involved in the “Fatty acid biosynthesis” and “pyruvate metabolism” were higher expressed (Figure 3d, Supplementary table S1) \textit{in vitro} relative to \textit{in situ} in the intestine. A similar pattern of \textit{in vitro} induction of “pyruvate metabolism” associated genes was observed. Interestingly, \textit{L. rhamnosus} growth in MRS that lacks a supplemented carbon source free, as well as the MRS supplemented with mannose did not induce these categories of genes (Figure 3d). This could relate to the lower bacterial growth rate under the latter two in vitro conditions, which could imply that growth rates in most of the intestinal tract samples is also lower as compared to the glucose or lactose supplemented MRS and milk grown \textit{in vitro} samples. It is important to notice that, the expression of carbon and other nutrient acquisition functions was in many cases activated during intestinal transit in a highly individual-dependent manner, probably
reflecting different diets and consequently different SI environments per individual, which probably underlies the observed variability of *in situ* gene expression (Figure 4a, Supplementary Figure S2). This is confirmed by the high coefficient of variation observed for the *in situ* expression values of specific genes related to nutrient acquisition and metabolism, such as (ABC) transporters (Figure 4a, I) and PTS systems (Figure 4a, II). For example, 9 out of the 18 PTS encoded by *L. rhamnosus* (Figure 4a, II) were, on average, expressed at a higher level *in situ* compared to *in vitro* conditions (predicted substrates include sucrose, β-glucoside, mannitol, cellobiose/diacerylchitobiose, mannose, sorbitol, galactitol, and ascorbate), but variable expression among the *in situ* samples is likely reflecting the differential availability of these substrates in an individuals’ SI environment (Figure 4a, I). Intriguingly, the global regulator involved in the regulation of many of the carbohydrate acquisition genes, Catabolite Control Protein A (*ccpA*, Figure 4b) was significantly higher expressed in the *in situ* samples compared to the *in vitro* samples, albeit with substantial fluctuation of expression in the *in situ* samples that could reflect overall carbon source availability variations *in situ*.

The sample variability of the metabolism associated functions observed in the intestinal samples is sharply contrasted by the highly consistent and homogeneous *in situ* induction of a subset of ribosomal protein encoding genes that are encoded in a gene cluster (*rpsK*, *rpsM*, *rpoO*, *rpmD*, *rpsE*, *rplR*, *rplF*, *rpsH*, *rplE*, *rplX*, *rplN*, *rpsQ*, *rpmC*, *rplP*, *rpsC*, *rplV*, *rpsS*, and *rplD*; Figure 4a, III). Conversely, three other ribosomal protein encoding genes (*rpsT*, *rpmF* and *rpmE2*) were consistently expressed at a higher level in the MRS grown cultures than *in situ* samples. These observations suggest that *L. rhamnosus* CNCM I-3690 consistently adjusts its translation machinery in these two distinct conditions, but not during growth in milk (Figure 4a, III).

**The small intestinal environment induces *L. rhamnosus* surface-protein alterations**

Surface-associated and extracellular proteins play an important role in the interactions with, and adaptations to the environment in bacteria. Therefore, SignalP 5.045 was employed to predict the complete secretome and surface-exposed proteome of *L. rhamnosus*, identifying 120 exported proteins. Strikingly, no less than 70 genes encoding these exported proteins were significantly differentially expressed when comparing the *in situ* (intestinal tract) and *in vitro* samples. Of these 70 genes, 41 and 29 were up- and down-regulated *in situ*, respectively. Several of these proteins are components of transport systems or PTS, but there were also many proteins of poorly defined function. Among the *in situ* upregulated genes many contained specific domains suggesting that they were involved in bacterial adhesion (Supplementary figure S3), i.e., fibronectin-binding, peptidoglycan-binding (LysM), sugar-moiety binding lectin, and two mucin binding (Mub B2 and MucBP) serine-rich adhesins. In addition, several proteins belonging to the sortase anchored cell-wall bound proteins based on the presence of the canonical LPXTG-motif in their protein sequence were similar to proteins that were reported to be important in adhesion to host cells and biofilm formation.46,47 Also, the genes encoding components of the *L. rhamnosus* pilus (*spaD-F*, Figure 4c) were upregulated *in situ*. These proteins form a surface bound multiprotein complex that forms an appendage on the cell-surface that has been shown to play a pivotal role in *L. rhamnosus* adhesion as well as immune-modulation.8 Taken together, the secretome gene expression was on average, higher *in situ* than *in vitro*, but was also quite variable between subjects and timepoints, analogous to what was concluded for the nutrient acquisition systems.

**L. rhamnosus metabolic contribution to the overall ecosystem**

Following the *L. rhamnosus* in situ transcriptome analysis, we wanted to evaluate the ecosystem contribution of this species by examining its relative contribution to the overall activity of the same functional category within the SI ecosystem. To this end, we calculated the “contribution enrichment factor” (see Materials and Methods for details) as a proxy for the contribution to pathway activity by *L. rhamnosus* focusing on the enriched KEGG categories described above (Figure 5a, b). This analysis revealed that while most of the KEGG category activity levels only
Figure 4. L. rhamnosus response to the small intestinal environment. Panel a: The expression values are displayed as edgeR derived, FDR < 0.05, logFC ≥ 2, displayed as a heat map representing fold change relative to the mean expression per row; blue, and red intensities reflect relative down- and up-regulation, white reflects mean-level expression. The variable environmental conditions encountered by L. rhamnosus in each individual is reflected by diverse in situ gene expression of nutrient acquisition genes (e.g., sugar import functions, panel a, parts I and II). Conversely, a ribosomal protein gene cluster is consistently induced in situ, while a few ribosomal protein transcripts are induced in MRS-cultures (panel a, part III), suggesting adjustment of translation machinery associated functions in these contrasting conditions. The degree of variation of in situ gene expression is illustrated by the coefficient of variation color scale at the left end of the heatmaps (white, red). The adjacent color scale (green, white, violet) indicates the average Log2FC in intestinal samples compared to in vitro samples. At the left end of the heatmap display a per category hierarchical clustering is displayed using linkage based on Euclidian distances. Panel b: expression of the global regulator Catabolite Control Protein A gene (ccpA) is induced in situ relative to in vitro, but variable in the intestinal samples (panels aI and aII). Panel c: variable in situ induction of the pilus encoding operon (spaD-F), which plays a role in adhesion and immune-related effects of L. rhamnosus. Significance of the differences in expression was assessed by FDR adjusted p-value <0.05; ****: FDR adjusted p-value <0.0001.
have a small contribution *L. rhamnosus* compared to the rest of the microbial community, the carbohydrate metabolism category displayed a significant overrepresentation of *L. rhamnosus* transcriptome activity relative to the overall community (Figure 5a). The contribution enrichment factors for “Carbohydrate metabolism” sub-pathways, revealed that activity contribution by *L. rhamnosus* was particularly pronounced for “Galactose metabolism”, “Fructose and mannose metabolism”, and “Inositol phosphate metabolism”. Conversely, the *L. rhamnosus* contributions to the ecosystem activity level of “Citrate cycle (TCA cycle)”, “C5-Branched dibasic acid metabolism”, and “Glyoxylate and dicarboxylate metabolism” (Figure 5b) were virtually negligible. Among the most prominent *L. rhamnosus* contributions to the ecosystem activity, the “Inositol phosphate metabolism” is of particular interest since it includes one of the Gut-Brain Modules (GBM) that were previously proposed to potentially influence the human neuronal system. Nineteen of these GBMs are encoded by *L. rhamnosus* (Supplementary Table S1), and two are significantly more expressed by the ingested bacteria than by the small intestinal endogenous microbiota, the “Inositol degradation” and “Glutamate biosynthesis II” modules. Moreover, the “Inositol degradation” module is among the highest contribution enrichment factors among the GBMs encoded by the *L. rhamnosus* genome, indicating that *L. rhamnosus* prominently contributes to the overall activity to this specific pathway (Supplementary Table S1, 2), which is in agreement with the observation that *L. rhamnosus* is responsible for most of the Inositol degradation expression in the small intestinal metatranscriptome (Figure 5d, mean 85.6% ± 7.9%). Moreover, most of the 13-gene inositol degradation operon of *L. rhamnosus* (LRHA10_v2_0256–0268) was strongly upregulated in situ relative to in vitro conditions (Supplementary Figure S4a, b, Figure S5e). While the Inositol degradation module is considered to be relatively rare in reference genomes of intestinal bacteria, with a prevalence of less than 10%, the Glutamate biosynthesis II module is more commonly found in the gut microbiota, being present in 76% of the bacterial population.

**Discussion**

The definition of probiotics by the FAO and WHO -live organisms that confer a health benefit to the host when consumed in sufficient amount leaves the viability and activity of the probiotic during gastrointestinal transit uncharacterized. However, what happens after ingestion, including the bacterial viability, adaptation, and response to the new environment is crucial to understand host-microbes interaction and their possible physiological effects on the host. Here, we demonstrate that *L. rhamnosus* CNCM I-3690 can effectively survive the passage of the human small intestine, and adjusts its gene expression based on the new environmental condition. We were able to consistently detect the strain in the ileostoma effluent for up to 12 h post-ingestion, with a peak in bacterial density observed in the 4–8-h interval and a high viability percentage within the *L. rhamnosus* population (average ~82%). Approximately 30% of the consumed bacteria were recovered from the effluent, indicating the metabolic contributions of the strain to the small intestinal ecosystem for at least 12 h. These findings align with existing literature that emphasized the robust survival capabilities within the upper gastrointestinal tract of members of the Lactobacillaceae family and that their ingestion does not induce significant changes to the endogenous SI microbiota, beyond the introduced strain, in high but variable relative abundance. The data collected in this study allowed us to estimate the community density during the 12 h of sample collection, which revealed a strikingly high degree of fluctuation. We noted considerable fluctuations in the community composition and density during sample collection, which reflect the microbial ecosystem’s response to nutrient availability and transit rate through the SI system, which plausibly affect transient selective pressure in the ecosystem as well as determine the growth rate and absolute abundance of its members. The dynamic nature of the human small intestine microbiota, following internal and external perturbations, has been reported in other studies, although never addressing such short timespan fluctuations as described in this study,
Figure 5. Ecosystem contribution by *L. rhamnosus* on the overall activity profile of the microbiota. The “contribution enrichment factor”, calculated as $\log_2$ of the fraction of all *L. rhamnosus* reads mapped to a category divided by the fraction of all other reads mapped to that category, was assessed for the main KEGG metabolism classes (panel a) and for the carbohydrate metabolism major categories (panel b). The analyses showed that *L. rhamnosus* dedicates more of its transcriptome compared with the endogenous
supporting the importance of carbohydrate availability as a determinant in rapidly shaping the small intestinal ecosystem.  

Recently, minimally invasive sampling capsules were successfully tested for the small intestine microbiome and metabolome analysis in human subjects.  These advancements can revolutionize gut microbiota research, by expanding the slope to the small intestine. However, although the capsules were enabling metabolite and microbiome composition analyses, there are still substantial challenges to overcome like adequate quenching procedures to stagnate further bacterial growth, activity changes, and metabolic conversions once the sample is internalized into the sampling capsule because the capsule remains at body temperature for hours afterward, while transiting through the remainder of the gut. Particularly metatranscriptome measurements will most likely be unreliable in such samples, due to the short lifetime of the average microbial mRNA as well as the very minute sample size that can be collected with these devices. This limited sample size, while capable of revealing valuable data, also raises concerns about representativeness, given the immense diversity and variability of the gut microbiota across different intestinal segments. Deploying multiple capsules could mitigate the latter issue and improve the samples’ representativeness. Although capsule sampling might not completely replace techniques aiming at obtaining larger sample volume sampling (e.g., ileostomy effluent) for comprehensive studies, it marks a significant advancement in gut microbiota investigations, which with further technical improvements may provide possibilities beyond microbiome composition and metabolism in the future.

The in situ transcriptome analysis revealed that more than half of all genes encoded by L. rhamnosus CNCM I-3690 were differentially expressed, primarily induced in situ, indicating that this strain is active during SI passage and drastically adjusts its gene expression profile in comparison to *in vitro* culturing conditions. Environmental conditions, particularly nutrient and carbon source availability, play a key role in modulating *L. rhamnosus* transcriptome. This is evidenced by the enrichment of differentially expressed genes related to carbohydrate metabolism and nutrient acquisition, which are crucial for bacterial survival during GI tract passage. Other lactobacilli, like *L. rhamnosus*, adapt their carbohydrate acquisition and surface protein expression to thrive in the variable dietary conditions in the human intestine. Carbon source availability may be highly diverse and dynamic in the small intestine due to variable dietary intake. This sharply contrasts the nutrient-rich environment and single carbon source provided in the laboratory media that were used in this study. The diversity of the small intestine environment is evidenced by varying expression of carbohydrate metabolism-associated genes across different individuals and timepoints. Importantly, the in situ expression of the global regulatory function CcpA is induced, signifying its role in regulating bacterial adaptation to these changing environmental conditions. CcpA controls the transcription of many genes involved in carbohydrate metabolism, growth, stress response, and metabolite production in many gram-positive bacteria and is a pivotal regulator in bacterial adaptation to environmental changes that orchestrates transitions between environmental (nutrient) specialization (e.g., in stable and constant laboratory conditions) and environmental (nutrient) generalization (e.g., in dynamic conditions like the intestinal tract). Notably, PTS systems and Pyrimidine metabolism, both highly expressed in situ, were reportedly involved in acid stress tolerance that links CcpA regulation to generic environmental fitness and stress tolerance.

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microbiota to “Carbohydrate metabolism”, “Galactose metabolism”, “Fructose and mannose metabolism”, and “Inositol phosphate metabolism” (Wilcoxon tests, Holm-Sidak Post hoc test * p-value < 0.05, ** p-value < 0.01, ***p-value < 0.005).

The “Inositol phosphate metabolism” and the “Glutamate biosynthesis II” are identified as two of the nine Gut-Brain Modules encoded by *L. rhamnosus* genome and they are significantly more expressed by *L. rhamnosus* than by the small intestinal endogenous microbiota (c, Mann–Whitney test, Holm-Sidak Post hoc test ***p-value <0.005, **** p-value <0.001). *L. rhamnosus* is responsible for most of the Inositol degradation expression (d, red represent in *L. rhamnosus* contribution, in blue the endogenous one) and that most of the 13-genes-long operon (e), encoding the entire Inositol degradation pathway, is highly expressed in situ compared with in vitro conditions.
We observed an opposite expression pattern for most of the fatty acid biosynthesis pathways (\textit{fabF-Z, accA-D}, and \textit{mleA}) involved in controlling the fatty acid composition of the bacterial membrane to maintain its stability and normal physiological function, and its expression is downregulated in media containing high amounts of saturated fatty acids.\textsuperscript{50,61} This suggests that \textit{L. rhamnosus} utilizes fatty acids that are available in the lumen of the small intestine, which is the main region of the intestinal tract for fat degradation and absorption, aligning with previous reports on \textit{Lactobacillus}'s influence on fat absorption.\textsuperscript{62}

The remarkable adaptability of \textit{L. rhamnosus} to the SI environment is exemplified by the consistent \textit{in situ} induction of one of the major ribosomal gene clusters, potentially regulated by the multifunctional ribosomal protein L4 RplD, which could play a critical role in the adaptation to the SI environment.\textsuperscript{63,64} Apart from protein biosynthesis, ribosomal proteins, especially the L4 protein, also contribute to DNA repair, cell death, transcription regulation, and environmental sensing, and was proposed to contribute to bacterial environmental adaptation by modulating mRNA composition under stress conditions through RNA degradation.\textsuperscript{63,65}

\textit{L. rhamnosus} CNCM I-3690 has been associated with various probiotic activities, including the ability to prevent and/or hamper dysfunctions related to the gut barrier. One of the bacterial functions responsible for this ability has been proposed to be the \textit{L. rhamnosus} CNCM I-3690 pilus (\textit{spaD-F}),\textsuperscript{8} a cell-associated extracellular proteinaceous appendage, homologues of which have been shown to influence bacterial adhesion and immune-modulation in various probiotic and commensal bacteria.\textsuperscript{66,67} The expression of the \textit{spaD-F} was significantly induced \textit{in situ}, demonstrating that this important host-microbe interaction function of \textit{L. rhamnosus} CNCM I-3690 is actively expressed in the small intestine, albeit with considerably fluctuating levels of expression in different individuals. The \textit{in situ} environment also influences the expression of other bacterial functions, such as surface and extracellular proteins, that are considered pivotal for host interaction and gut persistence. Specifically, mucus and extracellular matrix interaction genes are induced in situ, potentially influencing adhesion and biofilm formation.\textsuperscript{46,68,69} \textit{L. rhamnosus} CNCM I-3690 consumption significantly contributed to the ecosystem activity profile, especially on simple carbohydrate utilization pathways, including those for galactose, fructose and mannose. These pathways are relatively common among bacteria, and this finding is probably indicative of the competitive capacity of \textit{L. rhamnosus} to acquire these substrates for growth.

An intriguingly high enrichment factor was found for the "inositol phosphate metabolism" pathway, where in many samples the majority of this activity in the ecosystem could be assigned to \textit{L. rhamnosus} CNCM I-3690. Inositol, a widely present sugar especially in its Myo-inositol form, is critical for health, with aberrations linked to various metabolic imbalances and neurological disorders.\textsuperscript{70,71} In addition, inositol also is an important constituent of phosphatidylinositol (PI) and phosphoinositides (PIPs) that play important roles in lipid signaling, cell signaling and membrane trafficking, thereby affecting a range of functions in a variety of cell lineages and tissues. Notably, specific PIP derivatives have been associated with an expanding spectrum of neurological disorders (for a review see\textsuperscript{72}). Based on these pleiotropic consequences for health, and neurological functioning, the inositol degradation function of the microbiome has been identified as one of the Gut-Brain Modules (GBM) involved in the microbiome metabolism of neuro-active or neuro-modulatory compounds.\textsuperscript{43} Importantly, inositol degradation pathway is among the "rare" GBMs in microbial genomes, which is likely explaining the prominent contribution of \textit{L. rhamnosus} to this pathway’s activity level \textit{in situ} in the intestinal tract. The relevance of the high level \textit{in situ} activity of the inositol pathway in \textit{L. rhamnosus} remains to be deciphered, but based on the pleiotropic roles of inositol and its derivatives there may be various effects of the modulation of (local-mucosal) inositol levels by \textit{L. rhamnosus}. In addition, \textit{L. rhamnosus} has a significant contribution to the Glutamate synthesis II GBM in the SI ecosystem, which can affect glutamate balance in the gut and thereby influence the gut-brain axis due to the critical role that glutamate plays in the central nervous system.\textsuperscript{73} The significant contribution of
*L. rhamnosus* to the Glutamate synthesis II GBM activity *in situ* is particularly remarkable because this GBM is considered to be highly prevalent among intestinal bacteria. The prominent role of *L. rhamnosus* in the *in situ* expression of these rare and common GBMs warrants further investigation into its influence on the gut-brain axis and it is tempting to speculate that these *in situ* activities of *L. rhamnosus* CNCM I-3690 could underlie the effects of its consumption on academic stress in healthy adults.

Altogether, our results clearly demonstrate that *L. rhamnosus* CNCM I-3690 effectively survives SI passage in humans and persists in this niche for at least 12 h. During its residence in the SI *L. rhamnosus* CNCM I-3690 drastically adapts its transcriptional profile as compared to *in vitro* culture conditions, prominently including activation of carbohydrate utilization pathways, cell-surface exposed functions, and a specific ribosomal protein gene cluster. A striking ecosystem contribution of *L. rhamnosus* CNCM I-3690 to the activity of specific GBMs inspires further investigation of the potential role of this activity in the modulation of the gut-brain axis. Thereby, this study can support the probiotic role of *L. rhamnosus* CNCM I-3690, which may elicit at least some of its health-benefit effects in the small intestine where it is transiently and abundantly present in a viable and active form, expressing functions that can modulate mucosal biochemistry and/or responses that underlie observed (pre-)clinical effects.

**Materials and methods**

**Subjects**

All the adult ileostomy patients without comorbidities recruited for the INSIDE intervention study, registered in the US National Library of Medicine [http://www.clinicaltrials.gov](http://www.clinicaltrials.gov), ID NCT02920294), were asked to return to the laboratory facility within 4 weeks from the end of the study. As the additional test days extended the total duration of the INSIDE intervention, participants were asked for their separate consent for this part of the study. On the informed consent form, they indicated to consent to participation on these extra test days. Out of the 16 participants of the INSIDE study cohort, 10 participated also in the present study and gave written informed consent. Inclusion criteria did not filter for age, gender, or other demographic parameters due to the limited pool of available volunteers. The nature of our study necessitated working with ileostomy patients, of which there are few without comorbidities and willing to participate in such studies. Importantly, to avoid confounding effects on the study endpoints, study subjects were not allowed to consume pro-, pre- or symbiotics, fresh dairy fermented products (such as yogurt, cottage cheese, buttermilk, or soft raw cheeses) as well as other food products that are fermented (i.e., sauerkraut) during the study period and three months prior to participation in this trial (a list of forbidden products was provided). Due to the very limited availability of human intervention studies that quantitatively investigate microbial survival in the small intestine after their ingestion, no *a priori* sample size calculation could be performed.

**Study design and intervention products**

Study participants received 100 mL of milk fermented by *Lacticaseibacillus rhamnosus* CNCM I-3690 (*L. rhamnosus*) intervention product together with a standardized breakfast (bread and jam, coffee or tea at libitum). The intervention product was manufactured by Danone (Danone Nutricia Research, Palaiseau, France) and consisted of 100 mL of milk fermented by *Lacticaseibacillus rhamnosus* CNCM I-3690 (*L. rhamnosus*) at approx. 10$^{9.5}$×10$^{9}$ Colony Forming Units (CFU) per mL (i.e., total intake ~ 10$^{11.5}$×10$^{11}$ CFU). The CFU enumeration of the products was performed by the manufacturer using plating (in De Man, Rogosa and Sharpe, MRS agar supplemented with 1% lactose) and colony counting. Following this breakfast, participants stayed in Maastricht Medical Center for 12 h and collected the total ileostomy effluent during the time intervals of 0–4, 4–8, and 8–12 h after breakfast using 500 mL collection tubes (Corning, New York, United States). As a reference, an ileostomy effluent sample was taken just before product intake and breakfast (approximately 5 mL). Notably, the study lacks the 8–12 H sample of VOL01 because this participant could only take part in the first 8 h of the study. In addition, the 0–4 H and 4–8 H samples of VOL05
contained only very small effluent volumes (<5 mL), disallowing the *L. rhamnosus* CNCM I-3690 survival assessment, and only allowing the 16S analysis of the small intestine microbiome composition.

**Survival determination**

Ileostomy effluent volumes were determined, and the samples were kept on ice throughout the whole process. Initially, the samples were homogenized using Ultra-Turrax t50 (IKA, Germany), and when necessary defined volumes of sterile phosphate-buffered saline (PBS) solution were added to the sample to facilitate homogenization, and in the latter cases the consequential dilution factor was recorded. The viability of the *L. rhamnosus* population was determined using plating and colony enumeration, and using viability PCR (V-PCR). V-PCR is a molecular technique that combines qPCR and the PMAxx dye (Biotium, Fremont, CA, USA), a high affinity photoactive DNA-binding dye. Upon light exposure, PMAxx covalently binds to DNA, preventing it from being amplified by PCR. The dye is membrane-impermeant, thus, it binds only to free DNA or DNA of bacteria with compromised cell membranes, enabling the discrimination between live and dead (compromised) bacteria.  

Following homogenization, part of the sample was aliquoted in triplicate (2 mL) and processed for live/dead bacterial ‘staining’ using PMAxx (see below), while 1 mL was serial diluted (1:10) and plated in duplicate on MRS agar plates supplemented with vancomycin (200 mg/L; *Lactaseibacillus rhamnosus* CNCM I-3690 is naturally resistant to this antibiotic). After overnight incubating at 37°C, the colonies were counted, and the identity of the colonies was confirmed by strain-specific colony PCR using a random selection of 10–15 colonies per plate. The specificity of the primers used (FOR AS113: GTGACAACGCCAATCACTTG, REV AS114: TATCGGTGCCATTTGAGTGA), targeting a gene encoding a putative transcriptional regulator of the Cro/CI family, was verified both in silico via BLAST search paying particular attention to align the sequences with genomes of bacterial genera typically found in ileostomy effluents, and by performing PCR on DNA extracted from ileostomy effluents sampled prior *L. rhamnosus* product consumption.

For the samples designated for live-dead staining, debris was removed by slow-spin centrifugation (700 g, 15 min at 4°C), followed by harvesting the bacterial pellet (4000 g for 45 min at 4°C), which was subsequently washed in 1 mL of ice-cold sterile PBS, harvested again (4000 g for 45 min at 4°C) and finally resuspended in 800 μL of 10 mM Tris-Cl, pH 8.5. These samples were equally aliquoted into two vials (~400 μL each) and analyzed for total and intact (viable) bacterial counts. To determine intact (viable) bacteria, 2.2 μL of 20 mM PMAxx dye was added to the samples, followed by incubation in the dark for 10 min with periodic mixing, after which the samples were placed into the PMA-Lite LED Photolysis (Biotium, CA, USA) and exposed to the light for 17 min. This photoactivation step is essential for the dye to covalently bind to the bacterial DNA in cells that were penetrable by the dye (i.e., had compromised membrane integrity), thereby excluding this DNA from PCR-amplification. The matching sample aliquots with or without PMAxx were subjected to identical incubation and light exposure procedures. DNA was extracted using the DNA Faecal/Soil Microbe Kit (Zymo, CA, U.S.A) according to the manufacturer’s instructions. V-PCR was performed in triplicate for each treated and non-treated sample using iQ™ SYBR® Green buffer (Biorad, CA, U.S.A.) and the same primers as those used for the colony identification (FOR AS113: GTGACAACGCCAATCACTTG, REV AS114: TATCGGTGCCATTTGAGTGA). The reactions were initiated at 95°C (5 min), followed by 40 amplification cycles with denaturation at 95°C for 10 s and annealing 58°C for 45 s and extension at 72°C or 1 min. The amplification of a single product was confirmed by running a melting curve analysis for all the samples (65–95°C with 0.5°C intervals) and agarose gel electrophoresis analysis of the qPCR products. A threshold cycle (Ct) value was derived from the V-PCR assay, and the Ct value was converted to colony-forming unit (CFU/mL) using a standard curve. The calibration curve was obtained using purified genomic DNA, quantified via Qubit® Fluorimeter (Thermo Fisher Scientific, MA, U.S.A.), extracted from a suspension of *L. rhamnosus*, and serially diluted to yield a concentration range corresponding to 10^2–10^8 CFU/mL. Before this, we evaluated the possible effect that chemical and physical
characteristics of the ileostomy effluent may have on the limit of quantification, on the calibration curve quantification, and on the PMAXx efficacy. Therefore, known amounts of *L. rhamnosus* and of its genomic DNA were spiked in ileostomy effluents belonging to 3 different volunteers, sampled before *L. rhamnosus* product consumption. The V-PCR mentioned above protocol resulted from this optimization, and the limit of quantification with suitable precision and accuracy was set at LOG 3 CFU/ml. Calibration curves and conversion from Ct to CFU/ml were performed using CFX manager (Biorad, CA, U.S.A.) and GraphPad Prism® Version 9.0 (GraphPad Software, CA, USA). Congruency of V-PCR, indicating the similarity of V-PCR and plating results was calculated as:

$$\text{Congruency} = \frac{\text{viable counts by V-PCR} - \text{viable counts by plating}}{\text{viable counts by plating}} \times 100.$$ 

**Intestinal microbiota composition**

Homogenized ileostomy effluent samples (10 mL) were transferred to 15 mL collection tubes (screw cap feces container tubes, Sarstedt, Germany) containing a 2× concentrated DNA shield (Zymo Research, CA, USA) and stored at –20°C until further processing. Samples were thawed, and larger debris were removed by low-speed centrifugation (4°C, 500 g, 3 minutes, no breaks), followed by microbiota collection by high-speed centrifugation (at 4°C, 8000 g, 10 minutes). DNA was extracted from the pellets obtained using the DNA Faecal/Soil Microbe Kit (Zymo, CA, U.S. A), according to manufacturer’s instruction. The DNA quantity and quality were checked by gel electrophoresis and Nanodrop DeNovix DS-11 spectrophotometer (DeNovix Inc., USA). The production of paired-end sequencing of the Illumina 16S rRNA gene amplicon libraries (V3–V4 region, primer 341F: CCTAYGGGRBGCASCAG and primer 806R: GGACTACNNGGGTATCTAAT), was performed by Novogene (Hong Kong, China). The results were analyzed using CLC Genomics Workbench version v7.5.1 and the CLC Microbial Genomics Module version 1.5 (CLC bio, Arhus, Denmark). Briefly, the paired-end reads were merged into one high-quality representative (mismatch cost = 1, minimum score = 40, Gap Cost = 4, maximum unaligned end mismatches = 5), and primer and quality trimming processed (quality score = 0.05, trim ambiguous nucleotides: maximum number of ambiguities: 2, discards reads below length = 5). The remaining high-quality sequences were clustered into operational taxonomic units (OTU) using the SILVA 16S v132 97% database as the mapping reference database. A total number of paired-end, unique reads of ~1.6 M, with an average of 41,160 reads/samples (max: 58100, min: 26261), were clustered in 5101 operational taxonomic units (OTUs), of which 4566 with more than two counts.

The legitimate assignment of OTU-sequences to *L. rhamnosus* CNCM I-3690 was confirmed as previously described.\textsuperscript{15,35}

**In vitro and in situ transcriptome analyses**

For the transcriptome analysis of *in vitro* cultured *L. rhamnosus* CNCM I-3690, bacteria were inoculated in and grown overnight at 37°C in milk, or in house made MRS that lacked an added carbon source (MRS-C), which was supplemented with 1% glucose (MRS+G), lactose (MRS+L) or mannose (MRS+M). The next day the overnight cultures were subcultured (1:100) in triplicate in the same medium, and bacterial growth at 37°C was monitored in MRS by measurement of the optical density at 600 nm (OD600) with a Microplate Spectrophotometer (SpectraMax M5, Molecular Devices, San Jose, California, USA). The bacterial growth in milk was followed via time-series sample collection, dilution, and plating in MRS agar while recording the pH changes. The colonies were counted after 24 h at 37°C. The bacterial cultures (5 mL) were sampled when they reached mid-exponential phase: 6–7 h from subculturing; ~ pH 4.8 in milk, OD600 ~1.2 in MRS+G and MRS+L. Bacterial cells were harvested by centrifugation (4°C, 12000 g, 3 min) immediately after sample collection, and the pellet was snap-frozen in liquid nitrogen until further processing. Notably, *L. rhamnosus* grown in MRS-C and MRS+M never reached a high cell-density (final OD600 ~0.8) and were therefore sampled at a lower OD600 (~0.4) in higher volumes (20 mL). The *L. rhamnosus in situ* transcriptome, when it resides in the small intestine, was determined
by metatranscriptome analysis of the ileostomy effluent samples that were previously collected for that purpose. The bacterial pellets obtained from in vitro cultured and effluent samples were resuspended in 100 µl of phenol-chloroform – isoamyl alcohol (pH 6.5–8.0, Sigma, Germany). RNA was extracted using the RNeasy PowerMicrobiome Kit (Qiagen, Germany) following the manufacturer’s instructions. RNA quality and quantity were analyzed by agarose gel electrophoresis and the TapeStation 2200 (Agilent Technologies, CA, United States), and RNA samples were stored at −80°C. The 250 ~ 300 bp insert cDNA library with rRNA removal (Ribo-Zero™ Magnetic Kit, Illumina, USA) and sequencing was performed by Novogene (Hong Kong, China) using the Illumina HiSeq2500 platform (PE150, 12GB raw data/sample for the metatranscriptome analysis and 4 G raw data/samples for the in vitro transcriptome analysis).

Low-quality sequence regions and adapter-derived sequences were removed from the Illumina reads with fastp (v0.19.5) using default settings. The remaining rRNA sequences were removed with BBduk (v38.32) using parameters k = 31 and ref=riboKmers.fa.gz. The RNAsseq data obtained from in vitro cultured L. rhamnosus and the complete metatranscriptome RNAsseq data obtained from the ileostomy effluent samples (n = 12) were mapped against the L. rhamnosus CNCM I-3690 genome followed by determination of mappings per gene to obtain read counts per gene with BBmap default settings and its corresponding Reads per kilobase per million mapped reads (rpkm). All ORFs that were detected to be transcribed were functionally re-annotated using KofamKOALA with KEGG v.92. Signap 5.0 with the default settings was used to predict the presence of surface-associated and/or extracellular proteins in L. rhamnosus genome. For the remaining metatranscriptome reads that were not mapped to the L. rhamnosus CNCM I-3690 genome, the Human Gut Integrated Gene Catalog (IGC) was used for read mapping using BBmap with the following parameters: covstats, out, idhist, indelhist, ehist, sortscats, statsfil, rpkm, ambig=random, unpigt=t, machineout=t, intronlen = 20, maxindel = 20, strictmaxindel=t. The values in the rpkm files were used to further process the read counts per gene. For taxonomic classification of the genes in the IGC database, the online available classifications were used (https://db.cngb.org/microbiome/genecatalog/genecatalog_human).

Statistical analyses

The microbial compositional data was filtered per reads count (at least two reads in 10% of the samples), MicrobiomeAnalyst was used for the calculation of α-diversity indices, including Observed species, Shannon and Chao1. The β-diversity, distance-based redundancy analysis (db-RDA), principal component analyses (PCA), partial and non-redundancy analysis (pRDA), were obtained using the Canoco 5.10 software suite using 1000 permutations in (p-) RDAs to assess the significance, when needed, the relative abundance were log-transformed (Y’ = Y + 1000) and centered. The effect of variables such as gender and time was evaluated by RDA and pRDA separately and excluded as co-variates in further analyses as they did not significantly impact the microbiota composition. Differential expression and abundance analyses were performed via Empirical Analysis of Digital Gene Expression (edgeR) implemented in Network Analyst after trimmed means of M-values (TMM) transformation, and false discovery rate (FDR) was used to correct for multiple testing, and an FDR adjusted p-value < 0.05 was considered statistically significant. The Gene Set Enrichment Analysis was performed via GSEA-pro V3 (http://gseapro.molgenrug.nl, Groningen University, the Netherlands GOMixer version 1.7.5.0. (http://www.raeslab.org/gomixer/) was used for the dedicated functional analysis of the Gut-Brain modules with the default settings. Heatmaps were made in r, ComplexHeatmap, Bioconductor release 3.12, hierarchical clustering using complete linkage based on Euclidian distances. The average gene expression per volunteer was used to calculate the fold change from the row mean for each pathway or module displayed in the heatmaps. The Coefficient of Variation was calculated as the percent ratio of the standard deviation.
and the mean of the \textit{in situ} sample expression values. The “contribution enrichment factor” was calculated as log$_2$ of the fraction of all \textit{L. rhamnosus} reads mapped to a KEGG category over the fraction of all other reads mapped to that KEGG category. GraphPad Prism (9 for Windows, CA, USA) was used for graphical outputs and the Wilcoxon tests with Holm-Sidak Post hoc test and the Mann–Whitney test with Holm-Sidak post hoc test.

**Disclosure statement**

This author discloses the following: T. Smokvina and C. Chervaux are employees of Danone Nutricia Research. The remaining authors declare that they have no competing interests.

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**Availability of data and materials**

The Metatranscriptome dataset supporting the conclusions of this article is available in the DANS repository, on the following doi: https://doi.org/10.17026/dans-xvh-yww8. The 16S data and the \textit{In} vitro transcriptome data are available in the ENA repository under the following accession number: PRJEB61139 and PRJEB61087, respectively.

**Ethics approval and consent to participate**

This study was approved by the Medical Ethics Committee of Maastricht University (MU), Maastricht, the Netherlands, and performed in full accordance with the Declaration of Helsinki (latest amendment by the World Medical Association in Fortaleza, Brazil, in 2013) and Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998), as well as with the International Conference on Harmonisation – Good Clinical Practice (ICH-GCP) guidelines. The study was performed at MU from 27 October 2016 (screening visit, first subject) until 27 October 2017 (last visit last subject). This study was registered in the US National Library of Medicine (http://www.clinicaltrials.gov, ID NCT NCT02920294). All subjects gave written informed consent before screening.

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