Impact of lactobacilli on orally acquired listeriosis

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Listeria monocytogenes is a foodborne pathogen that crosses the intestinal barrier and disseminates within the host. Here, we report a unique comprehensive analysis of the impact of two *Lactobacillus* species, *Lactobacillus paracasei* CNCM I-3689 and *Lactobacillus casei* BL23, on *L. monocytogenes* and orally acquired listeriosis in a gnotobiotic humanized mouse model. We first assessed the effect of treatment with each *Lactobacillus* on *L. monocytogenes* counts in host tissues and showed that each decreases *L. monocytogenes* systemic dissemination in orally inoculated mice. A whole genome intestinal transcriptomic analysis revealed that each *Lactobacillus* changes expression of a specific subset of genes during infection, with IFN-stimulated genes (ISGs) being the most affected by both lactobacilli. We also examined microRNA (miR) expression and showed that three miRs (miR-192, miR-200b, and miR-215) are repressed during *L. monocytogenes* infection. Treatment with each *Lactobacillus* increased miR-192 expression, whereas only *L. casei* association increased miR-200b and miR-215 expression. Finally, we showed that treatment with each *Lactobacillus* significantly reshaped the *L. monocytogenes* transcriptome and up-regulated transcription of *L. monocytogenes* genes encoding enzymes allowing utilization of intestinal carbon and nitrogen sources in particular genes involved in propanediol and ethanolamine catabolism and cobalamin biosynthesis. Altogether, these data reveal that the modulation of *L. monocytogenes* infection by treatment with lactobacilli correlates with a decrease in host gene expression, in particular ISGs, miR regulation, and a dramatic reshaping of *L. monocytogenes* transcriptome.

probiotics | virulence | vitamin B12

The intestinal tract is colonized by the largest microbial community of the human body. The intestinal microbiota plays a key role in host nutrition, immune cell homeostasis, and defense against pathogens (1). Analysis of microbiota composition and its effect on the host have blossomed in the last decade owing to the development of high throughput sequencing and the use of germ-free and gnotobiotic animals (2). Comparison between healthy individuals and patients with various clinical conditions has revealed significant differences in microbiota composition (3). These results strongly suggest that intervention aimed at changing or restoring microbiota composition may have an impact on health (4). The term "probiotics" defines live microorganisms that when administered in adequate amounts confer a health benefit on the host (5). Most current probiotics are lactic acid bacteria that belong to the normal intestinal microflora and exert numerous positive effects on human health (6). However, the mechanisms underlying these effects remain poorly understood.

Listeria monocytogenes is the etiological agent of listeriosis (7). To disseminate within the host, *L. monocytogenes* has to cross three barriers: the intestinal, blood–brain, and/or placental barriers. Crossing the intestinal barrier involves the interaction of *L. monocytogenes* internalin (InlA) with luminally accessible E-cadherin (Ecad) on enterocytes and goblet cells (8). This interaction is species specific and mouse Ecad, in contrast to human Ecad, does not interact with InlA (9). In a transgenic *Fabpi–hEcad* mouse line expressing human Ecad and in a knock-in E16P mouse line ubiquitously expressing a "humanized" Ecad, *L. monocytogenes*

efficiently crosses the small intestine, cecum, and colon epithelial barriers (10, 11). The effects of the intestinal microbiota on *L. monocytogenes* infection have previously been assessed either in cell culture (12–14) or in vivo after *L. monocytogenes* i.p. or i.v. inoculation (15–18), two models that bypass the oral inoculation route. However, only limited information is available on the impact of lactobacilli on the pathophysiology of orally acquired listeriosis and on the mechanisms by which lactobacilli may be beneficial for the host in the context of an infection via the oral route (19, 20).

We took advantage of the knock-in E16P mouse line and of other recently developed tools such as mouse Affymetrix arrays containing miR probes and *Listeria* Affymetrix tiling arrays to perform a comprehensive analysis of the impact of two *Lactobacillus* species during orally acquired listeriosis. We analyzed the effect of *Lactobacillus paracasei* CNCM I-3689, a strain with in vitro antimicrobial and immunomodulatory properties (21) and *Lactobacillus casei* BL23, which mediates anti-inflammatory effects in a model of dextran sodium sulfate (DSS)-induced colitis in mice (22). We first established a germ-free colony of the E16P mouse line and showed that it develops systemic listeriosis upon oral challenge. We then showed that *Lactobacillus* administration decreases *L. monocytogenes* dissemination and down-regulates expression of immune genes normally induced upon *L. monocytogenes* infection, in particular IFN-stimulated genes (ISGs). We identified three miRs repressed upon *L. monocytogenes* infection in vivo and showed that their expression changes after treatment with the *Lactobacillus*. We also provide evidence that *L. monocytogenes* blocks gut IL-10 production induced by lactobacilli and enhances gut IL-22 production. Finally, we show that the two lactobacilli regulate *L. monocytogenes* genes and small RNA (sRNA) expression in particular genes involved in carbon and nitrogen utilization.

Results

Treatment with *Lactobacillus* Decreases *L. monocytogenes* Host Invasion. We derived the E16P mouse line (10) as germ-free. We first established that the two lactobacilli *L. paracasei* CNCM I-3689 and *L. casei* BL23 (hereafter called *L. paracasei* and *L. casei*) persisted in the intestine after intragastric gavage. Six days after three consecutive gavages of 2×10^9 lactobacilli, the number of lactobacilli was identical in the intestinal ($\sim 10^7$/g for each lactobacilli) and cecal luminal contents of gnotobiotic E16P mice ($\sim 10^8$/g), demonstrating that both strains can colonize ileum and cecum (Fig. S1A). Persistence in the intestine was still observed after 3 wk (Fig. S1B).

To determine whether *Lactobacillus* administration of germ-free E16P mice has an impact on *L. monocytogenes* translocation across the intestinal barrier and dissemination within the host, we evalu-

ated *L. monocytogenes* counts in the ileal and cecal luminal contents, within the ileal and cecal tissue, and in the mesenteric lymph nodes, spleen, and liver. This showed that intragastric gavage with 2×10^9 *L. paracasei* and *L. casei* per mouse for 3 consecutive days prior to *L. monocytogenes* inoculation (5×10^9) significantly decreased *L. monocytogenes* counts in the intestinal tissue (Fig. 1), but neither in the cecum, mesenteric lymph nodes, and liver nor in the ileal and cecal luminal contents (Fig. S1C). Strikingly, *L. paracasei* also reduced *L. monocytogenes* dissemination to the spleen. We noticed that *L. paracasei* counts in the intestinal lumen, in contrast to those of *L. casei*, significantly decreased after *L. monocytogenes* infection (Fig. S1A), suggesting a competition between *L. monocytogenes* and *L. paracasei* in intestinal colonization.

To demonstrate the relevance of our findings, we investigated whether lactobacilli are also able to limit *L. monocytogenes* colonization in conventional E16P mice and determined whether the two lactobacilli affect *L. monocytogenes* counts in mice: *L. paracasei* slightly but significantly decreased *L. monocytogenes* counts in the spleen, and *L. casei* significantly decreased *L. monocytogenes* counts in the spleen and the liver of conventional E16P mice (Fig. S1D).

Altogether these results establish that *L. paracasei* and *L. casei* can limit *L. monocytogenes* invasive infection. As *L. paracasei* and *L. casei* have similar inhibitory activity against *L. monocytogenes* in vitro (Fig. S2), their distinct effects probably rely on genomic differences and/or different interactions with the intestinal microbiota and the host.

Treatment with *Lactobacillus* Down-Regulates *L. monocytogenes*-Induced Immune Genes in the Intestine, in Particular IFN-Stimulated Genes.

We performed a whole genome transcriptional analysis of intestinal tissue in response to *L. casei*, *L. paracasei*, and *L. monocytogenes*, respectively, and compared it to that of germ-free E16P mice. We next analyzed whether treatment with each *Lactobacillus* affects host gene expression upon *L. monocytogenes* infection.

We found that expression of 87 and 71 genes significantly changed after 3 d of monoassociation with *L. paracasei* and *L. casei*, respectively (Fig. S3A). Only 18 genes were affected by both lactobacilli, suggesting that although the two species belong to the same genus, they do not behave identically. We next grouped these genes into canonical pathways using Ingenuity IPA software (IPA) (Fig. S4 A and B). Regulation of xenobiotic metabolism by cytochrome P450 and tryptophan metabolism were the only signaling pathways modulated by both lactobacilli. In conclusion, these results show that although *L. paracasei* and *L. casei* are taxonomically related, they each trigger a species-specific response.

*L. monocytogenes* alone was shown to affect the transcription of almost 1,000 genes 24 h postinfection: 493 genes were down-regulated and 494 were up-regulated in infected mice (Fig. S3B). Using IPA, we could show that these genes grouped in pathways involved in immune response, intracellular signaling, nuclear receptor signaling, and xenobiotic metabolism as well as amino acid, carbohydrate, and lipid metabolism (Fig. S4C). The most significantly induced genes (more than fivefold) were involved in immune responses, whereas genes related to host metabolism were highly repressed (more than fivefold). These results are in complete agreement with our initial study comparing the host response to *L. monocytogenes*, or to the related nonpathogenic noninvasive species *L. innocua*, or to *Bacteroides thetaiotaomicron*, a gut symbiont (23).

Strikingly, treatment with each *Lactobacillus* modulated expression of gene subsets during infection (Fig. S3C), with ISGs being the most affected by both lactobacilli (Fig. 2, *Left*). Among these 23 ISGs, 10 encode GTPases: 4 of the p47 immunity-related GTPase family, 4 guanylate-binding proteins of the p65 family (Gbps), and 2 immunity-related GTPases (M family). Other ISGs highly induced by *L. monocytogenes* and less induced in *L. monocytogenes*-infected mice treated with the two lactobacilli include *Ifit1* and *Ifit3* encoding IFN-induced proteins with tetratricopeptide repeats, *Ifi205* IFN-activated gene, *Oas2* 2′–5′ oligoadenylate synthetase 2 and *Mx2* encoding an IFN-induced GTP-binding protein. The differential expression of *Ifit1* and *Oas2* genes in the different conditions was confirmed by quantitative (q)RT-PCR (Fig. S5A). Some genes were modulated by only one of the two lactobacilli (Fig. S3 D and E): for instance, *L. paracasei* treatment affected expression of *B3galt5* (UDP-Gal:β-GlcNAc β-1,3-galactosyltransferase), *Car3* (carbonic anhydrase), *Gstm3* (GST), *Lrat* (lecithin-retinol acyltransferase), and *Nos2* (generation of reactive nitrogen species), whereas *L. casei* treatment affected expression of *Chd7* (chromodomain helicase), *Cubn* (cubulin), and *Lphn2* (latrophilin) (Fig. 2, *Right*). In conclusion, association with *Lactobacillus*, which alone only affects a small number of genes, modulates, in *L. monocytogenes*-infected mice, principally but not exclusively the expression of ISGs, which are among the most highly induced genes after *L. monocytogenes* infection.
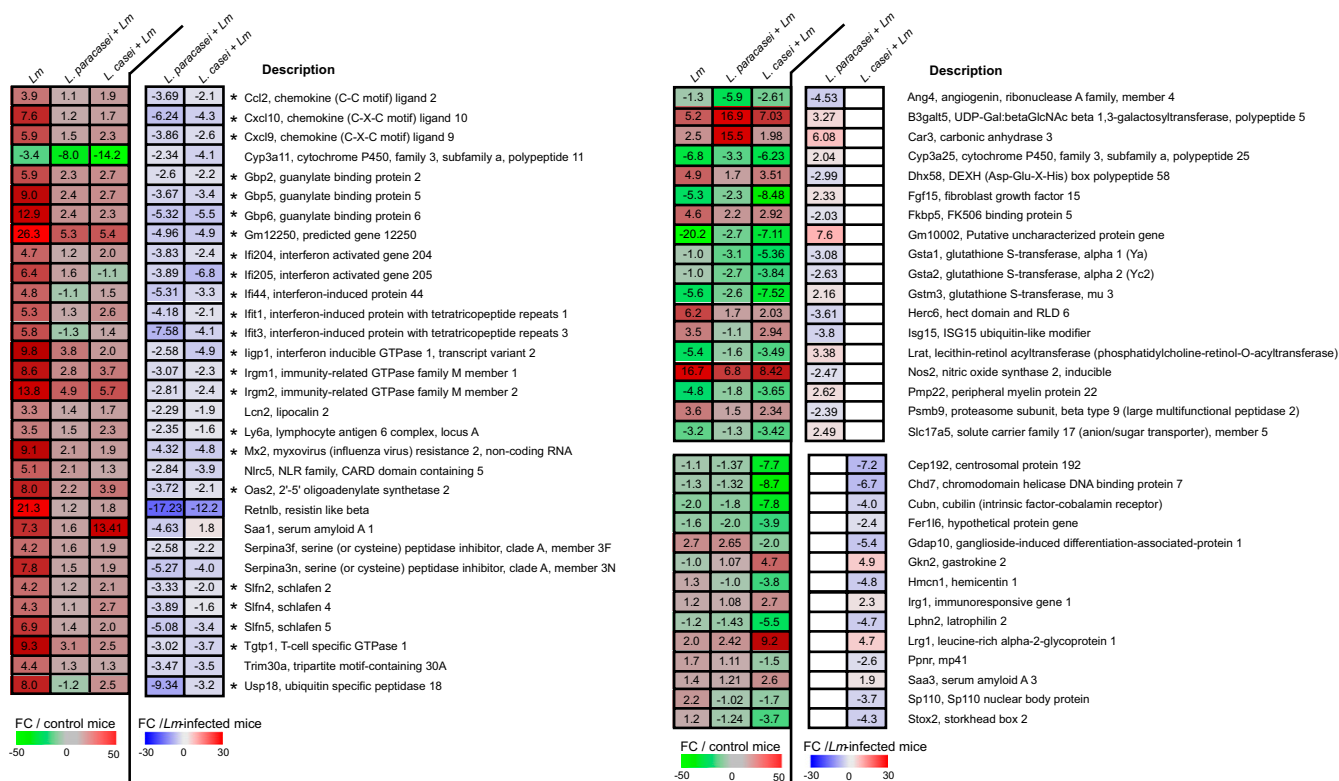
Treatment with *Lactobacillus* Modulates Expression of Several microRNAs During *L. monocytogenes* Infection.

microRNAs (miRs) are endogenous small RNAs regulating gene expression by base pairing to messenger RNAs. miRs are involved in a variety of functions such as cellular growth, apoptosis, metabolism, immunity, and cancer (24). The role of miRs in microbiota–host interactions has only recently started to be investigated (25, 26). We used the Affymetrix Mouse Gene 1.0 ST arrays to investigate miR expression. Although these arrays have been designed primarily to analyze expression of protein-coding genes, 97 miR probe sets are also present on this array. Our array data combined with qRT-PCR analysis showed that in absence of treatment with each *Lactobacillus*, expression of miR-192, miR-200b, and miR-215 was repressed during *L. monocytogenes* infection and that treatment with each *Lactobacillus* increased miR-192 expression in infected mice (Fig. 3). Only association with *L. casei* led to higher expression of miR-200b and miR-215. In addition, we detected one miR, mirR-181b, whose expression was not affected during *L. monocytogenes* infection alone but decreased during infection after treatment with each *Lactobacillus* (Fig. 3). In conclusion, miRs are regulated by *L. monocytogenes* infection in vivo and treatment with each *Lactobacillus*, suggesting that these miRs in turn regulate host gene expression.

Treatment with *Lactobacillus* Affects IFNγ Production in Spleen During Listeriosis.

Early production of IFNγ is a critical step of the immune response against *L. monocytogenes* (27). We detected IFNγ induction in the small intestine and spleen of *L. monocytogenes*-infected mice 24 h after infection (Fig. S6). A slight IFNγ production was detectable after 3 d of *Lactobacillus* monoassociation. Treatment with each *Lactobacillus* significantly reduced IFNγ production in the spleen of the *L. monocytogenes*-infected gnotobiotic mice but not in the small intestine (Fig. S6).

We also examined the production of IL-10, IL-22, and IL-2. IL-10 is an anti-inflammatory cytokine preventing excessive inflammation. IL-22 initiates an innate immune response against
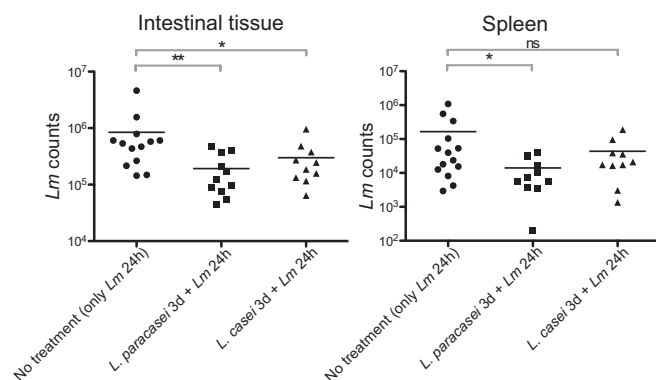
Fig. 1. Infection with *L. monocytogenes* (*Lm*) after *Lactobacillus* treatment. *Lm* counts in the intestinal tissue and in the spleen of gnotobiotic E16P mice that were monoassociated or not with the lactobacilli for 3 d and infected 3 d later with *Lm* for 24 h. Each dot represents one organ. Horizontal bars represent the mean for each condition. Statistical tests were performed using a Mann–Whitney test. Asterisks indicate a value considered statistically significant (*$P < 0.05$, **$P < 0.01$); NS, nonsignificant difference.

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Fig. 2. Transcriptomic analysis of the host response after *Lactobacillus* treatment and *L. monocytogenes* (*Lm*) infection. Effect of treatment with *Lactobacillus* on murine gene expression upon *Lm* infection. Mice were monoassociated or not monoassociated for 3 consecutive days (3 d) and infected 3 d later with *Lm* for 24 h. The heatmap presents a subset of host genes whose expression was significantly affected by the lactobacilli during *Lm* infection [false discovery rate, Benjamini and Hochberg approach (FDR-BH), $P < 0.05$]. *Left*, the three columns show values corresponding to the fold change (FC) of gene relative expression in *Lm*-infected mice (*Lm*), in mice treated with *L. paracasei* and infected by *Lm* (*L. paracasei* + *Lm*) and in mice treated with *L. casei* and infected by *Lm* (*L. casei* + *Lm*) compared with control mice (FC/control mice). *Right*, the two last columns show FC of gene relative expression in (*L. paracasei* + *Lm*) and (*L. casei* + *Lm*) mice compared with *Lm*-infected mice (FC/*Lm*-infected mice). White square in the heatmap indicates a gene whose expression was not significantly affected by the treatment with the lactobacilli. Asterisks indicate ISGs.

bacterial pathogens especially in epithelial cells such as enterocytes (28). IL-2, a pleiotropic cytokine, promotes the growth and differentiation of T cells and enhances the activity of natural killer cells (29). We showed that the level of IL-10 and IL-2 increased in the small intestine after 3 consecutive days of monoassociation with each *Lactobacillus* (Fig. S6). In contrast, IL-22 production was not detected in monoassociated mice. During *L. monocytogenes* infection without treatment with lactobacilli, the level of IL-22 increased, but not that of IL-10 and IL-2. Strikingly, *L. monocytogenes* infection down-regulated IL-10 and IL-2 production observed during monoassociation with *L. paracasei* and *L. casei*. However, *L. casei* treatment led to a higher IL-22 production in the small intestine of *L. monocytogenes*-infected mice (Fig. S6). Our data thus show that *L. monocytogenes* infection blocks IL-2 and IL-10 small intestinal production triggered after 3 d of monoassociation with the two lactobacilli, but promotes IL-22 production.

Treatment with *Lactobacillus* Reshapes the *L. monocytogenes* Protein-Coding Genes and sRNA Expression Program. We used *Listeria* Affymetrix tiling arrays and *L. monocytogenes* RNAs retrieved from the ileo-cecal content of E16P gnotobiotic mice treated or not treated by the two lactobacilli to determine whether *Lactobacillus* treatment modulates *L. monocytogenes* genes and also sRNA expression. Indeed *L. monocytogenes* possesses a large repertoire of both *cis*- and *trans*-encoded RNAs but relatively little is known about their role in *L. monocytogenes* pathogenesis (30–33). We have shown previously an extensive transcriptional reshaping upon intestinal infection when comparing gene expression levels in bacteria either present in the ileo-cecal content of germ-free mice or grown in broth medium (31). Here, we

compared *L. monocytogenes* gene expression in the ileo-cecal content of E16P germ-free mice during infection to that observed in broth medium: 520 *L. monocytogenes* protein-coding genes were significantly up-regulated (fold change, FC, from 2- to 130-fold) and 523 down-regulated (FC 2–102) (Fig. 4*A* and Fig. S7, first column); seven sRNAs were up-regulated (FC from 2- to 57-fold) and three sRNAs down-regulated (FC 2–10) (Fig. 4*B* and Fig. S8).

Treatment with each *Lactobacillus* affected the expression of the same 200 *L. monocytogenes* genes during infection. In addition, *L. paracasei* and *L. casei* also affected an additional specific set of 125 and 702 genes, respectively (Fig. S7). Remarkably, gene clusters involved in 1,2-propanediol and ethanolamine catabolism and in cobalamin (vitamin B12) biosynthesis were among the most significantly increased by the two lactobacilli (Fig. 4*A* and Fig. S5*B*).

Expression of several sRNAs, Rli15, Rli17, and that of Rli20 induced by *L. monocytogenes* infection, was higher after treatment with each *Lactobacillus* (Fig. 4*B*). We also found that expression of Rli12, Rli47, and Rli117, which was increased during infection, further increased after treatment with *L. casei*. On the contrary, we found examples like that of Rli116 whose expression was not affected by *L. monocytogenes* infection but increased after treatment with *Lactobacillus* (Fig. 4*B* and Fig. S5*C*).

Altogether our data show that treatment with each *Lactobacillus* decreases dissemination in the host, affects host gene expression, in particular ISGs, modulates miR regulation, and also triggers a significant reshaping in expression of *L. monocytogenes* protein-coding genes and sRNAs.

Discussion

We have performed a comprehensive study of the impact of two *Lactobacillus* species on orally acquired listeriosis. We show that

miR-181b

miR-192

miR-200b

miR-215

Fold change

No treatment
L. paracasei 3d
L. casei 3d
Lm 24h
L. paracasei 3d + Lm 24h
L. casei 3d + Lm 24h

☐ No treatment
▨ - Lm
■ + Lm
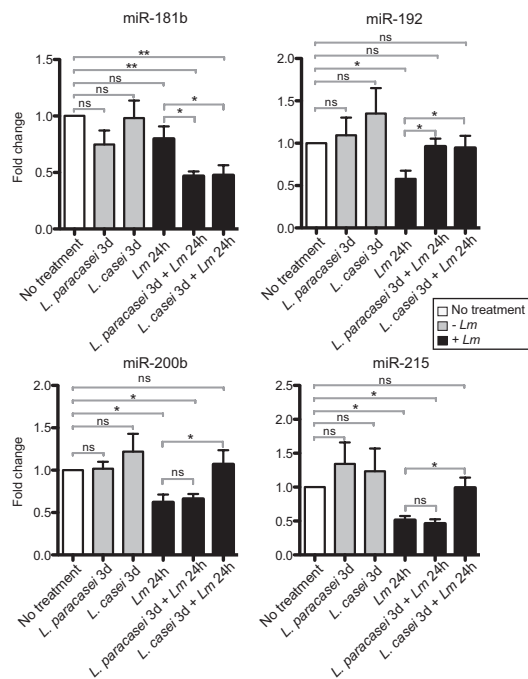
Fig. 3. Detection of miRNA during infection. Relative expression of miR181b, miR-192, miR-200b, and miR-215 in mice monoassociated for 3 consecutive days (3 d) with *L. paracasei* or *L. casei*, or in mice that were monoassociated or not with the lactobacilli for 3 d and infected 3 d later with *L. monocytogenes* (*Lm*) for 24 h. Fold change is presented after standardization to the small nuclear RNA U6 and using control mice that have been normalized to 1 as reference. Data are represented as mean with SEM. Statistical tests were performed using a two-tailed Student *t* test. Asterisks indicate a value considered statistically significant (*$P < 0.05$, **$P < 0.01$); NS, nonsignificant difference.

both *L. paracasei* and *L. casei* are able to limit *L. monocytogenes* dissemination in vivo. We then show that lactobacilli affect the expression of a subset of host genes, in particular ISGs. Moreover, lactobacilli modulate the expression of three miRs, miR-192, miR-200b, and miR-215, repressed by *L. monocytogenes* infection. We also show that *L. monocytogenes* infection decreases gut IL-2 and IL-10 production triggered by lactobacilli and stimulates IL-22 production in the small intestine. Finally, treatment with each *Lactobacillus* significantly reshapes the expression program of *L. monocytogenes* protein-coding genes and of sRNAs. To our knowledge, this is a unique genome-wide transcriptomic analysis investigating the host and pathogen responses simultaneously and the expression of protein- and small noncoding RNA-encoding genes.

Our study highlights that the two lactobacilli behave slightly differently. It is increasingly recognized that chemicals produced by bacteria and their hosts control bacterial interactions and/or communication in the environment and in the host (1). Although lactic acid production by the two lactobacilli may contribute to the decrease in *L. monocytogenes* counts in organs, their distinct effects on *L. monocytogenes* infection probably mainly result from genomic differences and/or different interactions with the intestinal microbiota and the host.
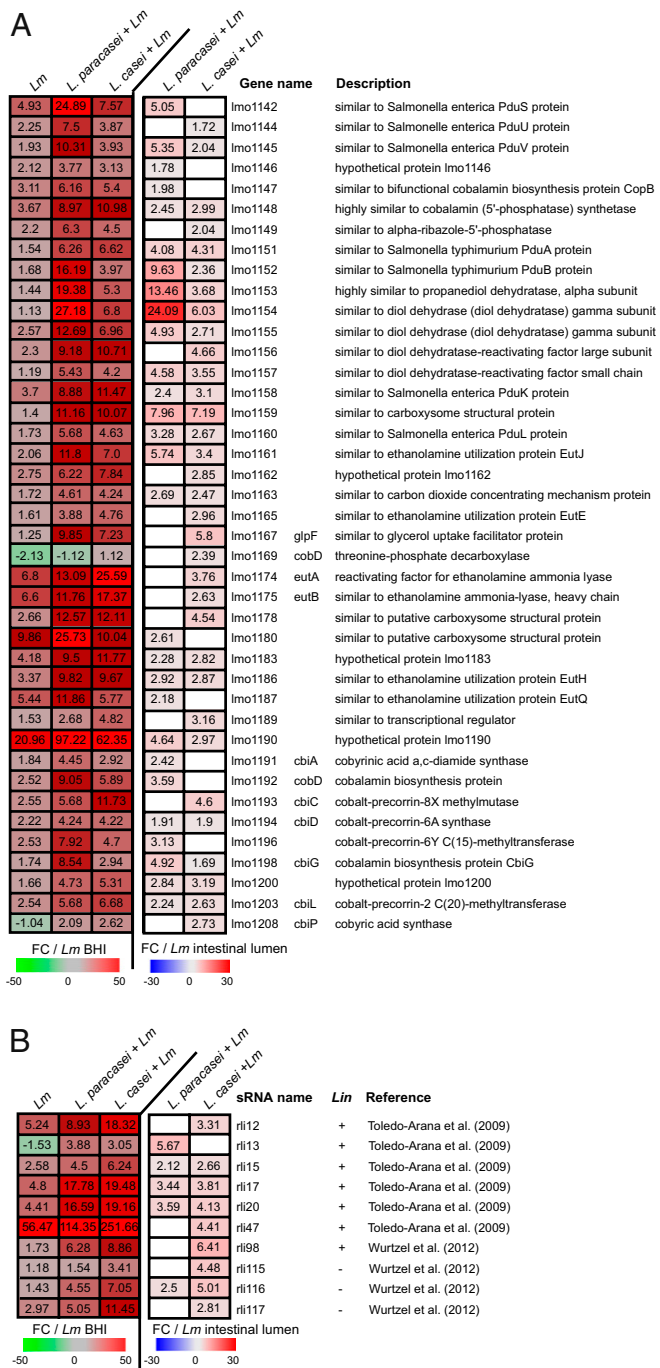
Lactobacillus treatment affects the host response to *L. monocytogenes* infection. We had previously shown that this response is characterized by an up-regulation of genes involved in immune responses and a down-regulation of genes involved in lipid, amino acid, and energy metabolism (23). In agreement with this initial study, we show here that the host response to *L. monocytogenes*, is characterized by an induction of genes involved in immune response, intracellular signaling, nuclear receptor signaling, and xenobiotic metabolism as well as amino acid, carbohydrate, and lipid metabolism. More specifically, we show that treatment with

Lactobacillus leads to a decrease of *L. monocytogenes* counts in host tissues and a down-regulated expression of ISGs at the intestinal level. Among *L. monocytogenes*-induced ISGs, genes encoding p47 GTPases have been shown to regulate innate immunity and inflammation (34) and their role in protecting *L. monocytogenes* infection has been recently highlighted (35). In contrast to type II IFN, which is beneficial to the host during listeriosis, type I IFN is detrimental (27, 36). More recently, type III IFN was shown to be produced upon *L. monocytogenes* infection (37, 38). It will thus be interesting to determine which type of IFN regulates the ISG subset affected by the *Lactobacillus*.

We also show in this study that treatment with both lactobacilli modulates expression of genes of the cytochrome P450 family (Cyp) and *L. paracasei* treatment affects expression of three glutathione S-transferases. Enterocytes have the ability to metabolize drugs or xenobiotics by numerous pathways involving phase I and II reactions (39). In phase I, enzymes such as Cyp P450 oxidases modify xenobiotics that are conjugated to polar compounds in phase II reactions catalyzed by enzymes such as GST before being processed and transported out from the cells. Gene expression in the Cyp P450 family is regulated by NF-κB and nuclear receptors such as CAR, PXR, RXR, PPAR, FXR, and LXR, which mediate gene regulation through chromatin remodeling and histone modifications (40, 41). Importantly, metabolism of xenobiotics by the Cyp P450 and LXR, PXR, and RXR signaling pathways were among the most significantly regulated pathways 24 h postinfection with *L. monocytogenes*: e.g., 19 *Cyp* genes from the P450 family and more than 30 genes encoding solute carrier proteins regulating transport of many substances. Altogether these data suggest that the so-called responses to xenobiotics mediated by the Cyp P450 and nuclear receptors could be involved in the control of host invasion by microbes. The Cyp P450 system consists of a large subfamily of enzymes that are genetically polymorphic. Because it has been proposed that this polymorphism may affect the capacity of individual patients to absorb and metabolize drugs (42), Cyp P450 polymorphism may also be involved in differences in susceptibility to infectious diseases.

The field of miRs and bacterial infections is still in its infancy. Two recent papers have reported that *L. monocytogenes* promotes changes in the level of several miRs in epithelial cells and in macrophages in vitro (43, 44). It has also been shown that miR-29 regulates immune responses to *L. monocytogenes* by targeting IFNγ mRNA in vivo (45). Here, we show that treatment with *Lactobacillus* leads to a higher expression of miR-200b, miR-215, and miR-192, which are normally repressed during *L. monocytogenes* infection and to a lower expression of miR-181b, which does not vary during infection. The miR-200 family has been reported to induce epithelial differentiation and suppress epithelial–mesenchymal transition by inhibiting translation of zinc finger E-box–binding homeobox ZEB1 and ZEB2, two transcriptional repressors of E-cadherin, in several types of cancers (46). Strikingly, using the Target Scan Mouse 6.0 software, we identified mRNAs predicted to be targeted by miR-200b, whose expression in our transcriptomic data anticorrelates with that of miR-200b: *Nlr5*, *Slf4*, *Ly6c*, *Ifi203*, *Gm12250*, and *Gbp6* encoding, respectively, the NOD-like receptor NLR5, a member of the Schlafen family involved in cell growth and T-cell development, the lymphocyte antigen 6, an IFN-induced protein, and the IrgB10 and Gbp6 GTPases, respectively. Interestingly, miR-192 which has the same "seed region" as miR-215 is also predicted to target *Nlr5*, *Gbp6*, and *Slf5* mRNAs as well as the GTPases encoded by the genes *Irgm2* and *Tgtp1*. Increase in expression of these miRs by one or two of the lactobacilli may be responsible for the lower expression of the corresponding genes after treatment with the *Lactobacillus*. It will be important to decipher among the hundreds of genes predicted to be targeted by these miRs (Fig. S9) those involved in lactobacilli-mediated effects.

We also demonstrate here that treatment with *Lactobacillus* reshapes *L. monocytogenes* transcriptome. Both protein-coding and sRNAs genes were affected. Work in progress addresses the mode of action of these sRNAs and their targets. We previously showed a role of the stress-responsive alternative σ factor, Sigma B (σB) in the expression of the invasion proteins, InlA and InlB, and

A

Lm	L. paracasei + Lm	L. casei + Lm	L. paracasei + Lm	L. casei + Lm	Gene name	Description
4.93	24.89	7.57	5.05		lmo1142	similar to Salmonella enterica PduS protein
2.25	7.5	3.87		1.72	lmo1144	similar to Salmonelle enterica PduU protein
1.93	10.31	3.93	5.35	2.04	lmo1145	similar to Salmonella enterica PduV protein
2.12	3.77	3.13	1.78		lmo1146	hypothetical protein lmo1146
3.11	6.16	5.4	1.98		lmo1147	similar to bifunctional cobalamin biosynthesis protein CopB
3.67	8.97	10.98	2.45	2.99	lmo1148	highly similar to cobalamin (5'-phosphatase) synthetase
2.2	6.3	4.5		2.04	lmo1149	similar to alpha-ribazole-5'-phosphatase
1.54	6.26	6.62	4.08	4.31	lmo1151	similar to Salmonella typhimurium PduA protein
1.68	16.19	3.97	9.63	2.36	lmo1152	similar to Salmonella typhimurium PduB protein
1.44	19.38	5.3	13.46	3.68	lmo1153	highly similar to propanediol dehydratase, alpha subunit
1.13	27.18	6.8	24.09	6.03	lmo1154	similar to diol dehydrase (diol dehydratase) gamma subunit
2.57	12.69	6.96	4.93	2.71	lmo1155	similar to diol dehydrase (diol dehydratase) gamma subunit
2.3	9.18	10.71		4.66	lmo1156	similar to diol dehydratase-reactivating factor large subunit
1.19	5.43	4.2	4.58	3.55	lmo1157	similar to diol dehydratase-reactivating factor small chain
3.7	8.88	11.47	2.4	3.1	lmo1158	similar to Salmonella enterica PduK protein
1.4	11.16	10.07	7.96	7.19	lmo1159	similar to carboxysome structural protein
1.73	5.68	4.63	3.28	2.67	lmo1160	similar to Salmonella enterica PduL protein
2.06	11.8	7.0	5.74	3.4	lmo1161	similar to ethanolamine utilization protein EutJ
2.75	6.22	7.84		2.85	lmo1162	hypothetical protein lmo1162
1.72	4.61	4.24	2.69	2.47	lmo1163	similar to carbon dioxide concentrating mechanism protein
1.61	3.88	4.76		2.96	lmo1165	similar to ethanolamine utilization protein EutE
1.25	9.85	7.23	5.8		lmo1167 glpF	similar to glycerol uptake facilitator protein
-2.13	-1.12	1.12		2.39	lmo1169 cobD	threonine-phosphate decarboxylase
6.8	13.09	25.59		3.76	lmo1174 eutA	reactivating factor for ethanolamine ammonia lyase
6.6	11.76	17.37		2.63	lmo1175 eutB	similar to ethanolamine ammonia-lyase, heavy chain
2.66	12.57	12.11	4.54		lmo1178	similar to putative carboxysome structural protein
9.86	25.73	10.04	2.61		lmo1180	similar to putative carboxysome structural protein
4.18	9.5	11.77	2.28	2.82	lmo1183	hypothetical protein lmo1183
3.37	9.82	9.67	2.92	2.87	lmo1186	similar to ethanolamine utilization protein EutH
5.44	11.88	5.77	2.18		lmo1187	similar to ethanolamine utilization protein EutQ
1.53	2.68	4.82		3.16	lmo1189	similar to transcriptional regulator
20.96	97.22	62.35	4.64	2.97	lmo1190	hypothetical protein lmo1190
1.84	4.45	2.92	2.42		lmo1191 cbiA	cobyrinic acid a,c-diamide synthase
2.52	9.05	5.89	3.59		lmo1192 cobD	cobalamin biosynthesis protein
2.55	5.68	11.73		4.6	lmo1193 cbiC	cobalt-precorrin-8X methylmutase
2.22	4.24	4.22	1.91	1.9	lmo1194 cbiD	cobalt-precorrin-6A synthase
2.53	7.92	4.7	3.13		lmo1196	cobalt-precorrin-6Y C(15)-methyltransferase
1.74	8.54	2.94	4.92	1.69	lmo1198 cbiG	cobalamin biosynthesis protein CbiG
1.66	4.73	5.31	2.84	3.19	lmo1200	hypothetical protein lmo1200
2.54	5.68	6.68	2.24	2.63	lmo1203 cbiL	cobalt-precorrin-2 C(20)-methyltransferase
-1.04	2.09	2.62		2.73	lmo1208 cbiP	cobyric acid synthase

FC / Lm BHI: -50 — 0 — 50 FC / Lm intestinal lumen: -30 — 0 — 30

B

Lm	L. paracasei + Lm	L. casei + Lm	L. paracasei + Lm	L. casei + Lm	sRNA name	Lin	Reference
5.24	8.93	18.32		3.31	rli12	+	Toledo-Arana et al. (2009)
-1.53	3.88	3.05	5.67		rli13	+	Toledo-Arana et al. (2009)
2.58	4.5	6.24	2.12	2.66	rli15	+	Toledo-Arana et al. (2009)
4.8	17.78	19.48	3.44	3.81	rli17	+	Toledo-Arana et al. (2009)
4.41	16.59	19.16	3.59	4.13	rli20	+	Toledo-Arana et al. (2009)
56.47	114.35	251.66		4.41	rli47	+	Toledo-Arana et al. (2009)
1.73	6.28	8.86		6.41	rli98	+	Wurtzel et al. (2012)
1.18	1.54	3.41		4.48	rli115	-	Wurtzel et al. (2012)
1.43	4.55	7.05	2.5	5.01	rli116	-	Wurtzel et al. (2012)
2.97	5.05	11.45		2.81	rli117	-	Wurtzel et al. (2012)

FC / Lm BHI: -50 — 0 — 50 FC / Lm intestinal lumen: -30 — 0 — 30

Fig. 4. *Listeria* tiling analysis. (*A*) Effect of treatment with *Lactobacillus* on *L. monocytogenes* (*Lm*) gene expression during infection. Mice were monoassociated or not monoassociated for 3 consecutive days (3 d) and infected 3 d later with *Lm* for 24 h. Heatmaps present a subset of *Lm* genes (*A*) and all sRNAs (*B*) whose expression was significantly affected by the lactobacilli during *Lm* infection (*t* test *P* < 0.05). *Left*, the three columns show values corresponding to the fold change (FC) of gene (*A*) and sRNA (*B*) relative expression in ileal-cecal content from *Lm*-infected mice (*Lm*), in mice treated with *L. paracasei* and infected by *Lm* (*L. paracasei* + *Lm*) and in mice treated with *L. casei* and infected by *Lm* (*L. casei* + *Lm*) compared with *Lm* growing in broth medium (FC/*Lm* BHI). *Right*, the two last columns show FC of gene relative expression in (*L. paracasei* + *Lm*) and (*L. casei* + *Lm*) mice compared with *Lm*-infected mice (FC/*Lm*-intestinal lumen). White square in the heatmap indicates a gene whose expression was not significantly affected by treatment with the lactobacilli. Absence (−) or presence (+) of sRNAs in *L. innocua* (*Lin*) is indicated.

other surface proteins (31). Here, we found that treatment with lactobacilli increases expression of such σB-regulated genes, illustrating the possible stress encountered by *L. monocytogenes* in the intestinal lumen in the presence of the two lactobacilli. More importantly, we identified several gene clusters encoding enzymes allowing *L. monocytogenes* to catabolize intestinal carbon and nitrogen sources such as 1,2-propanediol and ethanolamine and to synthesize cobalamin (vitamin B12), a cofactor for enzymes involved in ethanolamine and 1,2-propanediol degradation whose expression was increased by the presence of each *Lactobacillus*. 1,2-Propanediol is produced by the fermentation of plant sugars rhamnose and fucose (47). Ethanolamine is a compound mainly derived from phosphatidyl ethanolamine in epithelial cell membranes that only certain bacteria can use as a source of carbon and/or nitrogen (48). Many reports have shown that *L. monocytogenes*, *Clostridium perfringens*, *Enteroccocus faecalis*, and *Salmonella enterica* possess a highly similar organization of the ethanolamine gene cluster, which is absent in most other bacteria, suggesting that ethanolamine utilization contributes to virulence (49, 50). Interestingly, it has recently been shown that intestinal inflammation allows *Salmonella* to use ethanolamine and compete with microbiota (51). It is probably also the case for *L. monocytogenes*. In *L. monocytogenes*, a lack of ethanolamine ammonia lyase leads to an attenuated proliferation in epithelial cells (52) and all genes involved in ethanolamine catabolism are up-regulated in the intestine of infected gnotobiotic mice (31). However, our results suggest that in gnotobiotic mice, increasing the expression of these gene clusters does not confer a selective advantage to *L. monocytogenes* because treatment with both lactobacilli decreases *L. monocytogenes* translocation and dissemination in the host. It is possible that in the absence of lactobacilli, *L. monocytogenes* uses various carbon and energy sources including ethanolamine and 1,2-propanediol. After treatment with lactobacilli, a competition between *L. monocytogenes* and the lactobacilli is likely taking place for carbon and nitrogen availability forcing *L. monocytogenes* to use mostly ethanolamine not utilizable by lactobacilli (53), suggesting that the competition between *L. monocytogenes* and the lactobacilli for carbon and nitrogen availability is a critical step for a successful invasion.

To our knowledge, our study is a unique comprehensive approach that analyzes simultaneously in the host and the pathogen the molecular events occurring after treatment with two lactobacilli species. It paves the way for a thorough and systematic analysis of the key components mediating the protecting effect of lactobacilli.

Materials and Methods

Additional information on the strains and reagents and on the experimental protocols is provided in *SI Materials and Methods*.

Bacterial Strains. *L. monocytogenes* EGDe strain was grown in brain heart infusion (BHI) medium (Difco) at 37 °C. *L. paracasei* CNCM I-3689 and *L. casei* BL23 strains were grown in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid) at 37 °C.

Animals and Generation of a Germ-Free Mouse Line. All experiments involving mice were handled in accordance with the Pasteur Institute guidelines for animal welfare. We derived the knock-in E16P mouse line (10) as germ-free mice (Taconic).

Monoassociation and Treatment with *Lactobacillus*. For each *Lactobacillus*, mice were inoculated orally with 2×10^9 bacteria diluted in 200 μL of PBS. Mice (*n* ≥ 3 per group) were either (*i*) monoassociated with each *Lactobacillus* for 3 consecutive days (3 d) (or for 24 h when required) and killed 6 d after the first inoculation or (*ii*) monoassociated with the lactobacilli for 3 consecutive days and infected 3 d later with *L. monocytogenes* for 24 h.

Infection. Mice (*n* ≥ 3 per condition) were infected orally with 5×10^9 bacteria diluted in 200 μL of PBS supplemented with 300 μL of CaCO₃ (50 mg/mL) for 24 h. Serial dilutions of the inoculum were plated to control the number of *L. monocytogenes* inoculated in mice.

Bacterial Counts. The spleen was directly disrupted in PBS. Intestinal fragments were incubated 2 h in DMEM containing 100 µg/mL gentamicin and disrupted in PBS. Serial dilutions were plated on BHI plates and incubated for 2 d at 37 °C.

Antimicrobial Activity. The antimicrobial activity was tested as previously described (21).

Mouse Gene Chip Analysis. RNAs from the ileal tissue were extracted and purified using classical TRIzol/chloroform protocol. Labeled cDNA was synthesized from 200 ng total RNAs using NuGEN Applause WT-Amp Plus ST systems (NuGEN Technologies). Labeled samples were hybridized to Affymetrix MoGene 1.0 ST GeneChips and scanned with an Affymetrix GeneChip Scanner 3000, generating cell intensity files for each array. Gene-level expression values were derived from the CEL file probe-level hybridization intensities using the model-based robust multichip average algorithm (54). Canonical pathways have been identified using the Ingenuity IPA application using a P value calculated by Fisher's exact test, right tailed.

***Listeria* Tiling Array Analysis.** Total bacterial RNAs from the ileo-cecal content were extracted and treated as previously described (31); extracted RNAs from each mouse ($n = 3$ for each condition) were treated separately. Total RNAs (200 ng) were amplified using the MessageAmpII-Bacteria kit (Ambion) before fragmentation using the 5× fragmentation buffer. A total of 7.5 µg of amplified and fragmented RNA was used per chip. Sample preparation for each chip was then processed following the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev. 2) as previously described (31).

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