# Bacteriocin from epidemic *Listeria* strains alters the host intestinal microbiota to favor infection

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***Listeria monocytogenes* is responsible for gastroenteritis in healthy individuals and for a severe invasive disease in immunocompromised patients. Among the three identified *L. monocytogenes* evolutionary lineages, lineage I strains are overrepresented in epidemic listeriosis outbreaks, but the mechanisms underlying the higher virulence potential of strains of this lineage remain elusive. Here, we demonstrate that Listeriolysin S (LLS), a virulence factor only present in a subset of lineage I strains, is a bacteriocin highly expressed in the intestine of orally infected mice that alters the host intestinal microbiota and promotes intestinal colonization by *L. monocytogenes*, as well as deeper organ infection. To our knowledge, these results therefore identify LLS as the first bacteriocin described in *L. monocytogenes* and associate modulation of host microbiota by *L. monocytogenes* epidemic strains to increased virulence.**

*Listeria* | epidemic | infection | intestinal microbiota | bacteriocin

The gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen that causes foodborne infections in humans and animals. Upon consumption of contaminated food, *L. monocytogenes* reaches the intestinal lumen, crosses the intestinal barrier, and disseminates within the host. The clinical manifestations of listeriosis vary from a mild, self-limiting gastroenteritis to severe intestinal and systemic infections, with a fatality rate estimated to 20–30% of infected individuals (1). Host gut microbiota plays a critical role in resistance against colonization by invading pathogens within the intestine (2). The mechanisms of *L. monocytogenes* to compete with the host microbiota to survive in the intestine remain unknown.

During the last decades, the majority of *Listeria* studies in bacterial pathophysiology, cell biology, and immunology compared three pathogenic strains from lineage II: EGD, EGD-e, and 10403S (3). Interestingly, major listeriosis epidemics have been preferentially associated to *L. monocytogenes* clonal groups belonging to the evolutionary lineage I and, more specifically, to serotype 4b (4, 5), but the molecular mechanisms that contribute to the higher virulence potential of these bacterial strains have not been identified yet.

Bacteriocins are bacterially synthesized proteinaceous substances that target and inhibit the growth of closely related bacteria, allowing competition in diverse ecological niches, including the digestive tract (6, 7). Production of these antimicrobial peptides is widespread among bacterial species, and such production is made possible by biosynthetic machineries present in the genome, plasmids, or conjugative transposons (7). A conserved biosynthetic gene cluster for the production of bacteriocins displaying thiazole and oxazole heterocycles was discovered in 2008 in six microbial phyla (8). These gene clusters encode a toxin precursor and all indispensable proteins for toxin maturation in a mode similar to that associated with the bacteriocin microcin B17 (8). This gene cluster in *L .monocytogenes* was only present in a subset of lineage I strains responsible for the majority of *Listeria* epidemics (9). This *L. monocytogenes* toxin was designated Listeriolysin S (LLS) and was shown to produce a hemolytic and cytotoxic factor necessary for virulence in a murine intraperitoneal (i.p.) infection model (9).

The aim of the present work was to understand where LLS is produced in a murine oral infection model, as well as to investigate the function of this virulence factor. Interestingly, to our knowledge, we show that LLS is the first bacteriocin described in *L. monocytogenes*. This toxin that is specifically expressed in the intestine augments host colonization.

## Results

***L. monocytogenes* Lineage I Strain F2365 Is More Virulent than Reference Lineage II Strains EGD-e and 10403S in Orally Infected Mice.** To determine whether *L. monocytogenes* strains from lineage I are more virulent than *L. monocytogenes* strains belonging to lineage II, we orally infected conventional BALB/c mice with $5 \times 10^9$ *L. monocytogenes* of lineage I strain F2365 [clonal complex I *L. monocytogenes* responsible for the 1985 California outbreak (10, 11)] or of lineage II strains EGD-e and 10403S. We then evaluated bacterial burden in the intestinal content, small intestine, and spleen at 24 h and 48 h postinfection (p.i.). *L. monocytogenes* counts in the intestine and in the spleen at 24 h p.i. were significantly higher in mice infected with the F2365 strain than in mice infected with strains EGD-e

### Significance

***Listeria monocytogenes* is a bacterial pathogen responsible for listeriosis, a foodborne disease characterized by septicemia and abortion in pregnant women. The most severe listeriosis outbreaks are associated with a subset of bacterial epidemic clones, although the underlying virulence mechanisms of these clones remain elusive. Here, we demonstrate, to our knowledge for the first time, that these epidemic strains secrete a bacteriocin specifically in the gut and alter host intestinal microbiota, allowing *L. monocytogenes* colonization of the intestine and, consequently, invasion of deeper organs. Therefore, our work shows that epidemic listeriosis implicates not only interactions between *L. monocytogenes* and host cells, but also interactions between *L. monocytogenes* and the host intestinal microbiota that are critical for the establishment of infection.**

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The authors declare no conflict of interest.

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Data deposition: The read sequences from this study were deposited in the European Nucleotide Archive, www.ebi.ac.uk/ena (reference no. PRJEB13646). Datasets used for this study were deposited at: https://github.com/aghozlane/listeria_metagenomic.

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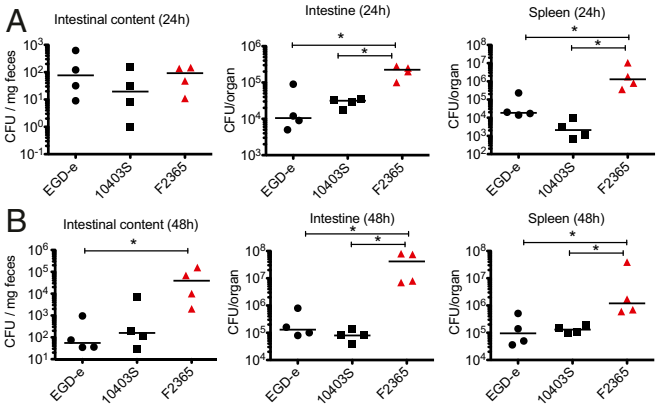


**Fig. 1.** Comparative virulence of lineage I strain F2365 and lineage II *Listeria monocytogenes* strains EGD-e and 10403S. cfu counts in the intestinal content, intestine, and spleen of BALB/c mice were measured at 24 h (*A*) and 48 h (*B*) after intragastric inoculation with $5 \times 10^9$ bacteria of the indicated strains. Each dot represents the value for one mouse. Statistically significant differences were evaluated by the Mann–Whitney test. $*P < 0.05$.

and 10403S (Fig. 1*A*). Strikingly, *L. monocytogenes* F2365 replicated and colonized the intestinal content at 48 h p.i. more efficiently than strains EGD-e and 10403S (Fig. 1*B*). *L. monocytogenes* counts in the intestine and spleen at 48h p.i. were also significantly higher in mice infected with the strain F2365 than in animals infected with the EGD-e and 10403S strains (Fig. 1*B*). These results show that the *L. monocytogenes* lineage I strain F2365 is more virulent than lineage II strains EGD-e and 10403S in a mouse oral infection model.

**LLS Contributes to Intestinal Survival and Virulence in a Murine Oral Infection Model.** Among the various virulence factors already identified in *L. monocytogenes*, LLS has been reported to be a secreted hemolytic toxin only present in a subset of isolates from the evolutionary lineage I (9). The LLS operon consists of a structural gene encoding for a peptide (*llsA*), three genes that are predicted to form a synthetase complex necessary for the maturation of the LLS (*llsB*, *llsY*, and *llsD*), an ABC transporter (*llsG* and *llsH*), a putative protease (*llsP*), and a gene of unknown function (*llsX*) (12). LLS contributes to virulence in a murine i.p. infection model (9); however, its precise toxicity mechanism in vivo is unknown. To elucidate the role of LLS during the natural route of *Listeria* infection, we infected mice orally and first compared the persistence in the intestinal content of the epidemic F2365 wild-type (WT) strain to that of a Δ*llsA* and a Δ*llsB* isogenic mutant strains. LlsB is an enzyme that putatively performs posttranslational modifications in the immature LLS peptide and whose deletion completely inactivates LLS in a blood agar test (9, 12, 13). In our murine oral infection model, the Δ*llsA* and Δ*llsB* mutant strains displayed significantly reduced bacterial loads in the intestinal content at 6 h p.i. compared with the WT strain, and these differences were also observed at 24 and 48 h p.i., indicating that LLS plays a role in the persistence of *L. monocytogenes* within the intestinal lumen (Fig. 2 *A*–*C*). The number of Δ*llsA* and Δ*llsB* bacteria was also significantly reduced in the small intestine tissue and spleen at 6, 24, and 48 h after inoculation, an indication that the lower numbers of the Δ*llsA* and Δ*llsB* strain in the intestinal content directly correlate with the reduced number of intracellular bacteria crossing the intestine and consequently reaching deeper organs such as the spleen (Fig. 2 *A*–*C*). No significant changes in the feces or diarrhea between groups were observed. We also tested the mutants Δ*llsA* and Δ*llsB* as well as their complemented strains (Δ*llsA* pPl2:*llsA* and Δ*llsB* pPl2:*llsB*), and we confirmed the results observed with the Δ*llsA* and Δ*llsB* mutants, detecting lower counts of the Δ*llsA* and Δ*llsB* strains in the intestinal content, in the small intestine and in the spleen compared with the WT strain (Fig. S1 *A*–*C*).

Two mice infected with the WT F2365 died before 48 h, whereas there were no deaths in the group of mice infected with the Δ*llsB* or Δ*llsB* pPl2:*llsB* strains (Fig. S1*C*). Complementation of Δ*llsA* and Δ*llsB* mutants with a plasmid containing *llsA* or *llsB*, respectively, partially restored virulence in the intestinal content and in the intestine (Fig. S1 *A*–*C*). However, the *llsA* and *llsB* complementation did not restore virulence in the spleen (Fig. S1 *A*–*C*). LLS expression is specifically triggered in the intestine, which could explain the lack of *llsA* and *llsB* complementation in the spleen, where the metabolic cost of carrying a pPl2 plasmid could be negative for bacterial fitness. Together, these results demonstrate that LLS plays a critical role in *L. monocytogenes* survival within the gastrointestinal tract, thereby favoring organ colonization.

**LLS Is Specifically Expressed in the Intestine of Orally Infected Mice.** Because LLS is not produced under routine laboratory growth conditions (9) and its role during in vivo infection is unknown, we investigated the organs in which the LLS gene is activated in the mouse during oral infection. For this purpose, we fused the LLS promoter to a Lux reporter plasmid and integrated it into the chromosome of the epidemic strain F2365 (F2365$^{llsA:lux}$). Upon oral infection of conventional BALB/c mice with $5 \times 10^9$ *L. monocytogenes* F2365$^{llsA:lux}$, a strong bioluminescent signal was detected in the abdomen of infected animals as early as 7 h p.i. (Fig. 3*A*). The bioluminescent signal was then continuously observed up to 96 h p.i. in the intestine specifically (Fig. 3 *A* and *B*) and was stronger after abdominal skin and peritoneum dissection (Fig. 3*B*). Interestingly, no other organ (including liver and spleen, which are the main organs targeted by *L. monocytogenes*) displayed bioluminescence on dissection, despite the presence of bacteria (cfu determined by plating organ homogenates) (Fig. 3*C*). To discard the possibility that the absence of bioluminescence in the liver and spleen could be due to a lower number of cfu in these organs compared with the intestine, we orally infected BALB/c with higher doses of F2365$^{llsA:lux}$, namely, $5 \times 10^{10}$ and $1 \times 10^{10}$ cfu per mouse. Mice orally infected with $5 \times 10^{10}$ cfu yield ~$1 \times 10^7$ cfu in the liver at 96 h p.i., and no bioluminescent signal was found in this organ in any of the mice analyzed ($n = 5$) (Fig. S2). For comparison, mice displaying a bioluminescent signal in the intestine contained between $1 \times 10^4$ and $1 \times 10^7$ cfu in the intestinal luminal content and ~$1 \times 10^4$ cfu in the

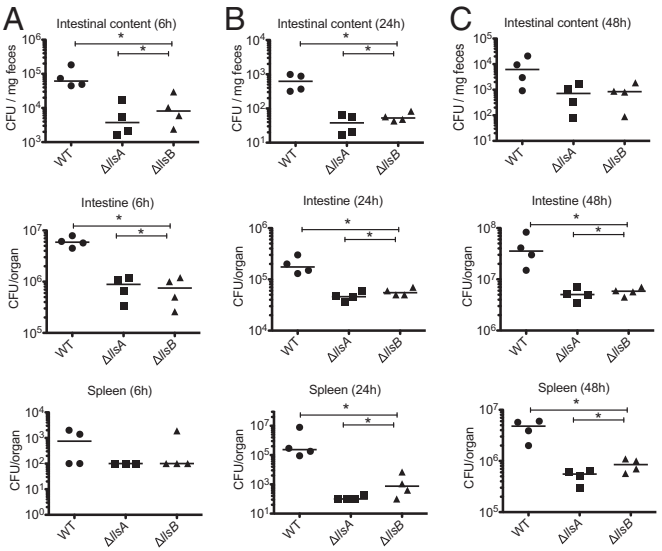


**Fig. 2.** Importance of LLS for the in vivo infection of a *L. monocytogenes* epidemic strain. BALB/c mice were intragastrically inoculated with $5 \times 10^9$ *L. monocytogenes* F2365 WT, Δ*llsA* or Δ*llsB* bacteria. Importantly, deletion of *llsB* renders LLS completely inactive in a blood agar test (13). cfu in the intestinal luminal content, the intestinal tissue (intestine), and spleen were assessed at 6 h (*A*), 24 h (*B*), and 48 h (*C*) p.i. Each dot represents the value for one mouse. Statistically significant differences were evaluated by the Mann–Whitney test. $*P < 0.05$.

intestinal tissue at 96 h p.i (Fig. 3*D* and Fig. S2). These results establish that the expression of LLS is specifically triggered in the intestine.

The LLS promoter induction could be attributed to host-derived signals, intestinal microbiota signals, or a combination of both. To elucidate the potential contribution of the intestinal microbiota to the LLS activation, we orally infected germ-free and conventional C57BL/6J mice with $5 \times 10^9$ *L. monocytogenes* F2365$^{llsA:lux}$. Bioluminescent signals at 24 h p.i. in the intestine of germ-free C57BL/6J mice were higher than in the conventional C57BL/6J mice (Figs. S3 and S4). The lower bioluminescent signal in the conventional C57BL/6J mice is probably due to the lower number of *L. monocytogenes* cfu in these mice compared with the germ-free ones (Fig. S5), which confirms the role played by the microbiota in protecting the host during orally acquired listeriosis as described (14). These results show that the host produces signals that activate the LLS promoter. We next examined different compounds or conditions found in the intestine that could be involved in transcriptional activation of the LLS promoter using *L. monocytogenes* F2365$^{llsA:lux}$. None of the compounds tested [mucin, gastric fluid (15), trypsin, pepsin, $NaHCO_3$, bile salts, detergents, succinic acid, butyric acid, propionic acid, valeric acid, octanoid acid, ethanolamine, and different antibiotics] or even 6% $O_2$ or intestinal content of mice added ex vivo could induce bioluminescence under in vitro conditions using the F2365$^{llsA:lux}$ strain. Furthermore, neither spent brain–heart infusion (BHI) with different concentrations of $H_2O_2$ or minimal medium with arabinose as the only sugar source [high glucose levels inhibit expression of *Streptococcus anginosus* SLS (16)] induced expression of the *lux* operon.

**LLS Is a Bacteriocin that Modulates the Growth of Other Bacteria.** We next investigated the contribution of LLS to the increased *L. monocytogenes* survival in the intestine of infected mice. The gastrointestinal tract is colonized by the largest microbial community of the body, and previous observations suggested that LLS sequence and operon are related to the class I bacteriocins family (9, 12). We thus hypothesized that LLS could behave as a bacteriocin. We first examined the potential inhibitory activity of *L. monocytogenes* F2365 WT, *L. monocytogenes* F2365 Δ*llsA*, and *L. monocytogenes* F2365 pHELP:*llsA* (a synthetic strain that constitutively expresses LLS) against different bacterial species or bioluminescent *L. monocytogenes* from other lineages that lack the LLS operon (Table S1). Briefly, diluted overnight cultures of *L. monocytogenes* F2365 strains and selected target strains were grown either alone or in coculture for 24 h in 6% $O_2$, and viable cfu were determined by using either bioluminescence or plating on BHI or Oxford agar plates. After 24 h of coculture, there was a significant reduction in the growth of *L. monocytogenes* 10403S$^{lux}$ [a strain that lacks the LLS operon (17)] (Fig. 4*A*), *L. monocytogenes* EGD$^{lux}$ [a strain that lacks the LLS operon (18)] (Fig. 4*B*), *Lactococcus lactis* (Fig. 4*C*), and *Staphylococcus aureus* (Fig. 4*D*) when cocultured with *L. monocytogenes* F2365 pHELP:*llsA*. No growth differences were observed among the different *L. monocytogenes* F2365 strains when cocultured with the LLS target strains (Fig. S6). However, no effect of LLS was detected on *Streptococcus thermophilus*, *Lactobacillus* spp., *Enterococcus faecalis*, *Escherichia coli*, or *Acinetobacter johnsonii*. These results show that LLS, to our knowledge, is the first reported *L. monocytogenes* bacteriocin able to modulate the growth of other bacteria.

**LLS Alters the Host Intestinal Microbiota and Increases *L. monocytogenes* Persistence.** Because LLS is a bacteriocin—specifically produced in the intestine of orally infected mice and necessary for *L. monocytogenes* survival in this organ—we reasoned that this toxin might influence the composition of the host intestinal microbiota and alter the intestinal environment, making this niche more beneficial to *L. monocytogenes*. To investigate this hypothesis, high-throughput 16S ribosomal DNA was used to examine the intestinal microbiota in mice orally infected with *L. monocytogenes* F2365 WT, *L. monocytogenes* F2365 Δ*llsA*, and its complemented strain at 24 h p.i. No significant changes in phyla abundance were observed at 24 h p.i. for any of the tested strains (Fig. S7). Microbial community compositions of the intestine were different in mice orally infected with the *L. monocytogenes* F2365 Δ*llsA* strain compared with the complemented and WT strains at 24 h p.i. ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 5*A*). A decrease in representatives of the *Allobaculum* ($P < 0.001$) and *Alloprevotella* ($P < 0.05$) genera was associated with LLS expression at 24 h p.i. (Fig. 5 *B* and *C*). *Allobaculum* genera include bacterial species that produce butyric acid, and *Alloprevotella* genera include species that produce acetic acid (19, 20). Butyrate is reported to inhibit virulence factor production in *L. monocytogenes* at the transcriptional level (21), whereas acetic acid was shown to inhibit *L. monocytogenes* growth (22). Together, these data support a model in which LLS alters the host intestinal microbiota, favoring an increase in *L. monocytogenes* persistence.

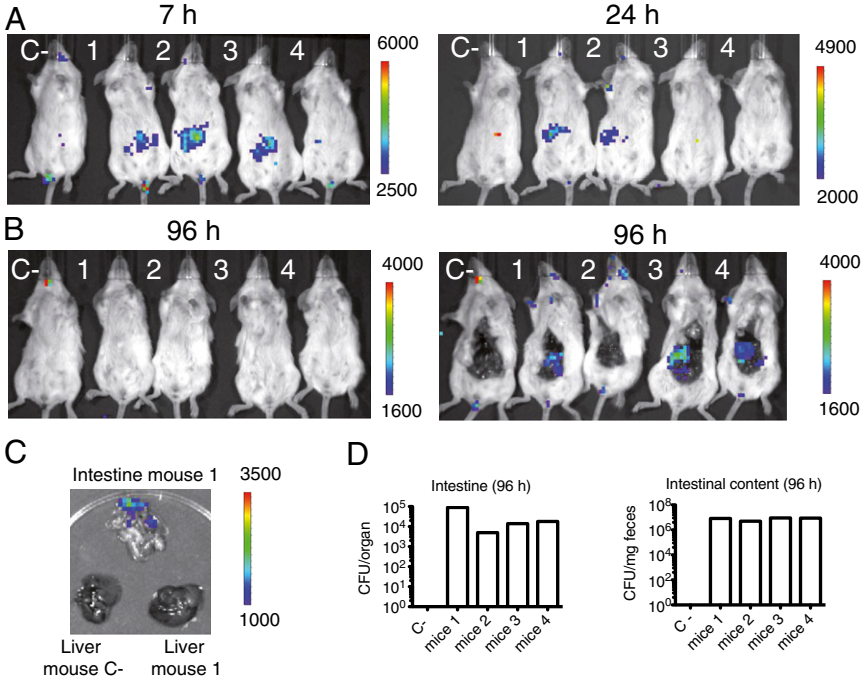


**Fig. 3.** Bioluminescence imaging of LLS promoter induction. (*A*) Induction of LLS promoter in the abdominal region after intragastric inoculation of four mice with $5 \times 10^9$ bacteria per BALB/c mouse. C, control mouse noninfected. Images were acquired at the indicated hours after infection with an IVIS Spectrum Imaging System. (*B*) Induction of LLS promoter specifically in the intestine of the same mice as in *A* but at 96 h p.i. Images were taken before (*Left*) and after (*Right*) abdominal skin and peritoneum dissection. (*C*) Ex vivo imaging of the liver and intestine removed from the mice in *B*. (*D*) Bacterial counts in the intestinal tissue (intestine) and intestinal luminal content of the same mice at 96 h p.i.

## Discussion

Since the discovery of *L. monocytogenes* in 1926 (23), its virulence factors have been shown to exert their activity by targeting host cells and tissues or by protecting the bacteria from host factors (1). The *L. monocytogenes* bile salt hydrolase was the first factor described to be involved in survival within the intestinal lumen (24). LLS was previously shown to be a hemolytic and cytotoxic factor (9) and has recently been associated with infectious potential in a molecular epidemiological and comparative genomic study of *L. monocytogenes* strains from different lineages (5).

In this study, we demonstrate not only that LLS is a *L. monocytogenes*-secreted bacteriocin, but we also show that this virulence factor is specifically produced in the intestine of infected animals and targets distinct genera of the intestinal microbiota. As a consequence, *L. monocytogenes* lacking LLS is impaired in its capacity to compete with intestinal microbiota and does not survive as efficiently as WT bacteria in the intestinal lumen, a critical step for the establishment of listeriosis. Interestingly, the strains EGD-e and EGD, which lack LLS, rarely cause human disease (5). The mechanisms by which these strains can colonize the intestine need to be assessed in future experiments.

A recent study showed that the opportunistic pathogen *Staphylococcus pseudintermedius* synthesizes BacSp222, a plasmid-encoded peptide that behaves as a bacteriocin and as a cytotoxic factor against eukaryotic cells (25). Because *S. pseudintermedius* colonizes the skin and mucosal surfaces of domestic animals, BacSp222 secretion could help to outcompete commensal bacteria inhabitants in these niches (25). This example (25) and the present work show how two different bacteria, *L. monocytogenes* and *S. pseudintermedius*, developed a common strategy to survive in the specific niches where they develop their pathogenic action: the intestine and the skin/mucosal surfaces, respectively. Our results show that the intestine is the only organ where LLS is expressed. This result does not discard the possibility that LLS could be expressed at lower levels, albeit not detectable by our bioluminescent system, in other organs such as spleen and liver, where a contribution of LLS in virulence after i.p. infection was previously described (9).

Our findings could explain why LLS-containing *L. monocytogenes* strains are those associated with the majority of epidemic outbreaks of listeriosis. The present results enhance our current understanding of epidemic listeriosis and highlight potential similarities to other enteric infectious diseases (6), paving the way for future studies to uncover the intestinal bacteria that control infection susceptibility of different groups within a population.

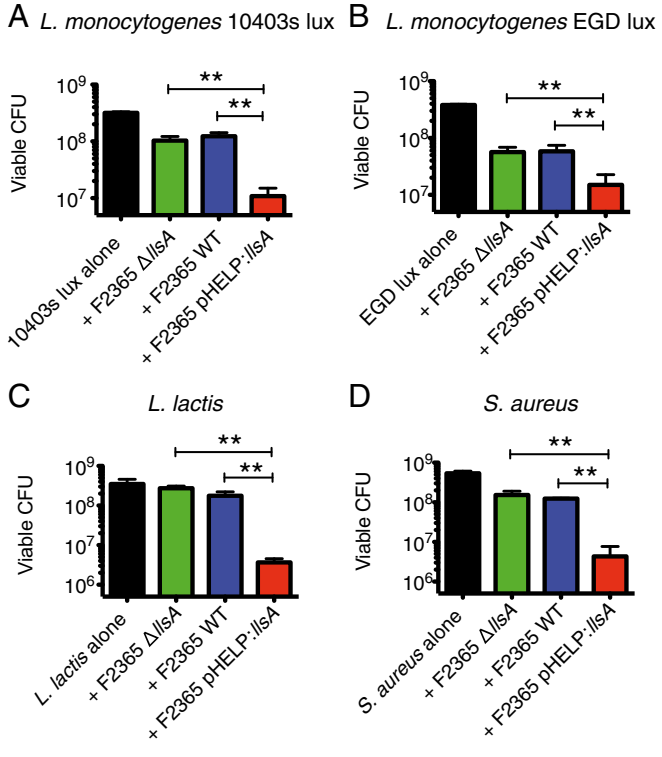


**Fig. 4.** LLS inhibits the growth of other bacteria in vitro. Viable *L. monocytogenes* 10403S$^{lux}$ (*A*), *L. monocytogenes* EGD$^{lux}$ (*B*), *L. lactis* (*C*), and *S. aureus* (*D*) at 24 h postinoculation either alone or in coculture with *L. monocytogenes* F2365 pHELP:LLS compared with *L. monocytogenes* F2365 WT or *L. monocytogenes* F2365 Δ*llsA*. Data from three independent experiments are presented. Error bar shows SD. Data were analyzed by using a Student's *t* test. $^{**}P < 0.01$.

## Materials and Methods

**Bacterial Strains and Growth Conditions.** Bacterial strains and plasmids that we used in this study are listed in Table S1. *L. monocytogenes* F2365 (10), *L. monocytogenes* EGD (3), *L. monocytogenes* EGD-e (3), *L. monocytogenes* 10403S (26), *S. aureus aureus*, *S. thermophilus*, *E. faecalis*, and *A. johnsonii* were grown in BHI broth (Difco). *L. lactis lactis*, *Lactobacillus paracasei paracasei*, *Lactobacillus paraplantarum*, *Lactobacillus plantarum plantarum*, and *Lactobacillus rhamnosus* were grown in de Man, Rogosa, and Sharpe broth. *E. coli* was grown in LB broth.

When required, antibiotics were added (chloramphenicol, 7 μg/mL for *Listeria* or 35 μg/mL for *E. coli*). Unless otherwise indicated, bacteria were grown with shaking at 200 rpm in tubes at 37 °C.

**Mutant Construction.** For the construction of the Δ*llsA and* Δ*llsB* deletion mutants, fragments of ∼500-bp DNA flanking each gene were amplified by PCR using chromosomal DNA of *L. monocytogenes* F2365 as template and finally ligated into the thermosensitive pMAD by using the BamHI/EcoRI or XmaI/SalI restriction sites (27). PCR primers are listed in Table S2. Allelic exchange was induced as described (27). All PCR amplifications for cloning were performed by using Phusion high-fidelity polymerase and reagents (Finnzymes, F-553) following the manufacturer's instructions. All plasmids and strains were confirmed by DNA sequencing. Quantitative real-time PCR discarded any polar effect of the *llsA* and *llsB* deletions on the expression of genes located in their vicinity. To construct a pPl2:*llsA* complementation plasmid, F2365 genomic DNA was used to amplify a fragment containing the *llsA* gene (oligos in Table S2), which was SalI–SmaI-digested and ligated into the pPL2 vector (28). To construct a pPL2:*llsB* complementation plasmid, we designed a chimeric construction composed of the LLS operon promoter (482 bp upstream of the ATG of the *llsA* gene) fused with the *llsB* gene and cloned into SalI–SmaI-digested pPL2 vector. Gene synthesis to construct pPL2:*llsB* was produced by Genecust.

**Mice Infections.** BALB/c mice were infected by intragastric inoculation with $5 \times 10^9$ bacteria of the indicated strains. Mice were anesthetized before oral gavage with isoflurane. The bacterial inoculum was prepared in a total volume of 200 μL. The bacterial inoculum was mixed with 300 μL of $CaCO_3$ (50 mg/mL) before oral gavage. Bacterial numbers in the inocula were verified by plating different dilutions onto BHI plates before and after injection. Mice were killed at subsequent time points, and intestines, spleens, and livers were removed. The small intestine was opened, and the luminal content was recovered in a 1.5-mL tube and weighed. The small intestine (duodenum, jejunum, and ileum) tissue was washed three times in DMEM (ThermoFisher), incubated for 2 h in DMEM supplemented with 40 μg/mL gentamycin, and finally washed three times in DMEM. All of the organs and the intestinal luminal content were homogenized, serially diluted, and plated onto BHI plates or Oxford plates (intestinal content) and grown overnight at 37 °C for 48–72 h. cfu were enumerated to assess bacterial load. Two independent experiments were carried out with four or five mice per group in each experiment. Statistically significant differences were evaluated by the Mann–Whitney test, and differences were considered statistically significant when *P* values were <0.05.

This study was carried out in strict accordance with the French national and European laws and conformed to the Council Directive on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/Eec). Experiments that relied on laboratory animals were performed in strict accordance with the Institut Pasteur's regulations for animal care and use protocol, which was approved by the Animal Experiment Committee of the Institut Pasteur (approval no. 03-49).

**Evaluation of *llsA* Promoter Expression with a Luciferase Reporter System.** The promoter of *llsA* was amplified by using oligos PllsAmonoFlong and PllsAmonInbluntR (Table S2), digested with XhoI, and cloned into SwaI–SalI-digested pPL2$^{lux}$ as described (29). The resultant plasmid pPL2$^{llsA:lux}$ was isolated from *E. coli* and introduced into *L. monocytogenes* F2365 generating F2365$^{llsA:lux}$.

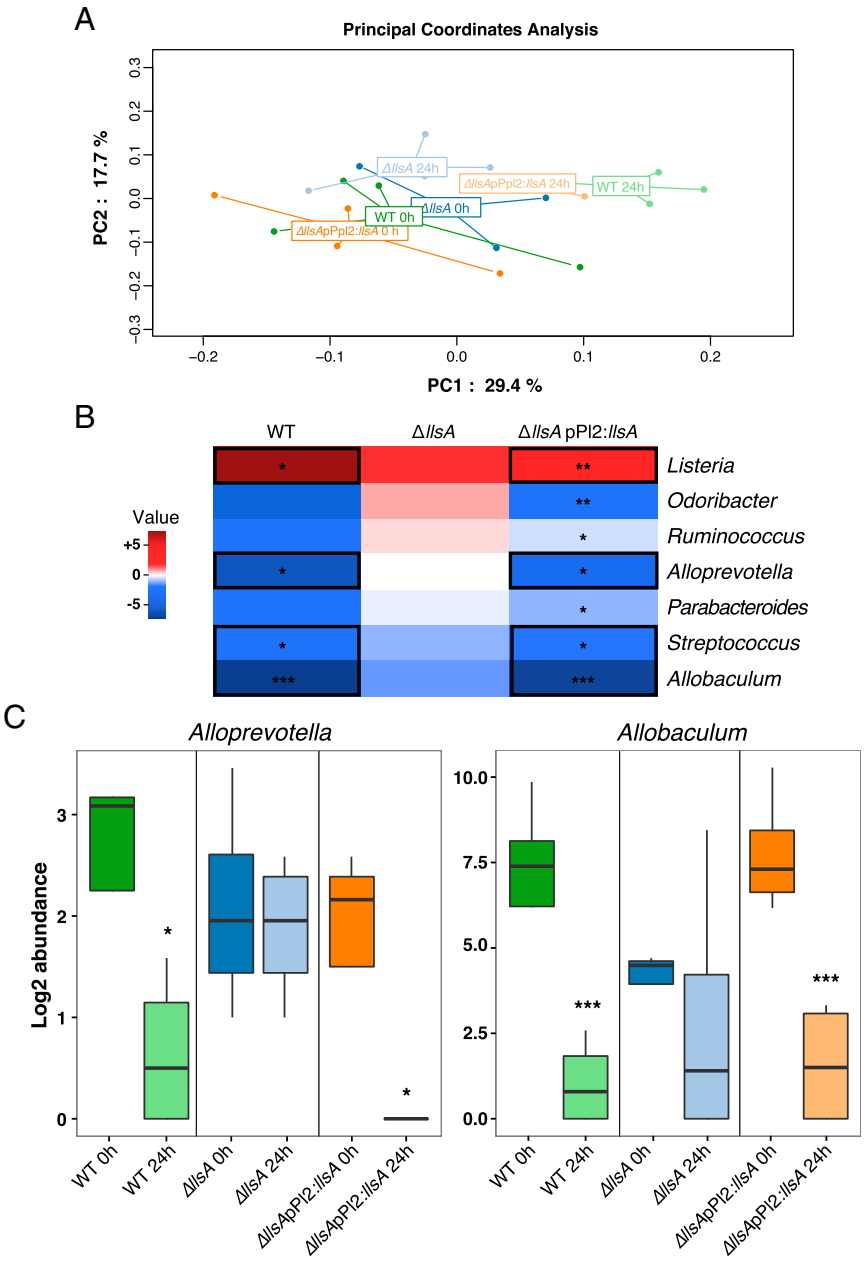


**Fig. 5.** LLS induces significant perturbations in the intestinal microbiome of infected animals. (*A*) Principal Coordinates Analysis results on Canberra distance matrices including sequence data from *L. monocytogenes* F2365 WT (green), *L. monocytogenes* F2365 Δ*llsA*-infected (blue), and *L. monocytogenes* F2365 Δ*llsA* pPl2:*llsA*-infected (orange) mice at 48 and 24 h before infection (averaged as time 0 h; dark color) as well as 24 h p.i. (light color). Absence of LLS expression correlates with no temporal changes in the intestinal microbiome, whereas LLS expression correlates with changes in the intestinal microbiome between time 0 and 24 h p.i. (*B*) Genera significantly altered in the gut microbiota of mice after 24 h of infection with *L. monocytogenes* strains F2365 WT, F2365 Δ*llsA*, and F2365 Δ*llsA* pPl2:*llsA*. *Alloprevotella, Streptococcus*, and *Allobaculum* populations decrease and *Listeria* populations increase (boxed areas) correlated with LLS expression. (*C*) Boxplots depicting changes in the *Alloprevotella* and *Allobaculum* populations in the microbiome of mice infected with the *L. monocytogenes* strains F2365 WT, F2365 Δ*llsA*, and F2365 Δ*llsA* pPl2:*llsA*. Significant *Alloprevotella* and *Allobaculum* changes correlate with LLS expression. Color codes are identical in *A* and *C*. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

For in vivo bioluminescence experiments, 8-wk-old female BALB/c mice were infected by intragastric inoculation with $5 \times 10^{10}$, $1 \times 10^{10}$ or $5 \times 10^{9}$ *L. monocytogenes* F2365$^{llsA:lux}$ grown in BHI broth to OD 1.0 at 37 °C. Bioluminescence imaging was performed by using an IVIS Spectrum in Vivo Imaging System (Perkin-Elmer) with a 5-min exposure time. Mice were anesthetized with isoflurane. For cfu determinations, whole luminal content from intestine or cecum, intestinal tissue, cecum, liver, and spleen were obtained, homogenized, serially diluted, and plated on Oxford agar plates (Oxoid).

For in vitro bioluminescence experiments, overnight bacteria cultures were washed and resuspended in an equivalent volume of BHI containing the compounds to test, which included hydrogen peroxide (2, 20, 50, or 100 mM), tripsin, pepsin, mucin, $NaHCO_3$, or bile salts at different concentrations (from 10 mg/mL to 0.6 ng/mL using fourfold dilutions). Triton X-100, Tween 20, and igepal were used in BHI at different concentrations, ranging from 10% (vol/vol) to 0.01% using 10-fold dilutions. Succinic acid, butyric acid, propionic acid, octanoid acid, and ethanolamine were assayed from 100 mM to 0.02 μM using fourfold dilutions. Gastric fluid was prepared as described (15). Minimal medium was prepared as described by Phan-Thanh and Gormon (30), but arabinose was added as the only sugar source. Antibiotics used included lincomycin, nalidixic acid, streptomycin, neomycin, doxycycline, vancomycin, rifampin, erythromycin, nisin, penicillin, trimethoprim, polymixin B, spectinomycin, ciprofloxacin, and levofloxacin at concentrations ranging from 100 μg/mL to 0.02 ng/mL using fourfold dilutions. In vitro bioluminescence was investigated in an IVIS Spectrum in Vivo Imaging System with a 5-min exposure time and with a plate-reading luminometer (Tristar LB 941; Berthold Technologies).

**Coculture Assays.** For coculture experiments, $5 \times 10^{7}$ bacteria from overnight cultures were inoculated into 5 mL of fresh BHI either in single culture or in coculture with another strain as indicated in the figures and incubated statically at 37 °C with 6% $O_2$. At 24 h after inoculation, cultures were serially diluted and plated on BHI and Oxford agar plates (Oxoid). *L. monocytogenes* F2365 was differentiated from *L. monocytogenes* 10403s$^{lux}$ and *L. monocytogenes* EGD$^{lux}$ by bioluminescence imaging using an IVIS Spectrum in Vivo Imaging System. Experiments were carried out three times independently. Data were analyzed by using Student's *t* test. Differences were considered statistically significant when *P* values were <0.05.

**Constitutive Expression of LLS.** To generate a strain that constitutively expresses LLS, we put the *lls* genes under the control of the strong constitutive promoter pHELP (31). Briefly, pHELP was fused between two 500-nt DNA

fragments flanking the start codon of *llsA*. This DNA construction was synthetically produced by gene synthesis (Eurofins Genomics) and cloned into SalI–EcoRI restriction sites of thermosensitive pMAD vector. Mutagenesis was performed by double recombination as described previously (27).

**Fecal Microbiota Analysis by 16S rRNA Gene Sequencing Using Illumina Technology.** BALB/c mice were orally infected with $5 \times 10^9$ cfu of each of the indicated strains (*L. monocytogenes* F2365 WT, Δ*llsA*, and Δ*llsA*pPL2:*llsA*). Six mice were used for each strain. Eight-week-old mice were kept together during 5 wk before infection to homogenize their intestinal microbiota. Two days before infection, each mouse was kept in an individual cage. Feces from each mouse were recovered 2 and 1 d before infection, as well at 6 and 24 h p.i. Feces were kept at −20 °C until DNA extraction was performed. The 16S rRNA gene amplification was performed by using the Nextflex 16s v1-v3 amplicon-seq kit. The 16S fecal DNA was sequenced by using Illumina Miseq, resulting in 287,000 ± 54,000 (mean ± SD) sequences of 300-base-long paired-end reads. Reads with a positive match with human or phiX174 phage were removed. Library adapters, primer sequences, and base pairs occurring at 5′ and 3′ ends with a Phred quality score <20 were trimmed off by using Alientrimmer (v0.4.0) (32). Filtered high-quality reads were merged into amplicons with Flash (v1.2.11) (33).

Resulting amplicons were clustered into operational taxonomic units (OTU) with VSEARCH (v1.4) (Rognes; https://github.com/torognes/vsearch). The process includes several steps for dereplication, singletons removal, and chimera detection (when aligned against the ChimeraSlayer reference database). The clustering was performed at 97% sequence identity threshold, producing 2,090 OTUs. The OTU taxonomic annotation was performed with the SILVA SSU (v123) database (34).

The input amplicons were then mapped against the OTU set to get an OTU abundance table containing the number of reads associated with each OTU. The first two columns of this table (corresponding to the two preinfection time points) were summed to strengthen the description of this initial state. All together, these stages are implemented in MASQUE software (Metagenomic AnalySis QUantitative pipeline. The statistical analyses were performed with SHAMAN (SHiny application for Metagenomic ANalysis (shaman.c3bi.pasteur.fr) based on R software (v3.1.1) and bioconductor packages (v2.14) (35). The matrix of OTU count data were normalized at the OTU level by using the normalization method included in the DESeq2 R package (v1.4.5) and described in ref. 36, as suggested in ref. 37. Normalized counts were then summed within genera to increase the power of the statistical analysis. The generalized linear model (GLM) implemented in the DESeq2 R package (38) was then applied to detect differences in abundance of genera between time points for each *L. monocytogenes* strain. We defined a GLM that included time, mice, and *L. monocytogenes* strains as main effects and interactions between time and strain as well as mice and strain. The later interaction was useful to model the pairing between successive measurements coming from the same mice. Resulting *P* values were adjusted according to the Benjamini and Hochberg procedure (39).

The PCOA plot was performed with the ade4 R package (v1.7.2), and plots in Fig. 4 were generated with ggplot2 (v1.0.1).

The read sequences from this study were deposited to European Nucleotide Archive (reference PRJEB13646; www.ebi.ac.uk/ena). Datasets used for this study are available at: github.com/aghozlane/listeria_metagenomic.

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