Genomic and epidemiological evidence of bacterial transmission from probiotic capsule to blood in ICU patients

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Probiotics are routinely administered to hospitalized patients for many potential indications(1) but have been associated with adverse effects that may outweigh their potential benefits(2–5). It is particularly alarming that probiotic strains can cause bacteremia(6), yet direct evidence for an ancestral link between blood isolates and administered probiotics is lacking. Here we report a markedly higher risk of Lactobacillus bacteremia for intensive care unit (ICU) patients treated with probiotics compared to those not treated, and provide genomics data that support the idea of direct clonal transmission of probiotics to the bloodstream. Whole-genome-based phylogeny showed that Lactobacilli isolated from treated patients’ blood were phylogenetically inseparable from Lactobacilli isolated from the associated probiotic product. Indeed, the minute genetic diversity among the blood isolates mostly mirrored pre-existing genetic heterogeneity found in the probiotic product. Some blood isolates also contained de novo mutations, including a non-synonymous SNP conferring antibiotic resistance in one patient. Our findings support that probiotic strains can directly cause bacteremia and adaptively evolve within ICU patients.

Probiotics are increasingly administered to hospitalized patients(1). These supplementary products have shown benefit in acute infectious diarrhea, antibiotic-associated diarrhea, and ulcerative colitis(6,13,14). In the intensive care unit (ICU), additional indications are being explored, including prevention of ventilator-associated pneumonia, pancreatitis, and sepsis(12–14). However, studies on the efficacy and adverse effects of probiotic treatment in ICU patients show conflicting results, and their use remains controversial(12–15). Adverse outcomes, including bacteremia, have been reported and may preclude their use in specific populations such as those with a compromised immune system or disorders of the gastrointestinal tract(15,16,17).

Bacteremia that appears during the course of probiotic treatment can involve Lactobacillus species similar to those in probiotics, yet as these species are also common in the human gastrointestinal microbiome, pinpointing the source of these infections has been challenging(18). Studies that use pulsed-field gel electrophoresis have previously revealed strain-level similarity between blood and probiotic isolates(19), but higher genomic resolution is required to establish direct clonal ancestry and the possibility of direct transmission of probiotic bacteria to the blood.

In the context of bacterial pathogens, whole-genome methods have been powerful in identifying transmission links and within-host adaptation(20–23). Constructing a single nucleotide polymorphism (SNP)-level phylogeny of isolates from patients can unravel ancestral links between lineages and likely paths of transmission. Whole-genome comparison of isolates can also reveal adaptive mutations important for the survival of the pathogen within the host(21,22). Yet, despite their established power, the use of these whole-genome approaches for tracing of probiotic strains has so far been limited. Here, we apply whole-genome analysis and phenotyping to blood isolates and probiotic strains administered to ICU patients.

Analysis of cases of Lactobacillus bacteremia in ICU patients at Boston Children’s Hospital showed that patients receiving Lactobacillus rhamnosus strain GG (LGG) probiotics had a markedly higher risk of developing Lactobacillus bacteremia than those who received no probiotics. Over a period of 5.5 years, a total of 22,174 patients were treated in an ICU, and 522 of these patients received LGG-containing probiotic—typically through a feeding tube—as part of their treatment. Analysis of recorded Lactobacillus bacteremia among these patients showed a significantly greater risk for patients who received the LGG-containing probiotic than for those who did not; 6 of the 522 patients had Lactobacillus bacteremia (1.1%, patients R1–R6; Supplementary Table 1a) compared to only 2 of the 21,652 patients who did not receive the LGG probiotic (0.009%, patients N1–N2; P = 4.8 × 10−9, Fisher’s exact test; Supplementary Table 1a). Furthermore, all six of the ICU blood isolates from patients receiving the LGG probiotic were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as Lactobacillus rhamnosus, while the two isolates from patients not receiving the LGG probiotic were identified as other Lactobacillus species (Supplementary Table 1a). The ICU patients receiving probiotics containing LGG are therefore at
markedly higher risk of developing *Lactobacillus rhamnosus* bacteremia (6 out of 522 compared to 0 out of 21,652; \(P = 1.8 \times 10^{-16}\), Fisher’s exact test). Further, the LGG probiotic bacteremia rate of 1.1% that we observed is also much higher than the annual rate of LGG probiotic bacteremia (0.00007%) reported in the general population\(^7\). Yet, *L. rhamnosus* bacteremia can occasionally also appear in patients not receiving these probiotics: during the study population\(^19\). Yet, of LGG probiotic bacteremia (0.00007%) reported in the general population\(^1\). We observed is also much higher than the annual rate of developing *Lactobacillus* bacteremia among approximately 93,000 non-ICU patients (Patients N3–N12, Supplementary Table 1b), and four of these ten isolates were identified by MALDI-TOF as *Lactobacillus rhamnosus* (Patients N5, N9–N11, Supplementary Table 1b). None of the ten non-ICU patients were receiving a probiotic at the time of the bacteremia. Taken together, these results suggest that ICU patients who receive probiotics containing LGG are at much higher risk of developing *Lactobacillus rhamnosus* bacteremia than patients who do not, but it is difficult to prove a direct causal relationship as occasional cases of *L. rhamnosus* bacteremia also appear in patients who do not receive these probiotics.

To achieve better ancestral resolution, we next used whole-genome sequencing to determine strain-level similarity among the blood and probiotic isolates. We performed whole-genome sequencing of all ten *L. rhamnosus* blood isolates (six from patients receiving probiotic and four from patients not receiving probiotic), as well as 16 isolates from each of three probiotic capsules of different lots (probiotic batches 1–3; Supplementary Table 2, Methods and Fig. 1a). To quantify strain-level relatedness among these isolates, we started by measuring their distance to all available *L. rhamnosus* genomes (GenBank, October 2017; Supplementary Table 3). Illumina reads of each isolate were aligned to each of these genomes, and the fraction of aligned reads, affected both by gene content similarity and SNP density, was quantified as a measure of similarity. We found that all six blood isolates and all probiotic product isolates shared the same closest reference genome—an LGG genome (GenBank chromosome ID: FM179322)—suggesting high relatedness between these two sets of isolates (Fig. 1b). By contrast, all four *L. rhamnosus* blood isolates from patients not receiving probiotics were more similar to other strains, indicating that they were not derived from the probiotic product (Fig. 1b).

To further increase genomic resolution, we next compared the genomes of the blood and LGG probiotic isolates by alignment to the reference genome. Analysis of gene content of the isolates identified only a single deletion; one of the probiotic isolates of batch 2 had a large deletion of a region which included 82 genes of the reference genome FM179322 (genes 384–465, Fig. 2). Strains were also almost identical at the single-nucleotide level; analysis of SNP-level variations identified a total of only 23 SNPs among all isolates (Methods; for the list of SNPs, see Supplementary Table 4). Indeed, the greatest SNP distance between any isolate and the last common ancestor was not more than six SNPs. Two SNPs were shared by all isolates, separating them from the reference genome, and indicating that the blood and probiotic isolates share a more recent last common ancestor than the LGG clone deposited in GenBank (Fig. 1c). Moreover, the blood and probiotic isolates were phylogenetically inseparable; there was no mutation that strictly separated these two groups (Fig. 1c and Supplementary Table 4).

Much of the genetic diversity among blood isolates mirrored pre-existing genetic diversity within the probiotic capsules. We identified 11 genomic positions that were polymorphic across blood isolates (Fig. 2). Three of these mutations, all of which were non-synonymous, were recurring mutations, observed in more than one blood isolate (H294Q in CamS, H248Y in G1vA, and Q1827R in SpCB; Fig. 2 and Supplementary Table 4). These repeatedly occurring blood isolate mutations were all shown to pre-exist in the probiotic product (these same loci were diverse within each of the three batches of the probiotic product, Fig. 2). Furthermore, the camS and
glvA SNPs always appeared together in both the blood and probiotic isolates. One other polymorphic locus, identified in a single blood isolate, was also found to pre-exist in the probiotic product (a D220G mutation in the ABC transporter CcmA). Overall, correspondence between blood isolate mutations and pre-existing diversity within the probiotic product further supports the likelihood of transmission of bacteria from probiotic to blood.

In addition to the six blood isolate mutations that were found to pre-exist in the probiotic product, we identified five blood isolate mutations that did not appear in the isolates from the probiotic product, suggesting de novo evolution within the patient (Fig. 2, Supplementary Table 5). These 5 mutations were not found in any of the 16 genomes isolated from each of the 3 capsules. To further test for their possible existence in the product, we deep-sequenced capsules from five different batches, obtained both from the hospital and from a commercial pharmacy, and identified diverse loci (batches 2–6, Methods; Fig. 2, Extended Data Fig. 1, Supplementary Table 6). No pre-existing genotypic diversity was found at the loci of the five blood-isolate-specific mutations (Fig. 2, Supplementary Table 7). One of these five mutations was in an intergenic promoter mutation, two were non-synonymous coding mutations (H487D in the RNA polymerase RpoB (Fig. 3a, Extended Data Fig. 2) and A259D near the active site of the RbsK ribokinase (Extended Data Fig. 3)), and two were synonymous mutations (at G44 of the YhfS transferase and at V132 of phosphoglucomutase). These mutations, existing in the blood but not identified in the probiotic product, could represent de novo mutations selected within the patient.

The blood-isolate-specific mutation in the rpoB RNA polymerase gene (H487D) appeared in an isolate from patient R1, who had received L. rhamnosus GG and the rifampin derivative rifaximin concurrently during the 3 months prior to bacteremia. This mutation, which changes a specific residue in the cleft of the RpoB DNA-binding site, is known to provide resistance to rifampin (Fig. 3a, Extended Data Fig. 3)), and two were synonymous mutations (at G44 of the YhfS transferase and at V132 of phosphoglucomutase). These mutations, existing in the blood but not identified in the probiotic product, could represent de novo mutations selected within the patient.

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position can retain fitness similar to that of the wild type\textsuperscript{25}. Indeed, the R1 isolate carrying the rpoB mutation showed no significant fitness cost compared with the probiotic strain containing no SNPs (Supplementary Table 9). The specificity of the rpoB mutation to the patient receiving rifampin, together with its associated resistance and growth phenotypes, further suggest that the probiotic strains may acquire adaptive mutations that increase their fitness in the host environment.

We further considered other adaptive phenotypes. Survival in serum or human whole blood was similar among the probiotic and blood isolates (Supplementary Fig. 1), whereas the level of biofilm formation (Extended Data Fig. 4), which may lead to increased adhesion to a central venous line (CVL) and/or enhanced survival in the gastrointestinal tract, was significantly higher in the LGG blood and probiotic isolates compared to the non-LGG blood isolates (from Patients N5, N9–N11) and to the probiotic isolate P2-1 containing an 82-gene deletion, which includes the spaCBA pilus genes critical for biofilm\textsuperscript{26} (Supplementary Table 10). These results suggest that biofilm is not required for bacteremia and that the LGG probiotic products may contain mutants with markedly different biofilm phenotypes.

Our patient population was critically ill, but the patients who developed LGG bacteremia while receiving probiotics did not have the typical risk factors for Lactobacillus bacteremia such as severe immune compromise or bowel disintegration. Furthermore, in a case-control study to compare potential risk factors for bacteremia in these 6 cases with 16 matched control ICU patients who received probiotics but did not have bacteremia (Methods), we found no significant differences in device utilization, vasopressor support, recent surgery, diarrhea, parenteral nutrition, or antibiotic exposure (Supplementary Table 11). Although the low number of patients in this case-control study may limit statistical power, our inclusion of a control group with case-control methodology represents a significant improvement over prior descriptive studies in understanding specific risk factors within the ICU. The lack of strong differences between the patients who had bacteremia and the control group that did not have bacteremia suggests that the ICU patients at risk for transmission of probiotics from product to blood may not be easily identifiable.

The exact mechanism of transmission from probiotic to blood is unclear. Nearly all of these patients had a central line, and direct contamination of the central line with a probiotic strain or with stool containing the probiotic strain could lead to the observed probiotic bacteremia. Alternatively, the probiotic bacteria could have translocated across the bowel wall. The antibiotic-resistance-related adaptation we observed could appear either prior to or immediately following the transmission of the bacteria to the blood. Our results suggest that these adaptive mutations are absent in the probiotic capsule and therefore evolved within the host environment. Nevertheless, given possible genomic variations among batches of the probiotic product, we cannot exclude the possibility that some of these presumably blood-specific mutations were present in the specific capsules given to each patient. In any case, whether they appear through rare mutations that already exist in the product or de novo during treatment, these emerging antibiotic-resistant probiotic bacteria could potentially undermine treatment efficacy. It would be interesting in future studies to explore the importance of other de novo mutations with additional in vitro phenotyping, or in animal models.

In summary, our epidemiological analysis uncovers a statistically and clinically significant risk for bacteremia associated with the use of probiotic Lactobacillus in the ICU, and genome-level analysis identified six independent cases of transmission of probiotics from capsule to blood in ICU patients treated with probiotics. Our results also provide evidence of within-host evolution of the probiotic, including acquisition of antibiotic resistance. Probiotics have shown significant benefits for acute infectious diarrhea, antibiotic-associated diarrhea, and ulcerative colitis\textsuperscript{2,16,11}. However, our findings highlight that as ICU patients have increased risk for probiotic-associated bacteremia, these potential benefits must be weighed against this risk when considering the continued use of probiotics in the ICU.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-019-0626-9.

Received: 11 April 2018; Accepted: 25 September 2019; Published online: 07 November 2019

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Eighteen cases of probiotic product batch 1, a capsule was streaked and frozen. To isolate individual bacteria from probiotic capsules, we used two selective techniques. For probiotic product batch 1, a capsule was streaked on MRS-agar plates, and the bacteria were frozen at the time of isolation by the clinical microbiology laboratory and were confirmed as potential contaminants or transient bacteremias and were examined for the presence of bacteria within 7 days of bacteremia. Notably, although we do note that these episodes of bacteremia manifested initially as bacteremia (only Patients R2, N1, and N5 did not), so nearly all of these episodes of non-LGG bacteremia were treated with a course of antibiotics directed at *Lactobacillus*. CVLs were removed during treatment from two of the five patients receiving probiotics who had a CVL and from nine of the ten patients not receiving probiotics who had a CVL. Notably, two of the six cases of LGG bacteremia (patients R2 and R4) and one of the four cases of non-LGG L. *rhamnosus* bacteremia (patient N5) were considered by their treating physicians as potential contaminants or transient bacteremias and were not specifically treated with a long course of antibiotics. However, as patient R4 had a CVL, the bacteremia was classified as a CLABSI. None of the patients had endocarditis, and none died within 7 days of bacteremia.

Isolation of bacteria from probiotic capsules and blood. Blood isolates were frozen at the time of isolation by the clinical microbiology laboratory and were isolated from MRS-agar plates, and the *Lactobacillus* species were identified by MALDI-TOF. To isolate DNA for whole-genome sequencing, single colonies were streaked on MRS-agar plates, and the bacteria were frozen. To isolate individual bacteria from probiotic capsules, we used two selective techniques. For probiotic product batch 1, a capsule was streaked on a CDC Anaerobe Blood Agar plate, bacterial lawn was scraped off the plate, frozen (−80°C, glycerol) and then streaked to single colonies on MRS-agar plates (BD Biosciences). Bacteria were cultured at 37°C at 5% CO2 incubated at 37°C at 5% CO2 for 48 h on Trypticase Soy Agar II with 5% sheep blood. The protein accession numbers of the RpoB protein (NCBI) were used in COBALT for amino acid alignment from NCBI (https://www.ncbi.nlm.nih.gov/tools/cobalt). The growth curve was determined by the PHYLIP parsimony algorithm, which carries out unrooted phylogenetic parsimony.

Gene content analysis. For each isolate, a ‘raw copy number’ for each gene was calculated as the median base coverage across the genome divided by the median coverage across the genome of the isolate. To remove gene-specific biases, this raw copy number was further normalized by the median raw value of the gene across all isolates, yielding the gene copy number used to identify deleted genes. For Fig. 1, a similar analysis of polymorphic SNPs was performed, in which each 50-bp region the mean read coverage was divided by the median coverage across the genome and normalized by the median of this region-specific value across isolates.

Genomic analysis of capsule deep sequencing. Reads were filtered and aligned to the reference genome as described for single-isolate analysis above (GenBank: FM179322). This resulted in coverage of >97% of the reference genome. Per batch, median coverage of these positions was 372–1,268× (Supplementary Table 2).

Bacterial strains and culture conditions for in vitro assays. *L. rhamnosus* bacteria were grown at 37°C with 5% CO2 for 48 h on Trypticase Soy Agar II with 5% sheep blood (BD Biosciences). Liquid culture was performed using MRS broth (Sigma-Aldrich) supplemented with 0.001% Tween 80 (MRST) (Sigma-Aldrich) at 37°C with 5% CO2, for 24 h statically. *Pseudomonas aeruginosa* PA01 gu5 mutant17 were grown at 37°C overnight on Trypticase Soy Agar II (BD Biosciences). Liquid culture was performed using LB broth (Miller) at 37°C at 200 rpm overnight. Modified TS (mTSB) medium consisted of 15 g 1−1 TSB (BD Biosciences) and 20 g l−1 of Racto-protease peptone no. 3 (BD Biosciences) was used for the biofilm assay.

Biofilm assay. The assay for biofilm formation was based on a previous report, with minor modifications17. In brief, 3×106 colony-forming units (c.f.u.) were added to 200 μl of mTSB in three replicates in flat bottom polystyrene 96-well plates (Costar) at 37°C with 5% CO2, for 72 h. Bacteria were removed by inverting the plate. The plate was then washed with water, and attached bacteria were stained for 30 min with 200 μl 0.1% (w/v) crystal violet in an isopropanol–methanol–PBS solution (volume ratio of 1:1:18). Plates were washed with water, left to dry for 15 min, and then 150 μl of 33% glacial acetic acid was added to each well. Biofilm was measured at 570 nm (Versa max, Molecular devices).

Antibiotic resistance testing. Disk diffusion susceptibility testing was performed by the Boston Children’s Hospital Infectious Diseases Diagnostic Laboratory using the standard methods for *Staphylococcus aureus* (specific disk diffusion methods for *Lactobacillus* are not available)31.

Competition assay. The single culture and competition assays were based on a previous report, with modifications18. In brief, bacteria of the probiotic strain containing no SNPs (P3-2) and of the blood isolate R1, from overnight MRST liquid cultures were adjusted to an OD600 of 0.05, either in single culture or mixed in a 1:1 ratio. During growth in MRST broth at 37°C with 5% CO2, for 24 h CFUs were determined every 2 h by serial dilutions on MRST-agar plates for the single cultures and on both MRST-agar and MRST-agar with 1 μg ml−1 rifampicin (Emerald Products International) for the competition cultures.

BLASTP of RpoB protein. The protein accession numbers of the RpoB protein from the bacteria LGG (CA888393.1), *S. aureus* M1112 (EWR38128.1), *Staphylococcus epidermidis* RP62A (AAW55380.1), *Enterococcus faecium* 343-3 (AA000730.1), *E. faecalis* 40-4 (AA000730.1), *Escherichia coli* K12 sub-str. MG1655 (NP_418414.1), *Bacillus velezensis* CC09 (AN47365.1) were used in COBALT for amino acid alignment from NCBI (https://www.ncbi.nlm.nih.gov/tools/covalt).

Whole blood killing assay. Bacteria were grown on trypticase soy agar (TSA) with 5% sheep blood (BD Biosciences) and incubated for 48 h at 37°C with 5% CO2. Overnight cultures in MRST medium were washed once in PBS (Boston Bio-products) and adjusted to give 107 CFU per 50 μl. Next, 50 μl of each strain were added to 450 μl of heparinized blood from a healthy donor. Inoculum c.f.u. values were determined by serial dilutions on TSA with 5% sheep blood. After 1 h and 3 h of rotation at 37°C, serial dilutions were plated to determine the number of surviving c.f.u. In parallel, static tubes were held at 37°C as a non-phagocytosis control for all time points (0 h, 1 h, and 3 h).

Serum sensitivity assays. Bacteria were grown on TSA with 5% sheep blood (BD Biosciences) and incubated 48 h at 37°C with 5% CO2. Static overnight cultures in
MRST medium at 37 °C with 5% CO2, were washed in PBS once and then diluted in PBS plus 1 mM CaCl2 and 1 mM MgCl2, and 100 μl aliquots were placed in a sterile 96-well plate to give a final inoculum of approximately 5 × 10^3 c.f.u. per well. Pooled male, AB human serum (Sigma-Aldrich) was diluted in PBS plus 1 mM CaCl2 and 1 mM MgCl2 to give twice the desired final concentration. Final serum concentrations that were used were 50% and 25%. Human serum (50%) that was heat-inactivated by incubation at 56 °C for 30 min, and 0% serum served as controls. Equal volumes (100 μl) of sera and bacterial suspensions were mixed and incubated at 37 °C for 1 h with gentle shaking. An aliquot from each well was serially diluted and then plated on TSA with 5% sheep blood after incubation for 48 h at 37 °C with 5% CO2, for enumeration. A serum-sensitive, rough lipopolysaccharide (LPS) strain of P. aeruginosa (PA01 galU') was grown on TSA at 37 °C overnight and then in liquid culture in LB at 37 °C overnight, was used as a positive control.

Case-control study methods. As all cases of Lactobacillus bacteremia in patients receiving probiotics occurred in an ICU, cases were matched with up to 3 control patients who had received probiotics in an ICU within 90 d of the case and had similar or longer ICU exposure prior to censoring. Controls were selected randomly using incidence density sampling. Censoring occurred on the date of bacteremia diagnosis or, for controls, on the date of discharge, death, or transfer from the ICU. Five cases had 3 controls identified, whereas 1 case with a particularly long ICU stay, had only 1 possible control identified. As a result, 6 cases were compared with 16 controls in our analysis.

Patient data for the 6 cases and 16 controls were collected retrospectively by chart review onto a standardized case report form. Temperature, white blood cell (WBC) count, C-reactive protein (CRP), and 30 d mortality were collected from the date of bacteremia or censoring. Clinical variables previously associated with either Lactobacillus bacteremia or with central line–associated bloodstream infections in general were collected for the 7 days prior to bacteremia, death or discharge.

Immunodeficiency was defined as active cancer diagnosis, solid organ or stem cell transplant, primary immunodeficiency, receipt of immunosuppressant within 6 weeks prior to censoring, or neutropenia (absolute neutrophil count (ANC) or total WBC count <500 occurring for at least two days and within three calendar days before or after the date of censoring). Medical device included endovascular prosthetic material, CVL, tracheostomy tube, gastrostomy tube, and urinary catheter. Gastrointestinal breakdown included documentation of mucositis, diarrhea, or skin breakdown around the gastrointestinal or jejunostomy insertion site. Diarrhea was identified by documentation in the physician or nursing notes or by stool output of >20 ml kg^-1 in a 24 h period as per the CDC definition of mucosal barrier injury. Antibiotic data included information on oral and intravenous antibiotics regardless of indication.

For the case-control study, odds ratios for continuous and categorical variables were generated by exact conditional logistic regression using SAS 9.4.

Statistical analysis. Fig. 3b: Kruskal–Wallis test (P = 0.0297, Kruskal–Wallis statistic = 13.99) followed by Dunn’s multiple comparisons test to P3-2 were performed. α = 0.05. Statistical results relating to Fig. 3b: See Supplementary Table 12a.

Extended Data Fig. 4: 3 independent experiments were performed on different days. In each experiment, each bacterial isolate had 3 technical replicates. P < 0.0001 by ANOVA with Tukey’s multiple comparisons test for the pairwise comparison of any of the isolates making biofilm (defined as OD570 > 0.1, Mann–Whitney U = 0.9999, Mann–Whitney U = 4, number of divisions: P > 0.9999, Mann–Whitney U = 4 by unpaired two-tailed Mann–Whitney test for the competition culture experiment, doubling time: P = 0.1, Mann–Whitney U = 0, no. of divisions: P = 0.1, Mann–Whitney U = 0 by unpaired two-tailed Mann–Whitney test.

Please refer to the Nature Research Reporting Summary for additional details.

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Acknowledgements

We thank T. Moniz who provided the probiotic administration data; J. Kinlay and P. Scanlon who noted the pattern of cases; A. Mello, who provided epidemiological data, and R. Marshall and E. Derderian, who performed strain analysis and antibiotic susceptibility testing on the clinical isolates. This work was funded in part by the Richard A. and Susan F. Smith President’s Innovation Award (to G.P.P.) and by funds for the Translational Research for Infection Prevention in Pediatric Anaesthesia and Critical Care (TRIPPACC) Program of the Department of Anaesthesiology, Critical Care and Pain Medicine at Boston Children’s Hospital (to G.P.P.), US National Institutes of Health grant R01 GM081617 (to R.K.), The Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine (to R.K.), and European Research Council FP7 ERC grant 281891 (to R.K.).

Author contributions

K.B.F., G.P.P., and T.J.S. conceived the study. R.K., I.Y., J.S., E.S., M.H., E.L., and P. McGann performed whole-genome sequencing and data analysis. C.M. performed
phenotypic experiments and molecular modeling studies along with data analysis. K.B.F., P. Mehrotra, and T.J.S. designed and analyzed the case-control study to evaluate clinical risk factors. I.Y., K.B.F., C.M., A.J.M., T.J.S., R.K., and G.P.P. interpreted the results. I.Y., K.B.F., C.M., R.K., and G.P.P. wrote the manuscript. All authors reviewed the manuscript and provided input.

**Competing interests**
The authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the interpretation of the article.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41591-019-0626-9.

Supplementary information is available for this paper at https://doi.org/10.1038/s41591-019-0626-9.

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Peer review information Alison Farrell is the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | Deep sequencing identifies loci of diversity across probiotic product batches. Five probiotic batches (batches P2-P6, see Supplementary Table 2) were sequenced at high depth together with a single colony. In each batch, for each position in the reference genome, a two-sided Fisher’s exact test was carried out to determine differences in diversity between the batch-derived sequences and the colony-derived ones, and the respective P values were plotted. Significant loci (P < 1.66 x 10^{-8}) are marked with labels A–O (for details see Supplementary Table 6). A single locus of increased diversity in the colony in comparison to only one of the probiotic batches (P3) was also observed (green).
Extended Data Fig. 2 | see figure caption on next page.
Extended Data Fig. 2 | The blood-isolate-specific rpoB SNP does not perturb the RpoB predicted structure but occurs near the DNA-binding site and is associated with rifampin resistance in other bacterial species. (a) Predicted structures of *L. rhamnosus* GG RNA polymerase β-subunit RpoB with histidine at position 487 seen in the probiotic (blue, left), aspartic acid at position 487 seen in the blood isolate from Patient R1 (magenta, middle), and overlap (right). (b) Predicted DNA-binding site amino acids are shown in white, with the histidine (blue) of the probiotic (left) and the aspartic acid (magenta) of blood isolate from Patient R1 (right) shown compared to the DNA-binding positions. (c) Amino acid (aa) sequence alignment of the rifampin cluster I of the RpoB protein from LGG and other genera. Numbering begins and ends at the first and last aa of the cluster; asterisks depict evolutionarily conserved aa residues; red asterisk shows the conservation across species of the histidine. In magenta, aa substitution H487D of the *L. rhamnosus* GG rifampin-resistant isolate (Patient R1) found in this study, H481D of *S. aureus* M1112 rifampin-resistant isolate, and H482D of *B. velezensis* rifampin-resistant isolate; in orange, substitution H481Y of *S. epidermidis* RP62A rifampin-resistant isolate, H489Y of *E. faecium* 343-3 rifampin-resistant isolate, H489Y of *E. coli* K-12 substr. MG1655 rifampin-resistant isolate, and H482Y of *B. velezensis* rifampin-resistant isolate; in lavender, substitution H489Q of *E. faecium* 38–15 rifampin-resistant isolate; in brown, substitution H482R of *B. velezensis* rifampin-resistant isolate; in turquoise, substitution H482C of *B. velezensis* rifampin-resistant isolate.
Extended Data Fig. 3 | The blood-isolate-specific ribokinase SNP does not perturb the predicted structure of ribokinase but occurs near the active site. (a) Predicted structures of probiotic ribokinase with A259 (blue, left), blood isolate from Patient R1 with ribokinase A259D SNP (magenta, middle) and overlap (right). (b) The predicted binding site amino acids of ribokinase for adenosine are shown in white, with the alanine 259 (blue) of the probiotic (left) and the aspartic acid (magenta) of blood isolate 1 (right) shown compared to the adenosine-binding positions.

(aa: 186, 222, 223, 224, 225, 227, 241, 242, 244, 246, 252, 253, 256, 279, 282, 286)

Adenosine-binding site of ribokinase

Probiotic (A259) | Blood (A259D)

(aa: 10, 12, 14, 39, 40, 41, 44, 108, 110, 139, 141, 250, 251, 254, 291)
Extended Data Fig. 4 | see figure caption on next page.
Extended Data Fig. 4 | Biofilm formation of probiotic and blood L. rhamnosus isolates. Blood isolates from patients receiving (R1-R6) and those not receiving probiotics (N5, N9, N10, N11), as well as selected probiotic isolates, were tested for biofilm formation. Isolates are grouped by similar mutations, as depicted in the grid below the isolate labels. Isogenic probiotic isolates from different probiotic capsules were used as controls, if available, as were controls for mutations found in blood isolates, when available. In Px-y, x is probiotic batch number, y is probiotic isolate number. Bars represent means of three independent experiments performed on different days, with three technical replicates per isolate in each experiment. Error bars depict the s.e.m. ****P < 0.0001 by ANOVA followed by Tukey’s multiple comparisons test for the pairwise comparison of any of the isolates making biofilm (defined as OD_{570} > 1) compared to either P2-1, N5, N9, N10, N11, or medium control. There were no statistically significant differences among the isolates making biofilm or among the isolates not making biofilm.
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Software and code

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- **Data collection**: Illumina HiSeq 2500 software (HCS version 2) was used for DNA sequencing.
- **Data analysis**: Illumina sequencing data analysis tools: Bowtie 1.2.1.1 for short read alignment, SAMtools and BCFtools 0.1.19 for SNP calling. Custom code was used to automate these tools and visualize output. COBALT was accessed on the NCBI website: https://www.ncbi.nlm.nih.gov/tools/cobalt

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Sequence data are available under BioProjectID PRJNA562050. BioSample accession numbers are: SAMN12632778-SAMN12632834. Figure 3, Extended Data Figure 4, Supplementary Table 9, and Supplementary Figure 1 have associated raw data. All other data are available from the corresponding authors upon reasonable request.
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| Sample size | All patients that developed Lactobacillus bacteremia while receiving probiotics during our study period were included. For the case-control analysis, a 3:1 matching scheme was chosen given the small sample size and potential variability in control subjects. Sample size calculations were not performed for the overall study. Given the infrequency of our outcome, all patients that developed Lactobacillus bacteremia while receiving probiotics during our study period (n=6) were included. The study period was chosen based on the availability and reliability of infection prevention and microbiology data during this time period. |
| Data exclusions | There were no data exclusions |
| Replication | All in vitro experiments were performed 2-3 times, and all findings were replicated. |
| Randomization | As a retrospective study, randomization was not performed. |
| Blinding | As a retrospective case-control study, blinding was not performed. Data analysis was done according to methods pre-specified before data collection to prevent bias during analysis. |

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Population characteristics

Six patients with Lactobacillus bacteremia and sixteen matched controls, all from the intensive care unit, had their charts reviewed for clinical factors that could be associated with the development of bacteremia. As a retrospective study, consent was waived by our IRB (protocol #P00018055). The covariate-relevant population characteristics of the participants in the case-control study are shown in Supplementary Table 11. For the whole blood killing assays, blood was obtained from human volunteers with informed consent on a separate IRB-approved protocol (protocol #P00025848).

Recruitment

See above.

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