Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways

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Probiotic bacteria, specific representatives of bacterial species that are a common part of the human microbiota, are proposed to deliver health benefits to the consumer by modulation of intestinal function through largely unknown molecular mechanisms. To explore in vivo mucosal responses of healthy adults to probiotics, we obtained transcriptomes in an intervention study after a double-blind placebo-controlled cross-over design. In the mucosa of the proximal small intestine of healthy volunteers, probiotic strains from the species *Lactobacillus acidophilus*, *L. casei*, and *L. rhamnosus* each induced differential gene-regulatory networks and pathways in the human mucosa. Comprehensive analyses revealed that these transcriptional networks regulate major basal mucosal processes and uncovered remarkable similarity to response profiles obtained for specific bioactive molecules and drugs. This study elucidates how intestinal mucosa of healthy humans perceives different probiotics and provides avenues for rationally designed tests of clinical applications.

gene regulation | host–microbe interactions | lactobacillus | transcriptomics | gut bacteria

It has become widely acknowledged that the interaction between normal microbiota and the human mucosa is essential for proper intestinal function. Metagenomic studies have shown that the gut microbiota influence the efficiency of energy harvest from the diet and fat storage to such extent that the gut microbiota may be of therapeutic and clinical relevance. In fact, microbiota may even offer targets for drugs that help to counter diseases such as obesity and Crohn's disease (1, 2). The microbiota also influence development and functioning of the immune system (3). For instance, a specific part of the microbiota has been shown to cooperate with the development of regulatory instead of inflammatory IL17-producing T helper cells in the small intestine (4). Besides the gut-resident bacteria, many bacteria transiently pass through our intestine as part of our diet. Studying the interactions of food-associated microbes and the intestinal mucosa in vivo in humans offers an interesting possibility to address host–microbe interactions in a reductionist approach. However, because the gut of adult humans is already colonized with an endogenous microbiota, interventions should involve large numbers of specific microbes to induce a measurable response, and these microbes should be supplied through food (supplements) in an experimental setting that relates as well as possible to normal food consumption. Use of *Lactobacillus* species, Gram-positive bacteria that play important roles in diverse food-fermentation processes, seems to fulfill these requirements and offer a plausible model to study the molecular impact of food-associated bacteria in human mucosal tissues.

One specific group among the lactobacilli are the probiotics, live microorganisms that, when administered in adequate amounts, are proposed to confer a health benefit on the host. Probiotics are typically supplied in large amounts of 10^8 to more than 10^9 viable bacteria per daily dose (5). The proposed health benefits include reduction of intestinal infection risk, allergies, and atopic eczema (6, 7) and relief from symptoms of inflammatory bowel disease (IBD) (8). However, placebo-controlled cross-over studies to scientifically support these health claims have shown different success rates (8–18).

L. acidophilus Lafti-L10 (DSM), *L. casei* CRL-431 (Hansen), and *L. rhamnosus* GG (Valio) are among the most commonly sold commercially available probiotic strains. *L. rhamnosus* GG bears the most substantial and scientifically convincing support for its clinical efficacy (14, 15, 19, 20). Clinical trials investigating how probiotics may influence human intestinal function should ideally include measurements at the whole-genome level. Using a placebo-controlled randomized double-blind cross-over design (21), we studied in vivo mucosal responses of healthy adults to the three aforementioned probiotic strains. The volunteers consumed all three probiotic preparations and a placebo control in a randomized order; the interventions were separated by a 2-wk wash-out period. After a 6-h period of consumption of bacterial or placebo preparations, biopsies were taken from the duodenum by standard flexible gastroduodenoscopy. RNA extracted from these biopsies was hybridized to Affymetrix whole-genome expression arrays. Comprehensive interpretation of the mucosal transcriptomes using advanced software tools revealed differential pathway modulation with strong biological implications, indicating that, notwithstanding a large person-to-person transcriptome variation, normally colonized human mucosa mounted fast and specific responses on perception of each bac-

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terial strain. Comparison of these specific expression profiles with response profiles associated with pharmaceutical and other biologically active compounds showed that the responses to probiotic strains correlated significantly with responses to compounds active in regulation of immune responses, the cell cycle, blood pressure, and water and ion homeostasis. Intriguingly, we found that these transcriptomes could explain some of the published results obtained in clinical probiotic trials. These comprehensive and comparative analyses may guide the design of rational strain-specific clinical tests involving application of probiotic supplementation in humans, possibly including persons suffering from specific intestinal conditions.

Results

Here, the biological context of differential in vivo transcriptional responses of seven healthy adult human volunteers to three probiotic strains is described at mucosal-tissue level. Each volunteer consumed 5.2×10^{10} *L. acidophilus*, 3.2×10^{10} *L. casei*, 1.68×10^{10} *L. rhamnosus*, or a placebo control. These dosages fall within the range of probiotic dosages used in clinical interventions or marketed as product. The trial was carried out according to a randomized double-blind cross-over design during a 6-wk experimental period with a 2-wk separation between interventions. None of the volunteers experienced any discomfort or complaint after the consumption of bacteria or placebo control. After the 6-h period, biopsies were taken by standard flexible gastroduodenoscopy, and total RNA was isolated and hybridized to whole-genome expression microarrays. Quality control of the hybridizations and primary data analysis were performed according to strict criteria (*SI Appendix*, *SI Materials and Methods* and *SI QC Report*) to ensure that the array data were of the highest possible quality.

Consumption of bacteria resulted in the differential expression of several hundred up to thousands of genes in vivo in human mucosa (*SI Appendix*, Table S1). As observed in a previous study (21), transcriptomes clustered largely per individual volunteer (Fig. 1), showing that variation between persons was the largest source of transcriptome variation. The array data were validated for seven genes using quantitative reverse-transcription PCR (QPCR), corroborating the differences in transcription measured on the array (*SI Appendix*, Figs. S1 and S2).

Because gene-set comparisons and pathway reconstruction from differentially expressed genes are far more informative than their tabulated up- or down-regulation (22), array data were provided with biological context by six complementary in silico approaches. These approaches relate changes in gene expression to functional changes that reflect which cellular pathways and processes were modulated by transcriptional networks and if these changes may have had clinical or pharmaceutical relevance (*SI Appendix*, *SI Materials and Methods*).

Mucosal Responses to *L. acidophilus* Involve Regulation of Immune Response, Hormonal Regulation of Tissue Growth and Development, and Ion Homeostasis. We first used the Bibliosphere (Genomatix) software program to visualize coexpressed genes clustered in a network structure according to protein–protein and protein–DNA interactions. Mucosal responses to consumption of *L. acidophilus* were associated with 10 regulatory nodes (*SI Appendix*, Fig. S3*A*), driving regulatory networks associated with ILs, IFN, insulin, hormones, and metabolism. The majority of the strongest induced genes were associated with up-regulation of IL-1β and included genes stimulating and regulating immune responses such as IL-17B (23), IL-1 receptor-activated kinase 2 (IRAK2), and multiple chemokine (C-C motif) ligands (CCLx) and chemokine (C-X-C motif) ligands (CXCx) cytokines (*SI Appendix*, Fig. S4 and Tables S1 and *S2*). Cytokines and IL-1 are potent stimulators of the immune-regulatory NF-κB signaling cascade, illustrated by NF-κB–associated gene-regulatory network nodes [e.g., genes coexpressed

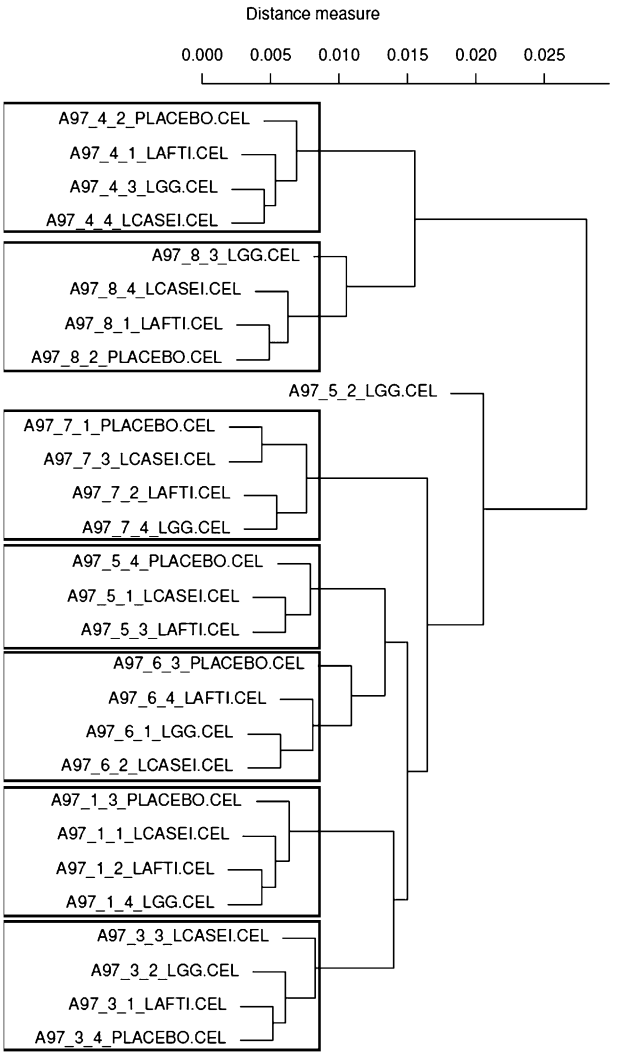

Fig. 1. Dendrogram visualizing similarity and distances between the microarray data. The second number of the array identification indicates each individual volunteer. Note that the clusters, representing the array datasets with higher similarity, are basically consisting of data from individual volunteers, not interventions. Clusters representing individuals are boxed. From the dendrogram, it is apparent that the differences between individuals, represented by the node-to-node distances, are larger than the differences between interventions.

through activity of Toll-like receptor 3 (TLR3) and one of its targets, TNF-related apoptosis inducing ligand TRAIL] (*SI Appendix*, Fig. S3*A*). The genes encoding cytokines and the intercellular adhesion molecule (ICAM-1) were likely induced by TLR3–NF-κB and TLR3–IFN regulatory factor (IRF) signaling pathways (24). One node included the NF-κB subunits NFKB2 and v-rel reticuloendotheliosis viral oncogene homolog B (RELB), which together may drive the transcription of genes involved in lymphogenesis and B cell maturation (25). These genes were up-regulated together with the NF-κB regulatory inhibitor IκB. Another distinct node included the angiogenesis- and water homeostasis-promoting hormones angiogenin (ANG) and oxytocin (OXT), blood pressure-regulating apelin (APLN), immunoregulatory proopiomelanocortin (POMC), and inflammatory cytokines-inhibiting urocortin (UCN). POMC-derived peptides and UCN are involved in modulation of immune tolerance (26). Induction of these hormonal signaling pathways and nodes containing the growth factors bone morphogenetic protein (BMP2) and insulin-like growth factor 1 (IGF1) indicated that multiple tran-

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scriptional networks involved in mucosal development and function were regulated.

The IL-23 signaling pathway mediating cell differentiation and inflammation (27) seemed to be regulated through up-regulation of the genes encoding IL-12 receptor β 1 (IL12RB1), Janus kinase 2 (JAK2) and one of its targets, signal transducer and activator of transcription (STAT)3, and the genes encoding suppressor of cytokine signaling (SOCS) 1 and 3 (*SI Appendix*, Fig. S4). The α (p19) subunit of IL-23 was down-regulated; the p40 subunit did not show differential expression.

The collective analyses indicate that consumption of *L. acidophilus* by healthy adults seemed to lead to altered mucosal gene-expression networks that stimulate and regulate immune responses, hormonal regulation of tissue growth and development, and water and ion homeostasis.

Mucosal Responses to *L. casei* Involve Proliferation, Th1–Th2 Balance, and Hormonal Regulation of Blood Pressure. Consumption of *L. casei* led to differential expression of genes regulating cellular homeostasis and metabolism together with genes involved in mitosis, growth, and proliferation. Up-regulated growth- and development-promoting transcriptional regulators such as jun oncogene (JUN) and the cell-cycle regulator NB WEE1 homolog take a central position in regulatory networks (*SI Appendix*, Fig. S3*B*). One regulatory node included the hormones POMC and OXT and the YY peptide (PYY) that are involved in immune tolerance, digestion, and cell proliferation (26). A third node included up-regulated genes involved in immune-response regulation such as TLR3, TLR9, IFN regulatory factors, and IFN-induced or -regulated genes. Other nodes contained genes involved in hormonal secretion, blood-vessel development, mitosis, and immune tolerance, such as endothelin 1 (EDN1), insulin receptor substrate 2 (IRS2), and the cytokine-inhibitory peptide adrenomedullin (ADM) (26). The up-regulation of genes encoding interleukins that regulate T, B, and dendritic cells and genes encoding lymphocyte surface receptors (*SI Appendix*, Fig. S5) suggests that consumption of *L. casei* also influenced regulation of Th2-type immune responses. Down-regulation of the TNFSF13B gene encoding TNF-related B-cell activating factor (BAFF) suggests that survival of mature B cells was not promoted (28).

Consumption of *L. casei* seemed to lead to mucosal gene-expression networks that regulate cell proliferation and balance between the Th1 and Th2 parts of the immune response, metabolism, and hormonal activity involved in blood pressure.

Mucosal Responses to *L. rhamnosus* Involve Wound Healing, IFN Response, and Ion Homeostasis. After consumption of *L. rhamnosus*, the major altered transcriptional networks and pathways were involved in cellular growth, proliferation, and development, with central roles for JUN, JAK2, STAT4, and IGF1 (*SI Appendix*, Fig. S3*C*). One regulatory node contained up-regulated genes controlled by IFN and genes that are coactivated and coregulated by the IFN-inducible eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2) (or protein kinase R, PKR) kinase. A second regulatory node, activated by JUN-JAK-STAT4, included genes involved in angiogenesis, proliferation, and wound repair [such as heparin-binding EGF-like growth factor (HBEGF), the zinc finger protein 135, zinc finger protein 135 (ZNF135), and the achaete-scute complex homolog-like 2 (ASCL2)] and genes encoding proteins involved in (possibly ubiquitin-directed) proteolytic activity [granzyme M (GZMM) and cathepsin G (CTSG)]. Reconstruction of the up-regulated genes resulted in transcriptional networks with important roles for (calcium-activated) kinases, G protein-coupled receptors (GPCRs), and Rho/Rab signaling proteins and nuclear (transcription) factors (*SI Appendix*, Fig. S6). In conclusion, consumption of *L. rhamnosus* seemed to lead to differential expression of genes participating in signaling networks involved in wound repair and healing, angiogenesis, IFN response, calcium signaling, and ion homeostasis.

Differentially Expressed Genes Include Genes with Highly Variable Between-Person Expression and Genes That Hardly Show Variable Expression. The pathways and gene-regulatory network reconstructions result in single images, suggesting that specific pathways and processes induced by one bacterial intervention do occur to the same extent in the mucosa of all volunteers. We reasoned that basal gene-regulatory networks should theoretically contain genes with low variation in expression when encoding regulatory-node proteins, such as transcription factors, whereas genes that are more to the tips of regulatory networks might encode bioactive factors of which the expression is driven by downstream, regulatory transcription factors, such as NF-κB or STAT family regulators. Genes that are regulated by other upstream genes can be expected to show more variable expression, because their regulation may be more often determined by multiple interacting downstream pathways. To evaluate this, we calculated the coefficient of variation (CoVar) (*SI Appendix*, *SI Materials and Methods*) as a measure of variability of gene expression between individual volunteers for all bacterial interventions (*SI Appendix*, Table S3). We then compared variability of gene expression for regulatory nodes and tip nodes that we found in protein–protein interaction networks reconstructed for cellular functions relevant for each specific bacterial intervention. Relevant biological context for each intervention was determined by gene ontology (GO) overrepresentation, gene-set enrichment analysis, and pathway analysis using Ingenuity Pathway Analysis (IPA) software. Fig. 2 shows a protein–protein interaction network generated using IPA for genes participating in the immune response and infectious and inflammatory disease. The CoVars for genes encoding central regulators (e.g., the transcription factors STAT3 and CCAAT/enhancer binding protein [C/EBP], delta [CEBPD]) were substantially lower (4–20 times lower) (Fig. 3) than genes with less central roles that were located towards the external boundary of the network area, genes encoding bioactive factors, or genes encoding proteins that interact with other proteins in multiple downstream pathways (e.g., most of the CCL chemokines, but also NFKBIA, the gene encoding the NF-κB inhibitor IκB) (Fig. 3). Similar findings were obtained for representative, specific networks for the mucosal responses after consumption of *L. rhamnosus* and *L. casei* (*SI Appendix*, *SI Results*). Based on these outcomes, we considered the reconstructed pathways and processes representative for a more general response. To further evaluate the biological context of the transcriptomes, we performed a cross-database analysis using the Connectivity-map pipeline (Broad Institute; see below) and compared our transcriptome data with existing data that describe transcriptional responses of cells to bioactive molecules that play roles in disease development and immunity.

Biologically Relevant Connections Between in Vivo Duodenal Responses to Probiotics, Bioactive Molecules, Human Conditions, and Clinical Trials. Connectivity-map (ConnMap) analysis enables the search for biologically relevant connections between gene-expression profiles and three different but strongly interconnected types of information: (*i*) high-throughput expression profiles of human cell lines after treatment with (*ii*) biologically active molecules that are known to (*iii*) influence certain diseases or conditions (29, 30). In ConnMap analysis, expression data serve as signatures or groups of gene identities that describe a specific condition or status. Statistical support for the strongest correlations (enrichment scores, *P* values, and specificity scores) is provided in *SI Appendix*, Table S5. The in vivo transcriptomes obtained after consumption of *L. acidophilus* shared the largest similarity to transcriptomes that were obtained by exposing cell lines to compounds used to treat hypertension, convulsions, and inflammation (Table 1). The negative association (*SI Appendix*, Table S5) with fludrocortisone, a compound that is used to increase blood pressure and regulate specific ion and water balance, suggests that consumption of *L. acidophilus* may support modulation of Na^+/K^+ ion and water balance. Consumption of

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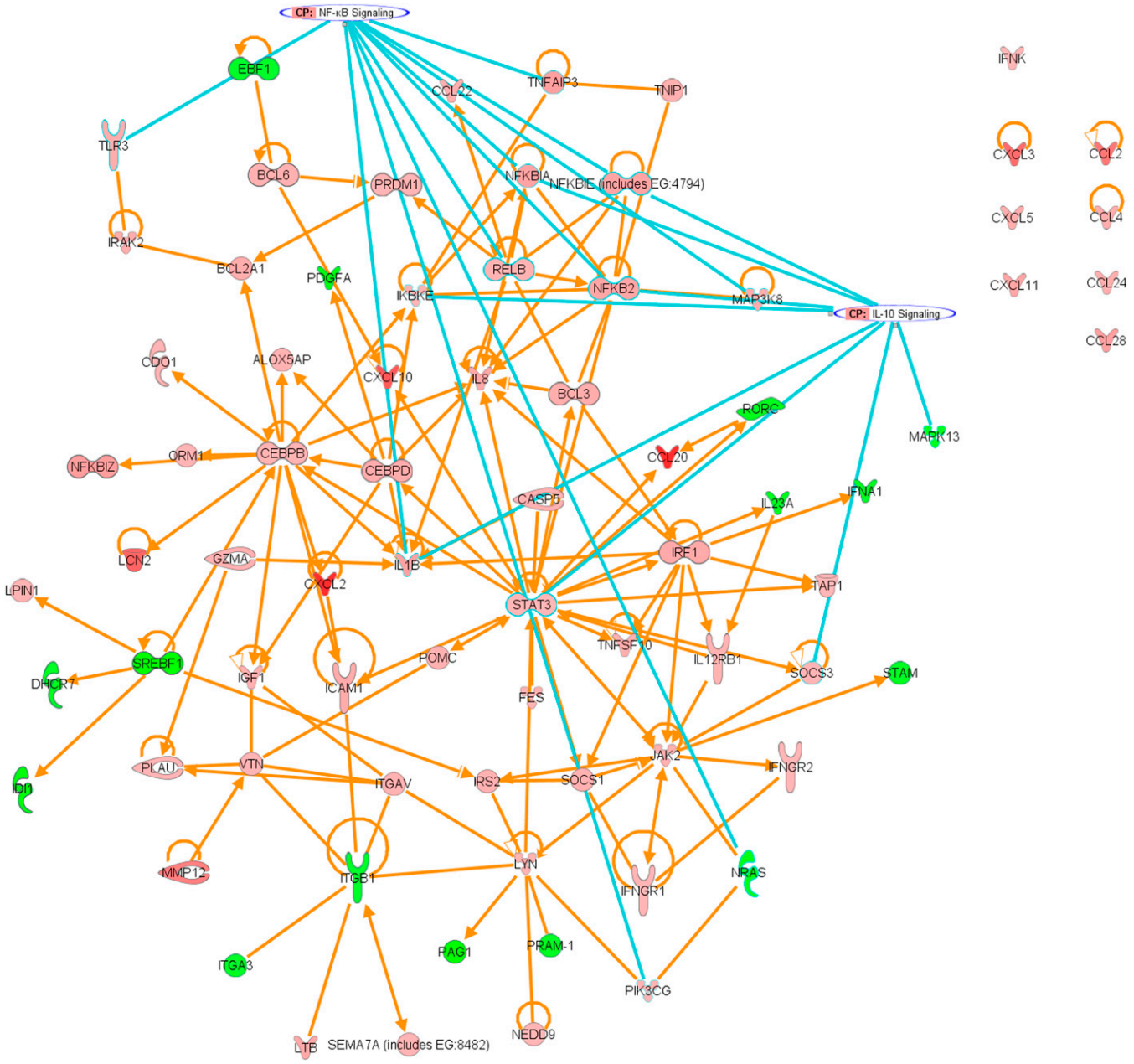

Fig. 2. Ingenuity protein–protein interaction network reflecting immune response-related transcriptome changes after consumption of *L. acidophilus*. Nodes in the interaction network are encoded by differentially expressed genes with the following functional annotations: immune response, infectious disease, and inflammatory disease. Interactions between the nodes represent protein–protein interactions (binding and phosphorylation) as well as regulation of gene transcription by transcription factors. Chemokines without direct interactions are depicted as well (upper right corner). Transcriptional information was projected onto the interaction map such that up-regulated genes are depicted in shades of red and down-regulated genes are in shades of green. From this interaction map, it can be seen that the up-regulated transcription factors STAT3, CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), CEBPD, RELB, and NFKB2 connect, by multiple outward pointing arrows, to multiple nodes. These nodes represent downstream genes that are known to be regulated by these transcription factors. Genes that participate in the immuno-regulatory pathways, NF-κB and IL-10 signaling, are indicated by the blue lines.

L. casei shared similarity to expression profiles that are typical for compounds that are used to treat muscle hypertension, water retention, and inflammation (Table 1). We found a positive correlation (enrichment score = 0.72, $P = 0.0012$) (*SI Appendix*, Table S5) with a corticosteroid-like compound. The correlations suggest that, in our in vivo study, consumption of *L. casei* may support stimulation of intestinal-muscle movement, regulation of water homeostasis to water retention, and avoidance of excessive immune stimulation. Consumption of *L. rhamnosus* was comparable with treatments with compounds that have activity as Na^+/K^+ ATPase inhibitors, compounds that are effective against amoebal infection and that amplify bowel movements, and a compound that controls apoptosis. The positive and negative associations suggest that consumption of *L. rhamnosus* may support regulation of intestinal-muscle movements through regulation of Na^+/K^+ homeostasis and that it may promote innate immune responses against intestinal infections (Table 1).

The information provided by the ConnMap analyses and databases provides possible explanations for transcriptional regulation of some of the findings obtained in clinical and medical intervention studies with human, mouse, rat, and cell-line models (*SI Appendix*, Table S4). All three bacteria stimulate expression of

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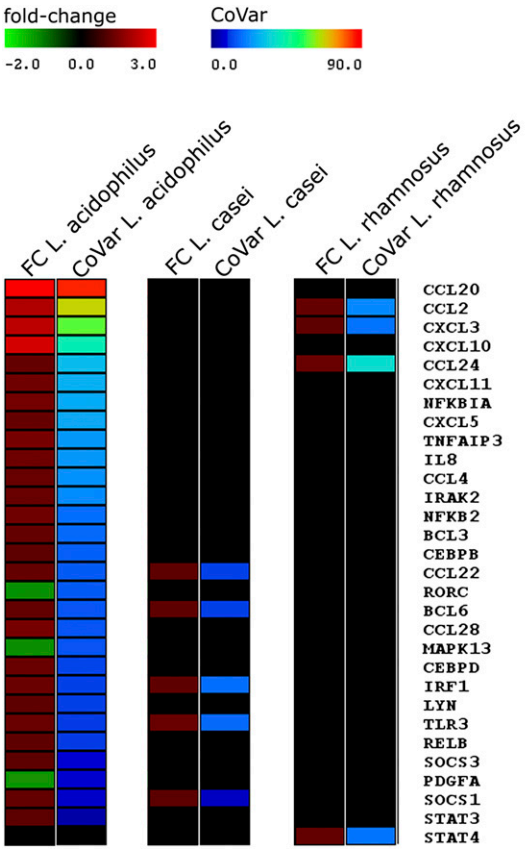

	L. acidophilus		*L. casei*		*L. rhamnosus*	
GeneSymbol	FC	CoVar	FC	CoVar	FC	CoVar
CCL20	10.86	85.23	-	-	-	-
CCL2	2.1	67.1	-	-	1.24	22.02
CXCL3	2.25	58.8	-	-	1.17	18.5
CXCL10	2.54	42.64	-	-	-	-
CCL24	1.24	30.88	-	-	1.28	37.7
CXCL11	1.37	28.54	-	-	-	-
NFKBIA	1.4	27.42	-	-	-	-
CXCL5	1.24	27.11	-	-	-	-
TNFAIP3	1.46	24.18	-	-	-	-
IL8	1.34	24.05	-	-	-	-
CCL4	1.25	23.14	-	-	-	-
IRAK2	1.25	22.36	-	-	-	-
NFKB2	1.37	17.89	-	-	-	-
BCL3	1.24	17.51	-	-	-	-
CEBPB	1.15	16.17	-	-	-	-
CCL22	1.24	15.45	1.13	11.87	-	-
RORC	-1.14	14.89	-	-	-	-
BCL6	1.2	14.82	1.14	11.57	-	-
CCL28	1.47	14.79	-	-	-	-
MAPK13	-1.13	14.12	-	-	-	-
CEBPD	1.28	12.14	-	-	-	-
IRF1	1.31	11.96	1.17	17.87	-	-
LYN	1.16	11.34	-	-	-	-
TLR3	1.27	11.08	1.26	17.46	-	-
RELB	1.18	10.95	-	-	-	-
SOCS3	1.14	8.26	-	-	-	-
PDGFA	-1.18	7.87	-	-	-	-
SOCS1	1.19	7.35	1.12	6.069	-	-
STAT3	1.11	3.35	-	-	-	-
STAT4	-	-	-	-	1.19	19

Fig. 3. Heat map visualization of transcriptional change (fold change) and coefficients of variation (CoVars) for those genes that encode the proteins that are represented in the interaction network depicted in Fig. 2. The values listed in the table correspond with the heat-map colors. An expression value represented in black indicates that the respective gene was not differentially expressed. From this, it can be seen that genes with functional annotations relating to the immune response are mainly regulated on consumption of *L. acidophilus*, not on consumption of the other two lactobacilli. Note that genes encoding proteins that occupy more central regulatory functions in the network of Fig. 2 tend to have lower CoVars compared with genes that encode proteins with more acute functions such as chemokines. This trend is also apparent in the responses to the other two lactobacilli (*SI Appendix*, *SI Results*, Figs. S8 and S9, and Tables S11 and S12).

genes involved in IFN-driven immune responses; *L. casei* also stimulates expression of immune-cell receptors and cytokines that modulate the balance between Th1 and Th2 immune responses. An increased expression of genes involved in maintaining ion homeostasis, blood pressure, and bowel movements has also been reported in clinical literature (*SI Appendix*, Table S4). Our data analyses describing the transcriptional duodenal responses to consumption of *L. rhamnosus* GG have identified, among others, pathways that can be driven by ERK3 and PKC-η (*SI Appendix*, Fig. S6). These genes may coregulate and drive Akt-dependent protection from apoptosis (31, 32), together with poly (ADP-ribose) polymerase family, member 14 (PARP14), B-cell CLL/lymphoma (BCL) 9, and JUN, which play more general roles in cell proliferation and survival (*SI Appendix*, Figs. S3*C* and S6). These genes and the cellular pathways that they are involved in (*SI Appendix*, Fig. S6) may exemplify how *L. rhamnosus* GG and its secreted proteins coregulate epithelial homeostasis and Akt-dependent protection from apoptosis, processes that have been commonly observed in clinical trials and in vitro experiments (*SI Appendix*, Table S4) in the human duodenum.

Discussion

In a previous study (21), we showed that transcriptomes representing the in vivo response to three growth stages of the species *L. plantarum* showed stage-specific promotion of NF-κB–driven gene regulatory networks and pathways. To help design future studies into probiotic mechanisms and human therapeutic trials and extend our basal knowledge on human in vivo responses to common lactobacilli, we obtained in vivo duodenal mucosal transcriptional responses of healthy adults to three widely used probiotic strains of different *Lactobacillus* species and a placebo control according to a randomized double-blind cross-over study design. The experimental conditions were chosen such that intestinal homeostasis was not lost. The amounts of bacteria consumed were about 10^{10}, dosages recommended to reach clinical usefulness (33). We measured acute responses to lactic acid bacteria, namely after 6 h of consumption, in the proximal part of the duodenum. It can be expected that the measured responses are less suitable to provide clues to possible probiotic effects in the more distal ileum or colon. At present, it is unknown how the acute responses that we did measure relate to prolonged consumption of probiotics. The modest changes in gene expression (at most, moderate fold changes up to 10 for a few genes encoding cytokines; usually lower fold changes, under or near 2) suggest that our interventions did not lead to loss of immune and metabolic homeostasis. We expect that up-regulated transcription of genes encoding factors involved in immunity will return to baseline levels if probiotics are consumed one time a day. It is, therefore, possible that the mucosal responses that were measured in this study may be triggered frequently in individuals, potentially on a daily basis. Standard probiotic therapy often involves daily consumption of at least a single portion of probiotics for multiple weeks. When designing clinical trials based on the findings in this manuscript, these issues should be taken into account whenever possible and ide-

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Table 1. Connectivity-map analysis results for the interventions of healthy adults with *L. acidophilus* Lafti L10, *L. casei* CRL-431, and *L. rhamnosus* GG

Species	Compound (medicine)	Corr*	Biochemical interactions	Therapeutic usage
L. acidophilus	Phenoxy-benzamine (Dibenzyline)	+	Antagonist of α-adrenergic receptor activity	Antihypertension (e.g., of blood vessels)
	8-azaguanine	+	Guanine antagonist	Treatment of acute leukemia
	Fludrocortisone (Florinef)	+	Synthetic corticosteroid	Replacement for aldosterone hormone in adrenal insufficiency
	Luteolin (Lutimax)	+	Flavonoid	Antioxidant, free-radical scavenger, preventer of inflammation, and immune-system modulator
	Tracazolate	+	Anxiolytic drug, modulation of GABA receptors	Anxiolytic and anticonvulsant effects
L. casei	Adiphenine (Trasentine)	–	Cholinergic blocking agent	(Smooth) muscle relaxant; can cause constipation
	Nadolol (Corgard)	–	Antagonist of β-adrenergic receptor activity	Inhibition of water retention and vasoconstriction, may increase levels of plasma triglycerides and decrease HDL cholesterol
	Viomycin (Viocin)	–	Antibiotic; translation inhibitor	Treatment of tuberculosis
	Etiocholanolone	–	Ketosteroid, metabolite of testosterone	Causes fever, immunostimulation, and leukocytosis
	Medrysone (Medrisone Opthalmic, HMS)	+	Corticosteroid	Treatment of (eye) inflammation caused by infections or injury
L. rhamnosus	Proscillaridin (Talusin)	–	Glycoside steroid, endogenous digitoxin-like	Increase heart contractions; Na^+/K^+ ATPase inhibitor
	Cephaeline (related to emetine)	+	Alkaloid	Promotes bowel movement, emetic, amoebicide
	Helveticoside	–	Glycoside	Digitalis-like; Na^+/K^+ ATPase inhibitor
	Emetine	+	Alkaloid; protein synthesis inhibitor in eukaryotic cells	Emetic, amoebicide; treatment of herpes zoster, protection against T-2 mycotoxin
	H-7	–	Protein kinase C inhibitor	Induction of apoptosis in human neuroblastoma cells through a p53-dependent pathway

*Correlation, indicated with a + or – sign, indicates if the corresponding compound induced (+) or repressed (–) the expression of the probe sets that feature as gene signatures in the ConnMap database. Statistics supporting significance and specificity of similarity can be found in *SI Appendix*, Table S5.

ally, involve measurements at multiple time points and different locations throughout the intestine.

In this study, we found that transcriptomes clustered per person, not per intervention, showing that person-to-person variation in gene expression was the largest determinant of differences between transcriptomes. Notwithstanding, consumption of different probiotic lactobacilli led to markedly different expression profiles in vivo in human mucosa, corroborating the notion that specific probiotic strains, potentially even the growth stage of bacteria in a preparation (21), induce specific responses in humans. Note that it is possible that the findings of this study do not apply to all probiotic strains of a given species. We reconstructed the mucosal expression profiles into comprehensive networks, annotated these with biological function, and transformed the gene networks into interconnected signaling pathways. Strikingly, the in vivo expression profiles bear significant similarity to expression profiles from high-throughput pharmaceutical experiments aimed at profiling responses of common cell lines treated with small molecules with known pharmaceutical impact and bioactivity, including several drugs. Genes that play central roles in regulatory networks show little variation between individuals, and their correlations may explain part of the probiotic effects observed in clinical trials. Note that our measured in vivo responses may be specific to the proximal duodenum and probably lead to local effects in intestinal mucosa, whereas most drugs act systemically in the bloodstream. The data and interpretations from this study can help to rationally design clinical trials involving human volunteers to measure effects resulting from probiotic treatments.

Consumption of *L. acidophilus* Lafti L10 resulted in modulation of transcriptional regulation of the mucosal IBD-associated IL-23 signaling pathway. In the healthy volunteers, the p40 subunit of IL-23 did not show differential expression, whereas expression of the p19 subunit was down-regulated. Expression of p40 and p19 was up-regulated in lamina propria of persons suffering from Crohn's disease (27), whereas a decrease in p19 ameliorated bacterial-induced inflammation in a mouse colitis model (34). The observed regulation of IL-23 signaling is, therefore, more consistent with a role in immune tolerance. Several Th1-specific IFN-induced chemokines such as CXCL10 and CXCL11 and IFN-responsive genes were up-regulated, indicating that consumption of *L. acidophilus* Lafti L10 may promote Th1 immune responses. In a mouse model, oral ingestion of *L. acidophilus* Lafti L10 led to a stimulation of innate immune responses, mainly through an increased IFN production (35). It may be of interest to test the effect of *L. acidophilus* in disease models that are characterized by a lack of Th1 response and associated loss of immune tolerance. It may also be of interest to investigate if consumption of *L. acidophilus* has a positive effect

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on relieving intestinal muscle hypertension and regulation of water and salt balance, as suggested by ConnMap analysis.

Consumption of *L. casei* CRL-431 may promote a shift in the Th1/Th2 balance to a Th2 type and/or Th17 type, the latter considering the observed up-regulation of IL-17D (syn. IL-22) and IL-21 (36). IL-15, IL-17D (IL-22), and IL-21 are also involved in development of natural killer cells (37–39), immune cells of which the more regulatory roles in mucosal immunology have only recently been recognized. We observed an increased expression of surface receptors that are typical for antibody-presenting cells. An increased expression of receptors was also observed in macrophages and dendritic cells in a mouse model after oral administration of *L. casei* CRL-431 (40). Antiinflammatory effects as inferred from ConnMap analyses have been reported for an *L. casei* strain in a human intestinal epithelial-cell infection model (41). The similarity to profiles induced by compounds that modulate water retention and salt homeostasis was exemplified by the increased expression of multiple ATPase transporters. It may be of interest to further investigate possible immune-modulatory, antiinflammatory, and water-regulatory properties of *L. casei*.

Consumption of *L. rhamnosus* GG has been associated with prevention or relief of allergic symptoms. In a randomized, placebo-controlled trial, *L. rhamnosus* GG did reduce the development of atopic eczema in neonatals and infants by one-half (15, 42), possibly by preventing excess production of Th2 effector cells (10). After 5 wk of daily oral intake of 2×10^9 *L. rhamnosus* GG by healthy adults, measurements of cytokine production by peripheral blood cells suggested that consumption of *L. rhamnosus* GG had altered the Treg vs. Th1/Th2 ratio and the Th1/Th2 balance (43). We found that consumption of *L. rhamnosus* GG induced, among others, the cytokine-encoding genes CCL24, CCL2, and CXCL3. The latter two are early-response genes (44) that are especially effective in stimulating Th1 responses. The up-regulation of several IFN-induced genes and STAT4 suggest that consumption of *L. rhamnosus* may have promoted expression of genes that stimulate Th1 effector-cell development (45, 46). In two different microarray studies, one using a mouse cell line and one profiling intestinal responses of humans suffering from esophagitis, the major modulated response pathways to *L. rhamnosus* GG participated in regulation of the immune response, apoptosis, and cell growth and differentiation (47, 48) (*SI Appendix*, *SI Results*), suggesting that different hosts exhibit at least a few similar responses to this bacterial strain.

Overall, there seems to be a remarkable correspondence between the human mucosal in vivo transcriptional networks altered after consumption of probiotic bacteria, high-throughput experiments profiling responses to bioactive molecules including commercial medicine, and the scientific literature (*SI Appendix*, Table S4). Although this study could only include a modest amount of volunteers, we consider that the response pathways induced by the specific bacterial interventions may be induced more generally. We infer this from the observation that bacterial treatment-specific response pathways were identified across all volunteers, despite the large variation between transcriptomes obtained from the individual volunteers. Moreover, regulatory genes with central roles in networks showed markedly less variable expression between persons than genes that occurred less central in networks and that could be modulated directly and indirectly by multiple networks. We found hundreds of differentially expressed genes that participate in (the regulation of) basal mucosal pathways, some with clinical relevance. This shows that investigating the effect of specific bacterial strains in cross-over trials using human volunteers may yield clinically relevant results. The more central, regulatory genes that were differentially transcribed with low variation in expression could lead to the development of biomarkers for healthy duodenal function. The results from this study may also contribute to the identification of the bacterial molecules that are involved in coregulating human mucosal function. Such molecules do indeed exist, as evidenced by studies where secreted *L. rhamnosus* GG proteins were found to avoid TNF-induced epithelial cell damage and promote intestinal epithelial healing and homeostasis (49, 50). We consider that probiotics research might eventually deliver therapeutic interventions that correct mild deviations from normal intestinal metabolism and may contribute to maintenance of intestinal health under conditions of mild stress, such as physical exercise. Research into probiotics might use a similar approach as nutrigenomics research (51) that is based on the idea that nutrition should focus primarily on health and disease prevention and be complementary to medical therapy that is used to prevent or cure more progressed disease (52). The large person-to-person variation in response transcriptomes that we observed in this study, together with the high CoVars for those genes that encode bioactive molecules including immune cell-attracting and -activating chemokines, helps to explain why probiotic supplementation may lead to measurable effects in some persons but not in others. We anticipate that responsiveness to probiotics is not only determined by characteristics of the consumed bacterial strain but also by genetic background, resident microbiota, diet, and lifestyle. This study could, therefore, be among the first steps to investigate the interplay between microbiota, probiotic, or other nutritional supplements and human genetics tow personalized nutrition.

Materials and Methods

Preparation of Bacteria. Lactobacilli were cultured at 37 °C in Man, Rogosa and Sharpe (MRS) medium (Merck). To obtain stationary-phase cultures, bacteria were cultured overnight. Maltodextrin and glucose were added to a final concentration of 20% and 2% (wt/vol), respectively, to obtain bacterial preparations; placebo controls only contained the two sugars. Bacteria and placebo materials were prepared such that they contained similar final sugar concentrations. Detailed protocols for culturing, harvesting, freeze-drying, storing, and viable count determining of *Lactobacillus* species can be found in *SI Appendix*, *SI Materials and Methods*.

Volunteers and Interventions. This human-intervention study was approved by the University Hospital Maastricht Ethical Committee and conducted in full accordance with the principles of the Declaration of Helsinki. All subjects gave their written informed consent before their inclusion in the study. Seven healthy nonsmoking volunteers (24 ± 4 y), without a history of gastrointestinal symptoms and free of any form of medication, were investigated on four separate occasions (three bacterial interventions and one placebo control, randomly chosen) in a randomized placebo-controlled cross-over study. Apart from a fasting period starting the evening before the actual interventions, the volunteers did not change their diet. Dairy products (including cheese and yogurt products) were a regular part of the diet. Interventions were separated by a 2-wk wash-out period; this 2-wk period did allow for complete healing of the biopsy-sampling region. The total experimental period was 6 wk. Volunteers fasted overnight (without breakfast) and were administered 1 × 150 mL of maltodextrin solution at the start of the intervention, after which they were provided every 30 min with a preparation containing reconstituted freeze-dried bacteria resuspended in 100 mL maltodextrin solution just before consumption or a preparation only containing the maltodextrin solution (the placebo control) for a period of 6 h. Both the volunteers and the researchers providing the preparations did not know whether a subject received a bacterial preparation or a placebo control (double-blind study); the vials containing bacteria or placebo control were nontransparent. After this 6-h period, four to five tissue samples were obtained from the horizontal part of the duodenum by standard flexible gastroduodenoscopy at ~15 cm distal to the pylorus.

Transcriptome Analysis. Total RNA was extracted from the biopsies, labeled, and hybridized to human Genome U133 Plus 2.0 arrays (Affymetrix) using well-established methods (*SI Appendix*, *SI Materials and Methods*). Transcriptome datasets were processed using diverse statistical and functional analyses, starting with extensive quality control and ending with pathway analysis and gene-signature comparisons that are described in *SI Appendix, SI Materials and Methods*. Detailed protocols for RNA labeling as well as the primer and probe pairs to be used in QPCR amplifications are in *SI Appendix*, *SI Materials and Methods*.

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