Pathogen elimination by probiotic Bacillus via signalling interference

There is increasing appreciation of the key role that the intestinal microbiota play in preventing the colonization and overgrowth of pathogens. The mechanisms that have been implicated in this beneficial function of probiotic bacteria are mostly indirect, and include modulation of the immune system, enhancement of the intestinal epithelial barrier, or competition with pathogens for nutrients. Whether there is direct interference between probiotic and pathogenic bacteria is less clear. Some probiotic strains produce bacteriocin proteins, which can kill phylogenetically related pathogenic bacteria, and it has been shown that a bacteriocin-producing Escherichia coli strain inhibits colonization by related pathogenic bacteria in the inflamed gut of mice. However, no evidence has been obtained to indicate that such mechanisms matter or are widespread in humans. Furthermore, it is not known whether there are mechanisms for direct probiotic bacterial interference that are not mediated by bacteriocins.

The genus Bacillus comprises different species of soil bacteria that form endospores with the ability to survive harsh environmental conditions, such as high temperatures encountered during cooking procedures. Bacillus spores are commonly ingested with vegetables. They can subsequently germinate to form metabolically active, vegetative cells, which can temporarily colonize the intestinal tract. Given the variability in dietary customs, the concentration of Bacillus spores in human faeces is also highly variable. It has been reported to be around 10^5 colony-forming units (CFU) per gram on average, occasionally reaching up to 10^6 CFU per gram. Several probiotic formulae contain Bacillus species, which are thought to reduce pathogen colonization by mechanisms that—except for a described immune-stimulatory effect of epithelial cells—remain poorly defined.

Staphylococcus aureus is a widespread and dangerous human pathogen that can cause a variety of diseases, ranging from moderately severe skin infections to fatal pneumonia and sepsis. Treatment of S. aureus infections is severely complicated by antibiotic resistance, such as in methicillin-resistant S. aureus (MRSA), and there is no working S. aureus vaccine. Therefore, alternative strategies to combat S. aureus infections are eagerly sought. Because S. aureus infections commonly originate from previous asymptomatic colonization and decolonization has recently gained considerable attention as a possible means to fight S. aureus infections in a preventive manner. While the nares (nostrils) have traditionally been considered the primary S. aureus colonization site, there is increasing evidence that the intestinal tract is also commonly colonized by S. aureus and forms an important reservoir for outbreaks of infectious S. aureus disease. Several studies have reported levels of S. aureus in the faeces of human adults of around 10^3–10^4 CFU per gram. Possibly, intestinal S. aureus colonization explains the failure of previous topical decolonization efforts aimed solely at the nose.

Here we hypothesized that the composition of the human gut microbiota affects intestinal colonization with S. aureus. To evaluate that hypothesis, we collected faecal samples from 200 healthy individuals from rural populations in Thailand (Fig. 1a). This exemplary population was selected in order to rule out, as much as possible, the food sterilization and antibiotic usage that are common in highly developed urban areas, which potentially could diminish the abundance of probiotic bacteria in the food and intestinal tracts of the participating subjects. Our analysis revealed a comprehensive Bacillus-mediated S. aureus exclusion effect in the human population. By demonstrating that quorum sensing is indispensable for S. aureus to colonize the intestine, and discovering that secreted Bacillus fengycin lipopeptides function as quorum-sensing blockers to achieve complete eradication of intestinal S. aureus, we provide evidence that strongly suggests that this pathogen-exclusion effect in humans is due to a widespread and efficient probiotic-mediated mechanism that inhibits pathogen quorum-sensing signalling.

S. aureus exclusion by Bacillus

We found that 25/200 (12.5%) of human subjects carried S. aureus in their intestines, as determined by growth from faecal samples. Nasal
 carriage was similar in frequency (26/200; 13%), a result that is in accordance with previous findings showing a correlation between nasal and intestinal colonization\(^{32}\). These rates are considerably lower than those commonly found in adult populations during cross-sectional culture-based surveys that were performed mainly in hospital-admitted individuals in urbanized areas (on average, 20% for intestinal and 40% for nasal carriage)\(^{16,21,22}\).

To examine the hypothesis that bacterial interactions in the gut determine intestinal \textit{S. aureus} colonization, we first analysed the composition of the gut microbiome by 16S ribosomal RNA sequencing. However, we did not detect substantial differences in the composition of the microbiome between \textit{S. aureus} carriers and non-carriers (Extended Data Fig. 1).

By contrast, we found a striking correlation between the presence of \textit{Bacillus} bacteria and the absence of \textit{S. aureus}. \textit{Bacillus} species (mostly \textit{B. subtilis}; Extended Data Table 1) were found in 101/200 (50.5%) of subject samples. \textit{S. aureus} was never detected in faecal samples when \textit{Bacillus} species were present (\(P < 0.0001\), Fisher’s exact test; Fig. 1b).

Furthermore, this pathogen-exclusion effect was not limited to the site of interaction—the gut—but extended to \textit{S. aureus} colonization in a general fashion. While \textit{Bacillus} was generally absent from nasal samples, \textit{S. aureus} nasal colonization was never detected when intestinal \textit{Bacillus} was present (\(P < 0.0001\), Fisher’s exact test; Fig. 1c). Notably, the levels of \textit{S. aureus} colonization that we found in non-\textit{Bacillus}-colonized individuals from rural Thailand approximately match those reported—using similar culture-based assays—in urbanized Western areas. These findings indicate a widespread mechanism exerted by \textit{Bacillus} species that comprehensively inhibits colonization with \textit{S. aureus}. Moreover, they suggest that \textit{S. aureus} colonization is increased in urban populations because of the lack of a probiotic, \textit{Bacillus}-containing diet. Of particular note, the results also indicate that the intestinal site has a previously underappreciated role in determining general \textit{S. aureus} colonization, a notion in accordance with findings attributing a key role to faecal transmission in MRSA recolonization\(^{28}\).

When we analysed data from previous 16S rRNA-sequencing-based microbiome studies, we found strongly variant results and no correlation between the absence of \textit{S. aureus} and the presence of \textit{B. subtilis}: studies that reported considerable \textit{B. subtilis} or \textit{S. aureus} numbers (samples with more than 10% colonization by either species) did not reveal exclusion phenomena (average 14.89 ± 15.69% colonization by both species) (Extended Data Table 2). However, although we did not find a correlation, this might be due to the fact that such sequencing-based analyses are set up to detect high-order taxonomic shifts rather than specific differences on the species or genus level.

### Quorum sensing and colonization

Our results, which show no substantial high-order taxonomic differences in the microbiome composition between \textit{S. aureus} carriers and non-carriers, exclude an indirect effect of \textit{Bacillus} on the microbiome composition. Rather, we hypothesized that the \textit{Bacillus} isolates produce a substance that directly and specifically inhibits intestinal colonization by \textit{S. aureus}. We first analysed whether there is a growth-inhibitory effect of the \textit{Bacillus} isolates on \textit{S. aureus}. However, only a minor growth inhibition occurred in just 6 out of 105 isolates (we saw a maximal 1-mm inhibition zone when using an agar diffusion test with a five-times-concentrated culture filtrate). Therefore, a growth-inhibitory effect fails to explain the observed complete correlation between the presence of \textit{Bacillus} and the absence of \textit{S. aureus}, and rules out a bacteriocin-mediated phenomenon.

The factors that are important for \textit{S. aureus} intestinal colonization are poorly understood. One study in mice has implicated teichoic acids found in the bacterial cell wall, as well as the cell-surface protein clumping factor A (ClfA)\(^{29}\). Prompted by our previous finding that ClfA is positively regulated by the accessory gene regulator (Agr) quorum-sensing system\(^{30}\), we hypothesized that the \textit{Bacillus} isolates secrete a substance that interferes with quorum-sensing signalling. Quorum sensing is responsible for sensing the density of the bacterial population (the ‘quorum’) and controlling a concomitant alteration in cell physiology\(^{31}\). Because quorum-sensing signals and sensors differ between different types of bacteria\(^{31}\), an underlying quorum-quenching mechanism could explain the specificity of the inhibitory effect that we detected.

Because the role of quorum sensing in \textit{S. aureus} intestinal colonization is unknown, we first used a mouse model of \textit{S. aureus} intestinal colonization to test whether Agr-based quorum sensing is involved (Fig. 2a). In all mouse models in our study, we included: first, a human faecal isolate belonging to a sequence type (ST) that was frequently detected in the faecal isolates that we obtained (ST2196),...
Fig. 2 | Quorum-sensing dependence of \textit{S. aureus} intestinal colonization. \textbf{a}, Experimental set-up of the mouse intestinal colonization model. Mice received, by oral gavage, either 100 \(\mu\)l containing 10\(^4\) CFU ml\(^{-1}\) of wild-type (WT) \textit{S. aureus} strain ST2196 F12 and another 100 \(\mu\)l of 10\(^6\) CFU ml\(^{-1}\) of the corresponding isogenic \textit{agr} mutant (\(n = 5\) per group; competitive experiment, shown in \(b\)); or 200 \(\mu\)l containing 10\(^6\) CFU ml\(^{-1}\) wild-type, isogenic \textit{agr} mutant or \textit{Agr} (RNAIII)-complemented \textit{agr} mutant (\(n = 5\) per group; non-competitive experiment, shown in \(c\)). CFU in the faeces were determined two, four and six days after infection. At the end of the experiment (day seven), CFU in the small and large intestines were determined. \textbf{b}, Competitive experiment. Total obtained CFU are shown as dot plots; also shown are mean \(\pm\) s.d. Bars show the percentage of wild-type among total determined CFU, of which 100 were analysed for tetracycline resistance (which is present only in the \textit{agr} mutant). No \textit{agr} mutants were detected in any experiment;

according to multi-locus sequence typing (MLST) that we performed (Supplementary Table 1); second, a mouse infection isolate (ST88)\(^\text{32}\); and third, a human infection isolate of the highly virulent MRSA type USA300\(^\text{35}\). In competition experiments with equal amounts of wild-type and isogenic \textit{agr} mutant strains, only wild-type \textit{S. aureus} was detected in the faeces and colonized the large and small intestines at the end of the experiment (competition index \(\geq 100\)) (Fig. 2b and Extended Data Fig. 2a, b). Furthermore, in a non-competitive experimental set-up, only those bacteria expressing the intracellular \textit{Agr} effector RNAIII\(^\text{36}\) achieved colonization; \textit{agr}-negative control strains never did (Fig. 2c and Extended Data Fig. 2c). These data show that, in addition to its well-known role in infection\(^\text{30,34}\), the \textit{Agr} quorum-sensing system is absolutely indispensable for intestinal colonization.

\textbf{Fengycin quorum quenchers}  

Having established that the \textit{Agr} quorum-sensing regulatory system is essential for \textit{S. aureus} intestinal colonization, we next analysed whether culture filtrates of the \textit{Bacillus} isolates collected from human faeces can inhibit \textit{Agr}. To that end, we used an \textit{S. aureus} reporter strain, into the genome of which we had transferred the luminescence-conferring \textit{luxABCD} operon under the control of the \textit{Agr} P3 promoter\(^\text{34}\), which controls production of RNAIII. Remarkably, culture filtrates from all 105 isolates reduced \textit{Agr} activity in the \textit{S. aureus} reporter strain by at least 80\% (Fig. 3a and Extended Data Table 1). No growth effects were observed, substantiating that growth inhibition does not underlie the inhibitory phenotype. Furthermore, a culture filtrate from a reference \textit{B. subtilis} strain suppressed the production of key \textit{Agr}-regulated virulence factors (phenol-soluble modulins, \(\alpha\)-toxin and Panton–Valentine leucocidin; Fig. 3b, c and Supplementary Fig. 1). These results indicate that the inhibitory effect of the \textit{Bacillus} isolates on \textit{S. aureus} colonization is due to a secreted substance that inhibits \textit{Agr} signalling.

To characterize the \textit{Agr}-inhibitory substance(s), we performed experiments with culture filtrate of the reference \textit{B. subtilis} strain. We found that the substance in question was thermostable and resistant to protease digestion (Extended Data Fig. 3a). In reversed-phase high-performance chromatography (RP-HPLC) (Extended Data Fig. 3b), substantial \textit{Agr}-inhibiting activity was associated with two peaks, which we analysed by RP-HPLC/electrospray ionization mass spectrometry (ESI-MS) (Extended Data Fig. 3c). This analysis, together with the elution behaviour and published literature\(^\text{36}\), allowed us to identify the \textit{Agr}-inhibiting substances as members of the fengycin cyclic lipopeptide family. Because fengycins can differ in specific amino acids and in the length of the attached fatty acid, which usually is \(\beta\)-hydroxylated (\(\beta\)-OH), and because different \textit{Bacillus} strains produce different fengycin species\(^\text{37}\), we used further tandem mass spectrometric fragmentation analysis (MS/MS) to identify the specific fengycins present in the two active peaks (Extended Data Fig. 3d). Fengycins in the first peak were identified as \(\beta\)-OH-C17-fengycin A and \(\beta\)-OH-C16-fengycin B. The second peak consisted of one fengycin species, \(\beta\)-OH-C17-fengycin B. According to RP-HPLC/ESI-MS analysis, smaller, adjacent peaks also contained fengycin species, which we tentatively identified as \(\beta\)-OH-C17-fengycin A and the dehydroxylated versions of the identified three major fengycins (Extended Data Fig. 3e). For further analyses, we purified higher amounts of \(\beta\)-OH-C17-fengycin B to homogeneity from culture filtrate and verified the dose-dependent \textit{Agr}-inhibiting activity of this pure substance (Extended Data Fig. 4).

Using RP-HPLC/ESI-MS analysis, we found fengycin production in all isolates, substantiating the general character of the inhibitory interaction (Extended Data Table 1). Although the production pattern of different fengycins varied between the analysed isolates, in many of them \(\beta\)-OH-C17-fengycin B was the most strongly produced type. Notably, almost complete inhibition of \textit{Agr} was detected at a concentration of about 1.4 \(\mu\)M total fengycin (Fig. 3d). This corresponds to the median concentration of total fengycin (1.5 \(\mu\)M) produced by stationary-phase cultures of the \textit{Bacillus} isolates (Fig. 3e).
To provide definitive evidence that fengycin production underlies the Agr-inhibiting capacity of Bacillus, we produced an isogenic mutant in the reference B. subtilis strain of the fenA gene, which is essential for fengycin production.\(^8\) RP-HPLC/ESI-MS showed a specific absence of fengycins in that mutant strain, whereas surfactins—the predominant Bacillus lipopeptides—were still present (Extended Data Fig. 3f). Culture filtrate of the fenA mutant strain was devoid of Agr-inhibiting activity, in contrast to that of the isogenic wild-type strain (Fig. 3f). We also measured an isogenic surfactin-negative mutant strain, which showed Agr-inhibiting activity similar to that of the wild-type strain (Fig. 3f). These results confirmed that fengycin production is the source of the observed Agr inhibition.
found complete Agr inhibition (Fig. 3d). These findings indicate that fengycins inhibit Agr signal transduction by efficient competitive inhibition as structural analogues of AIPs.

The fact that AgrC–AIP interaction differs according to Agr subtype raises the question of whether fengycins have a general ability to inhibit Agr. We found that purified β-OH-C-17 fengycin B inhibited Agr in members of all S. aureus subtypes, as well as in S. epidermidis (Fig. 4e). Furthermore, the S. aureus strains used in our mouse experiments belong to different Agr subtypes (strain USA300, type I; strain ST88, type III; strain ST2196, type I). These results indicate that fengycins have broad-spectrum Agr-inhibiting activity.

**Bacillus spores eradicates S. aureus**

To validate our findings in vivo and demonstrate the specific role of fengycins in the inhibition of S. aureus intestinal colonization, we compared the impact of the B. subtilis wild-type reference strain and its isogenic fenA mutant on S. aureus colonization in a mouse intestinal colonization model. We first performed a control experiment to analyse the colonization kinetics of B. subtilis when given as spores, which corresponds to the form in which Bacillus would be taken up with food or probiotic formulae (Extended Data Fig. 5b). We observed transient colonization that strongly declined within two days. Importantly, colonization by the B. subtilis fenA mutant was not different to that by the wild-type strain, ruling out the possibility that fengycin production as such affects B. subtilis colonization.

Feeding mice B. subtilis spores completely abrogated colonization of all tested S. aureus strains in the faeces and intestines, in experimental set-ups with or without antibiotic pretreatment to eliminate the pre-existing microbiota. (Fig. 5b, c and Extended Data Fig. 5c–f). By contrast, spores of the fenA mutant had no notable effect on colonization of any S. aureus test strain. As Bacillus intestinal colonization in humans has been shown to reach much higher levels than that by S. aureus⁴—a situation likely to be even more pronounced in the tested rural population—our mouse data obtained with S. aureus numbers approximately equal to or exceeding those of applied Bacillus spores suggest that fengycin-mediated interference in quorum sensing contributes to the exclusion of S. aureus colonization that we observed in humans.

**Conclusions**

Scientific evidence to support the frequent claims that probiotic nutrients improve human health is scarce. However, this study provides evidence for a molecular mechanism by which probiotic bacteria found in food could directly interfere with pathogen colonization. In particular, our data underscore the often-debated probiotic value of B. subtilis. Notably, we found the responsible agents to work by quorum quenching, demonstrating that pathogen exclusion in the gut may work by inhibition of a pathogen signalling system. Furthermore, our findings emphasize the importance of quorum sensing for pathogen colonization.

Our study suggests several valuable translational applications regarding alternative strategies to combat antibiotic-resistant S. aureus. First, the quorum-quenching fengycins—which previously had been known only for their antifungal activity—could potentially be used as quorum-sensing blockers in eagerly sought antivirulence-based efforts to treat staphylococcal infections. Second, Bacillus-containing probiotics could be used for simple and safe S. aureus decolonization strategies. In that regard, it is particularly noteworthy that our human data indicate that probiotic Bacillus can comprehensively eradicate intestinal as well as nasal S. aureus colonization. Such a probiotic approach would have numerous advantages over the present standard topical strategy involving antibiotics, which is aimed exclusively at decolonizing the nasal cavity.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0616-y.
To construct the agrBD deletion mutant of strain LAC P3-lux, we used a 4.8-kilobase PCR product from USA300 genomic DNA that included the agrBDCA operon as well as 1 kb upstream and 1 kb downstream; we cloned this product into the Smal site of plasmid pIMAY64 and used inverse PCR to delete agrBD. Alleric exchange was then performed, and the chromosomal deletion was confirmed by PCR using one primer outside of the 1-kb homology arm, followed by sequencing of the PCR product. See Supplementary Table 2 for the oligonucleotides used.

In order to construct the tetracycline-resistant derivatives of S. aureus ST88 and ST21196, we carried out pI1-phage-mediated transduction as described in order to transfer the tetracycline cassette in the donor strain (S. aureus RN4220 with integrated pL92) to S. aureus strains ST88 and ST19665.

To construct the B. subtilis fengycin mutant strain, SPP1-phage-mediated transduction66 was performed to transfer the fena deletion present in the donor strain (BKE18340, a fenA/pfpsA-cemm in B. subtilis strain 168 obtained from the Bacillus Genetic Stock Center) to B. subtilis strain ZK3814. This was necessary as B. subtilis strain 168 bears a mutation in the sfp gene, abolishing lipopeptide production.

Bacteria were generally grown in tryptic soy broth (TSB) with shaking unless otherwise indicated.

**Typing of S. aureus isolates.** S. aureus isolates were typed by MLST as described44, PCR amplification of seven S. aureus housekeeping genes (arcC, 16S rRNA, gmrG, gmk, tpi, tpi1 and yqiL) were obtained from chromosomal DNA and their sequences compared with those available from the PubMLST database (https://pubmlst.org/saureus/). Previously undescribed alleles (arcC 520–521 and gmk 337) and sequence types (ST4630–ST5638) were deposited to the website. The Agr subtype of S. aureus strains was determined using a modified multiplex quantitative reverse transcription PCR (qRT–PCR) protocol67. Two duplex qRT–PCR protocols, using the respective described primer sets and two coloured probes each, were set up for Agr types I and II, and III and IV, respectively. Isolates for which the Agr type could not be determined by that method were analysed for the type of AIP production using RP-HPLC/ESI-MS with the chromatography method also used for PSM detection (see below), integrating the three major ms2 peaks for each AIP type.

**Microbiome analysis.** Genomic DNA from each faecal sample was extracted using a QIAamp DNA stool MiniKit (Qiagen) according to the manufacturer’s instructions. The DNA was quantified using a Nanodrop spectrophotometer, and 16S rDNA paired-end sequencing of the V4 region of 16S rDNA was performed by Illumina using an Illumina MiSeq system as described68.

For all obtained paired-end sequences, the abundance of operational taxonomic units (OTUs) and alpha and beta diversity were identified using quantitative insights into microbial ecology (QIIME 1.1.9)69. This study used the Nephle (release 1.6) platform from the National Institute of Allergy and Infectious Diseases (NIAID) Office of Cyber Infrastructure and Computational Biology (OCICB) in Bethesda, Maryland, USA. The sequences were assigned to OTUs with the QIIME’s usearch function using a 97% identity cut-off and a maximum of 1000 iterations. The chloroplast reference set60 at 99% similarity. Alpha diversity was calculated using Chao1 and Shannon analyses64 and compared across groups using a non-parametric t-test with 999 permutations.

**Growth-inhibition analysis.** Growth inhibition of S. aureus by Bacillus culture filtrates was tested with an agar diffusion assay. To that end, 10 μl of Bacillus culture filtrate from each isolate was spotted on sterile filter disks. The filters were left to dry and the procedure was repeated four times, after which filters were laid on agar plates containing S. aureus, resulting in the analysis of five-times concentrated culture filtrate.

**Fengycin purification.** To identify the Agr-inhibiting active substance, 10 ml of culture filtrate from the B. subtilis reference strain grown for 48 h in TSB were applied to a Zorbax SB-C18 9.4 mm × 25 cm reversed-phase column (Agilent) using an AKTA Purifier 100 system (GE Healthcare). After washing with three column volumes of 100% buffer A (0.1% trifluoroacetic acid (TFA) in water) and five column volumes of 30% buffer B (0.1% TFA in acetonitrile), a 20-column volume gradient from 30% to 100% buffer B was applied. The column was run at a flow rate of 3 ml min⁻¹. Peak fractionation was performed using the absorbance of 214 nm, and fractions were subjected to further analysis by RP-HPLC/ESI-MS and MS/MS and tested for Agr inhibition (see below).

To purify larger amounts of the main active peak containing 3-OH-C17–fengycin B, we added acetonitrile to 200 ml filtrate from cultures grown under the same conditions to a final concentration of 10%; precipitated material was removed by centrifugation for 10 min at 3,700g using a Sorvall Legend RT centrifuge, and the obtained cleared supernatant was applied to a self-packed HR 16/10 column filled with Resource Phe (GE Healthcare) material (column volume 17 ml). After sample application, the column was washed with 10% buffer B for three column volumes followed by a 20-column volume wash step with 35% buffer B and 25% buffer B. Five column volumes with a gradient of 15 column volumes from 25% to 60% buffer B was applied. We collected 10-ml fractions and lyophilized positive fractions (as determined by RP-HPLC/ESI-MS). The lyophilisate was redisolved in 2 ml acetonitrile. We added 6 ml of water and...
removed the precipitated material through a 5-min centrifugation in a table-top centrifuge at maximum speed. The cleared supernatant was then further purified on a Zorbas SB-C18 9.4 mm × 25 cm reverse-phase column as described above. PSM and lipopeptide detection by RP-HPLC/ESI-MS. PSMs were analysed by RP-HPLC/ESI-MS using an Agilent 1260 Infinity chromatography system coupled to a 6120 Quadrupole LC/MS in principle as described88, but with a shorter column and a method that was adjusted accordingly. A 2.1 mm × 5 mm Perkin-Elmer SPP C8 (2.7 μm) guard column was used at a flow rate of 0.5 ml min−1. After sample injection, the column was washed for 0.5 min with 90% buffer A and 10% buffer B, then for 3 min with 25% buffer B. Next, an elution gradient was applied from 25% to 100% buffer B in 2.5 min, after which the column was subjected to 2.5 min of 100% buffer B to finalize elution.

Bacillus culture filtrates or (partially) purified fractions containing lipopeptides (fengycins and surfactins) were analysed using the same column, system and elution conditions. To quantify the production of different fengycins, we used the two most abundant peaks, corresponding to double- and triple-charged ions, for the integration. Agilent mass hunter quantitative analysis version B.07.00 was used for quantification.

Measurement of Agr activity. To determine the Agr-inhibiting activity of Bacillus culture filtrates or purified fractions, we measured luminescence emitted by an Agr P3 promoter–luxABCD reporter fusion construct that was inserted into the genome of S. aureus strain LAC44. Strain LAC P3–luxABCD was diluted 100-fold from a preculture grown overnight in TSB before distribution into a 96-well microtitre plate. To 100 μl of that dilution, we added 100 μl of sterilized culture filtrate sample, unless otherwise indicated. Plates were incubated at 37 °C with shaking. Luminescence was measured with a GloMax Explorer luminometer (Promega) every 2 h for a total of 6 h. Inhibition was considered significant if the 4-h sample and control values differed by at least a factor of two. Of note, the quorum-quenching effect exerted by the one-time initial dose of fengycin or fengycin-containing culture filtrates was transient and was overcome at later times by the increasing intrinsic AIP production. The Agr-inhibiting activity of purified fengycin was also measured using quantitative real-time PCR of RNAIII as described88.

To determine the Agr-inhibiting activity with target strains other than LAC (Agr subtype I), we measured the production of h-toxin, for which the gene is embedded in the Agr intracellular effector RNA, RNAIII, in most staphylococci. Promega of h-toxin was measured by RP-HPLC/ESI-MS as described above. Strains LAC (Agr subtype I), A950085 (Agr subtype II), MW2 (Agr subtype III) and A970377 (Agr subtype IV) were used for testing the effect of β-OH-C17-fengycin B on S. aureus of different Agr subgroups. Strain 1457 was used for S. epidermidis. All strains were diluted 100-fold from a preculture grown in TSB, β-OH-C17-fengycin B dissolved in dimethylsulfoxide (DMSO) was added to each sample to a final concentration of 20 μM and 100 μM. All samples were incubated at 37 °C with shaking for 4 h. Samples were centrifuged and supernatant was collected for RP-HPLC/ESI-MS detection.

Analysis of PVL and α-toxin expression. S. aureus strain LAC was diluted 100-fold from a preculture grown in TSB and inoculated into 500 μl TSB. Then, 250 μl of B. subtilis culture filtrate was added into the sample. Samples were incubated at 37 °C with shaking for 4 h. Samples were centrifuged and supernatant was collected for RP-HPLC/ESI-MS detection.

Preparation of Bacillus spores. B. subtilis wild-type or isogenic fengycin mutant strains were inoculated from a preculture (1:100) into 1 litre of 2× SG medium63 and allowed to sporulate for 96 h. Cells were pelleted, washed with water, and resuspended in 20% metrizic acid (Sigma). Five different concentrations (w/v) of metrizic acid (60% to 20%) were added stepwise to a 50-ml centrifuge tube to obtain a density gradient. A cell suspension was added to the top of the gradient, and was followed by centrifugation at 40,000 g for 60 min at 4 °C (as described previously88). Spores were found in the middle layers and were collected. They were washed three times with 10 ml water. The total obtained number of viable spores per ml was counted by serial dilution, plating on TSA and counting of CFU. The total number of heat-resistant spores per ml was determined by submerging the spores in a water bath at 80 °C for 20 min, followed by serial dilution and quantification of CFU per ml as described above.

Mouse intestinal colonization model. In vivo studies were approved by the Institutional Animal Care and Use Committee of the NIAID. Animal work was conducted by certified staff in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All of the animal work adhered to the institution’s guidelines for animal use and followed the guidelines and basic principles in the US Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals.

All C57BL/6J mice were female and six to eight weeks of age at the time of use. In one set-up, before S. aureus was given by oral gavage, mice were pretreated to eradicate the pre-existing intestinal microbiota using an antibiotic mix consisting of ampicillin (1 g l−1), metronidazole (1 g l−1), neomycin trisulfate (1 g l−1) and vancomycin (1 g l−1) in the drinking water. The last day before gavage, antibiotics were omitted from the drinking water. No bacteria could be found in the faeces or intestines of mice for seven days after this treatment in a control experiment. In another set-up, antibiotic pretreatment was omitted. In all set-ups, S. aureus strains were given in an exponential growth phase, washed, and resuspended in sterile phosphate-buffered saline (PBS) at 10⁷ CFU ml−1. Mice were inoculated by oral gavage with 200 μl of a 10⁷ CFU ml−1 suspension of the indicated S. aureus strains, or 1:1 mixtures of wild-type and isogenic agr mutants to reach the same final concentration and volume. For the experiments with strains containing plasmids of the pKX2 type, mice received kanamycin (0.2 g l−1) in the drinking water during the experiment to maintain plasmids. For the B. subtilis spore inhibition experiment, oral gavage with 200 μl of spores of wild-type Bacillus or its isogenic Δfengycin mutant (10⁴ CFU ml−1 sterile PBS) was performed on the following day. In the experiments without antibiotic pretreatment, extracts were plated on TSA plates containing 4 μg ml−1 oxacillin (for strain USA300 LAC) or 3 μg ml−1 tetracycline (for tetracycline-resistant derivatives of strains ST88 and ST2196), incubated for 48 h at 37 °C, and enumerated.

Statistics. Statistical analysis was performed using GraphPad Prism version 6.05 with one-way or two-way ANOVA, or Fisher’s exact test, as appropriate, except for the experiments shown in Figs. 2c, 5b, c, and Extended Data Figs. 2b, c, 5c–f, for which Stata Release 15 and Poisson regression were used, owing to the exclusive presence of 0 values in one group (no variance). For ANOVAs, Tukey post-tests were used, which correctly perform multiple comparisons using statistical hypothesis testing. All data show the mean and standard deviation (s.d.). All replicates are biological.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability Microbiome sequencing data are available from Bioproject with accession number 483343. All other data generated or analysed during this study are included in the published Article or in the Supplementary Information.

46. Miranda, C. A., Martins, O. B. & Clementino, M. M. Species-level identification of Bacillus strains isolates from marine sediments by conventional biochemical, 16S rRNA gene sequencing and inter-tRNA gene sequence lengths analysis. Antonie van Leeuwenhoek 93, 297–304 (2008).
47. Carrel, M., Perencevich, E. N. & David, M. Z. USA300 methicillin-resistant Staphylococcus aureus, United States, 2000–2013. Emerg. Infect. Dis. 21, 1973–1980 (2015).
48. Wang, R. et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA, Nat. Med. 13, 1510–1514 (2007).
49. Gauger, T. et al. Intracellular monitoring of target protein production in Staphylococcus aureus by peptide tag-induced reporter fluorescence. Microbiol. Biotechnol. 5, 129–134 (2012).
50. Queck, S. V. et al. RNAII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. Mol. Cell. 32, 150–158 (2008).
51. Monk, I. R., Shah, I. M., Xu, M., Tan, M. W. & Foster, T. J. Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis. MBio 3, e00277–11 (2012).
52. Luong, T. T. & Lee, C. Y. Improved single-copy integration vectors for Staphylococcus aureus. J. Microbiol. Methods 70, 186–190 (2007).
53. Yasbin, R. E. & Young, F. E. The production of Bacillus subtilis by bacteriophage SP1. J. Virol. 14, 1343–1348 (1974).
54. Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J. & Spratt, B. G. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *J. Clin. Microbiol.* **38**, 1008–1015 (2000).

55. François, P. et al. Rapid *Staphylococcus aureus* agr type determination by a novel multiplex real-time quantitative PCR assay. *J. Clin. Microbiol.* **44**, 1892–1895 (2006).

56. Caporaso, J. G. et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **6**, 1621–1624 (2012).

57. Caporaso, J. G. et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335–336 (2010).

58. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461 (2010).

59. Rideout, J. R. et al. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* **2**, e545 (2014).

60. McDonald, D. et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **6**, 610–618 (2012).

61. Lozupone, C. A., Hamady, M., Kelley, S. T. & Knight, R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* **73**, 1576–1585 (2007).

62. Joo, H. S. & Otto, M. The isolation and analysis of phenol-soluble modulins of *Staphylococcus epidermidis*. *Methods Mol. Biol.* **1106**, 93–100 (2014).

63. Nicholson, W. L. & Setlow, P. in *Molecular Biological Methods for Bacillus* (eds Harwood, C. R. & Cutting, S. M.) 391–450 (John Wiley, Chichester, 1990).

64. Fukushima, T. et al. Characterization of a polysaccharide deacetylase gene homologue (*pdaB*) on sporulation of *Bacillus subtilis*. *J. Biochem.* **136**, 283–291 (2004).
Extended Data Fig. 1 | Microbiome analysis of *S. aureus* carriers versus non-carriers. The microbiota of \( n = 20 \) randomly selected *S. aureus* carriers (red) and \( n = 20 \) non-carriers (blue) were analysed in faecal samples. a–c, Rarefaction (species-richness) curves based on 16S rRNA gene sequences. Data are mean ± s.d. a, Shannon index. b, Observed species. c, Chao1 index. d, Comparison of relative taxa abundance between *S. aureus* carriers (red) and non-carriers (blue). e, f, Beta diversity, represented by a principal coordinate analysis plot based on unweighted UniFrac (e) and weighted UniFrac (f) metrics for samples from *S. aureus* carriers (red) and non-carriers (blue).
Extended Data Fig. 2 | Quorum-sensing dependence of *S. aureus* intestinal colonization. Data from strains USA300 LAC and ST88 JSNZ. The experimental set-up is the same as in Fig. 2: mice received by oral gavage either 100 μl containing 10^8 CFU ml^-1 of wild-type *S. aureus* strain USA300 LAC or ST88 JSNZ plus another 100 μl of 10^8 CFU ml^-1 of the corresponding isogenic agr mutant (*n* = 5 per group; competitive experiment shown in a, b); or 200 μl containing 10^8 CFU ml^-1 wild-type, isogenic agr mutant or Agr (RNAIII)-complemented agr mutant (*n* = 5 per group, non-competitive experiment shown in c). CFU in the faeces were determined two, four and six days after infection. At the end of the experiment (day seven), CFU in the small and large intestines were determined. a, b, Competitive experiment. Total obtained CFU are shown as dot plots; also shown are mean ± s.d. Bars show the percentage of wild-type among total determined CFU, of which 100 were analysed for tetracycline resistance that is present only in the agr mutant. No agr mutants were detected in any experiment; thus, all bars show 100%. Given that 100 isolates were tested, the competitive index wild-type/agr mutant in all cases is ≥100. c, Non-competitive experiment with genetically complemented strains. Wild-type and isogenic agr mutant strains all harboured the pKX316 control plasmid; Agr-complemented strains harboured pKX3RNAIII and thus constitutively expressed RNAIII, which is the intracellular effector of Agr. During the experiment, mice received 200 μg ml^-1 kanamycin in their drinking water to maintain plasmids. Statistical analysis was performed using Poisson regression versus values obtained with the agr mutant strains. *P* < 0.0001. Data are mean ± s.d. Note that no bacteria were found in the faeces or intestines of any mouse receiving *S. aureus Δagr* with vector control. The corresponding zero values are plotted on the *x* axis of the logarithmic scale.
Extended Data Fig. 3 | Analysis of Agr-inhibitory substances.

a, Influence of heat and proteases on Agr inhibition. *B. subtilis* culture filtrate was subjected to heat (95 °C for 20 min) or digestion with proteinase K (50 μg ml⁻¹, 37 °C, 1 h) and the effect on inhibition of Agr activity was measured using the luminescence assay with the USA300 P3–luxABCDE reporter strain (see Fig. 3a). RLU, relative light units. The experiment was performed with *n* = 2 independent biological samples. Lines connect the means. (The observed additional suppression of Agr activity in the proteinase-K-treated sample at 6 h, compared with the *B. subtilis* culture filtrate sample, is expected owing to proteolytic inactivation of intrinsic AIP.)
b, Preparative RP chromatography of *B. subtilis* culture filtrate to determine the Agr-inhibiting substance. The peaks labelled 2 and 3 showed substantial Agr-inhibiting activities in the Agr-activity assay and were identified as fengycins using subsequent RP-HPLC/ESI-MS and MS/MS analysis (see c, d). The peaks labelled 1 and 4–6 also contained fengycin species (see e). AU, arbitrary units. The applied gradient (% buffer B) is shown in green.
c, Fractions corresponding to Agr-inhibitory peaks 2 and 3 from the preparative RP run (b) were subjected to RP-HPLC/ESI-MS. Top, total ion chromatograms (TICs) of the RP-HPLC/ESI-MS runs; bottom, ESI mass spectrogram of the major peaks. d, MS/MS analysis of the peak 2 and 3 fractions. Peaks that are characteristic of a given fengycin subtype (A or B in this case) are marked in colour. ‘Parent’ refers to the relevant numbered peak in the spectrograms above.
e, Analysis of further fengycin-containing fractions. Peaks 1, 4, 5 and 6 from the preparative RP run (b) were also found to contain fengycin species as determined by subsequent RP-HPLC/ESI-MS analysis. Shown are the mass spectrograms of the major peaks of those runs and the tentative characterization for fengycin type. The preparative and analytical chromatography and RP-HPLC/ESI-MS analyses (as shown in b, d) were repeated multiple (more than ten) times for fengycin purification, with similar results. MS/MS analyses were not repeated.
f, Analysis of fengycin and surfactin lipopeptide expression by the *B. subtilis* wild-type strain and its isogenic ΔfenA mutant.
Extended Data Fig. 4 | Assessment of purity and functionality of purified β-OH-C17-fengycin B. a, RP-HPLC run. b, Agr inhibition at different concentrations in the luminescence assay. RLU, relative light units. Statistical analysis was by two-way ANOVA with Tukey’s post-test. Comparisons shown are those versus DMSO control. c, Agr inhibition as measured by inhibition of expression of RNAIII by qRT–PCR. *P < 0.0001 (one-way ANOVA with Tukey’s post-test; comparisons shown are those versus 0μM value). The experiments in b, c were performed with n = 3 independent biological samples. Data are mean ± s.d.
Extended Data Fig. 5 | Inhibition of *S. aureus* colonization by dietary fengycin-producing *Bacillus* spores in a mouse model. a, Concentration of AIP-I during *S. aureus* growth. Strain LAC (USA300) was grown in TSB, and AIP-I concentrations were measured by RP-HPLC/ESI-MS. Calibration was performed using synthetic AIP-I. The detection limit of this assay is around 0.3 μM. The experiment was performed with *n* = 3 independent biological samples. Data are mean ± s.d. b, *B. subtilis* colonization kinetics in the mouse intestinal colonization experiment. Mice (*n* = 5) received 200 μl of a 10⁸ CFU ml⁻¹ suspension of wild-type *B. subtilis* or ΔfenA mutant spores by oral gavage; CFU in the faeces were analysed up to five days afterwards. Data are mean ± s.d. c–f, Inhibition mouse model with strains USA300 LAC and ST88 JSNZ. The experimental set-up was as shown in Fig. 5a. In brief, *n* = 4 or 5 mice per group received 200 μl of 10⁸ CFU ml⁻¹ *S. aureus* strains USA300 LAC or ST88 JSNZ by oral gavage. On the next day and every following second day, the mice received 200 μl of 10⁸ CFU ml⁻¹ spores of wild-type *B. subtilis* or its isogenic fenA mutant, also by oral gavage. CFU in the faeces were determined two, four and six days after infection. At the end of the experiment (day seven), CFU in the small and large intestines were determined. The experiment was performed with (c, d) or without (e, f) antibiotic pretreatment. Statistical analysis was performed using Poisson regression versus values obtained with wild-type *B. subtilis* spore samples. *P* < 0.0001. Data are mean ± s.d. Note that no *S. aureus* were found in the faeces or intestines of any mouse challenged with any *S. aureus* strain that also received *Bacillus* wild-type spores. The corresponding zero values are plotted on the x axis of the logarithmic scale.
### Extended Data Table 1 | Fengycin production and Agr-inhibition potency of *Bacillus* faecal isolates

| Bacillus species | Fengycin production (nM) | Agr-inhibition potency (%) |
|-----------------|--------------------------|---------------------------|
| *B. subtilis*   | 100                      | 50                        |
| *B. amyloliquefaciens* | 200                    | 50                        |
| *B. cereus*     | 300                      | 50                        |
| *B. megaterium* | 400                      | 50                        |
| *B. stearothermophilus* | 500                  | 50                        |
| *B. thuringiensis* | 600                    | 50                        |
| *B. subtilis*   | 700                      | 50                        |
| *B. amyloliquefaciens* | 800                    | 50                        |
| *B. cereus*     | 900                      | 50                        |
| *B. megaterium* | 1000                     | 50                       |
| *B. stearothermophilus* | 1100                   | 50                       |
| *B. thuringiensis* | 1200                   | 50                       |

**Notes:**
- *Bacillus* species were determined by sequencing 16S rRNA encoding DNA, as specified in the Methods.
- The percentage of Agr inhibition was determined by dividing the 4-h value obtained in the luminescence assay for the sample (using 100 μl of culture filtrate) by that obtained for the control, and multiplying by 100.
Extended Data Table 2 | Analysis of previous microbiome studies for correlation between the presence of *S. aureus* and *B. subtilis* in the human intestinal tract

| Study ID | Study Name                                                                 | Samples | Only *B. subtilis* | Only *S. aureus* | Both | Neither |
|----------|-----------------------------------------------------------------------------|---------|-------------------|------------------|------|---------|
| ERP012803 | American Gut Project                                                        | 6635    | 1 (0.015%)        | 304 (4.58%)      | 0    | 6330 (95.4%) |
| ERP011001 | Human gut bacteria that rescue growth and metabolic defects transmitted by microbiota from undernourished children 16S sequencing of Malawian children | 1732    | 408 (23.61%)      | 70 (4.05%)       | 71 (4.11%) | 1179 (68.23%) |
| ERP005437 | 16S sequencing of Malawian children                                           | 1515    | 118 (7.79%)       | 6 (0.4%)         | 4 (0.26%) | 1387 (91.55%) |
| SRP049113 | Human gut microbiota from the ALADDIN study                                  | 664     | 2 (0.30%)         | 61 (9.19%)       | 7 (1.05%) | 594 (89.46%) |
| ERP019564 | Role of Gut Microbiota in Pathophysiology of Parkinson's Disease             | 481     | 8 (1.66%)         | 7 (1.45%)        | 0    | 466 (96.88%) |
| SRP073172 | DNA from FIT can replace stool for microbiota-based colorectal sequencing   | 408     | 63 (15.44%)       | 71 (17.40%)      | 99 (24.26%) | 175 (42.89%) |
| SRP068240 | Human feces metagenome 16s rDNA sequencing                                   | 350     | 52 (14.85%)       | 189 (54%)        | 89 (25.43%) | 20 (5.71%) |
| SRP064846 | Homo sapiens fecal microbiome transplant                                     | 271     | 20 (7.38%)        | 47 (17.34%)      | 6 (2.21%) | 198 (73.06%) |
| SRP065497 | Human gut environment Targeted loci environmental                            | 270     | 54 (20%)          | 8 (2.96%)        | 19 (7.04%) | 189 (70%) |
| ERP021093 | Gut microbiome from patients obtained by 16s rRNA sequencing                | 268     | 88 (32.84%)       | 14 (5.22%)       | 57 (21.27%) | 109 (40.67%) |
| ERP010229 | Gut microbial succession follows acute secretory diarrhea in humans         | 260     | 12 (4.62%)        | 92 (35.38%)      | 122 (46.92%) | 34 (13.08%) |
| ERP010458 | Gut microbiota of stroke patients differentiates from healthy controls       | 233     | 3 (1.29%)         | 32 (13.73%)      | 4 (1.72%) | 194 (83.26%) |

We included in our analysis all studies found on the EBI Metagenomics website (https://www.ebi.ac.uk/metagenomics/) that had more than 200 participants (independent samples) and which used Illumina Miseq instruments. We pooled raw 16S rRNA sequencing data from the EBI Metagenomics website, and used taxonomic assignment (TSV) files for analysis. The number of sequence reads was used to analyze how many samples contained *S. aureus* or *B. subtilis*. Samples with a read number of more than 0 were defined as colonized. When there were no reads, samples were designated as noncolonized.
Experimental design

1. Sample size
Describe how sample size was determined.

Sample sizes for in-vitro experiments, usually n=3, were chosen as they are common for experiments of that type. Sample sizes for animal experiments were chosen according to preliminary pilot studies using n=5 animals. The sample size for the human colonization experiment (n=200) was chosen based on published Staphylococcus aureus colonization rates.

2. Data exclusions
Describe any data exclusions.

No data were excluded.

3. Replication
Describe whether the experimental findings were reliably reproduced.

When experiments were repeated in the same fashion, they yielded comparable results.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

This study only contains one study with humans subject, which only formed one group to be analyzed. There were no treatment/control or other group distinctions that would require randomization. Random selection of samples to be analyzed in the microbiome study was performed by a blinded person.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

No blinding was used.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

Confirmed

☐ ☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

☐ ☐ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ ☐ A statement indicating how many times each experiment was replicated

☐ ☐ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

☐ ☐ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

☐ ☐ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

☐ ☐ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

☐ ☐ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Details of mice used are described in methods. All C57BL/6J mice were female and six to eight weeks of age at the time of use.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

described in methods:

All participants were over 20 years old (age range: 20-87, median 57 ±14.5; 131 women and 69 men) and without history of intestinal disease. None had received any antibiotic treatment or stayed at a hospital within at least three months prior to the study.