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Pathogen elimination by probiotic *Bacillus* via signalling interference

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Probiotic nutrition is frequently claimed to improve human health. In particular, live probiotic bacteria obtained with food are thought to reduce intestinal colonization by pathogens, and thus to reduce susceptibility to infection. However, the mechanisms that underlie these effects remain poorly understood. Here we report that the consumption of probiotic *Bacillus* bacteria comprehensively abolished colonization by the dangerous pathogen *Staphylococcus aureus* in a rural Thai population. We show that a widespread class of *Bacillus* lipopeptides, the fengycins, eliminates *S. aureus* by inhibiting *S. aureus* quorum sensing—a process through which bacteria respond to their population density by altering gene regulation. Our study presents a detailed molecular mechanism that underlines the importance of probiotic nutrition in reducing infectious disease. We also provide evidence that supports the biological significance of probiotic bacterial interference in humans, and show that such interference can be achieved by blocking a pathogen's signalling system. Furthermore, our findings suggest a probiotic-based method for *S. aureus* decolonization and new ways to fight *S. aureus* infections.

There is increasing appreciation of the key role that the intestinal microbiota play in preventing the colonization and overgrowth of pathogens[1,2]. 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[2–5]. 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[2], and it has been shown that a bacteriocin-producing *Escherichia coli* strain inhibits colonization by related pathogenic bacteria in the inflamed gut of mice[6]. 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 the high temperatures encountered during cooking procedures. *Bacillus* spores are commonly ingested with vegetables[7]. They can subsequently germinate to form metabolically active, vegetative cells[8], which can temporarily colonize the intestinal tract[9]. 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 10^8 CFU per gram[7]. Several probiotic formulae contain *Bacillus* species[10], which are thought to reduce pathogen colonization by mechanisms that—except for a described immune-stimulatory effect on epithelial cells[11]—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[12]. Treatment of *S. aureus* infections is severely complicated by antibiotic resistance[13], such as in methicillin-resistant *S. aureus* (MRSA), and there is no working *S. aureus* vaccine[14]. Therefore, alternative strategies to combat *S. aureus* infections are eagerly sought[15]. Because *S. aureus* infections commonly originate from previous asymptomatic colonization[16,17], decolonization has recently gained considerable attention as a possible means to fight *S. aureus* infections in a preventive manner[18]. While the nares (nostrils) have traditionally been considered the primary *S. aureus* colonization site[19], there is increasing evidence that the intestinal tract is also commonly colonized by *S. aureus*[20–22] and forms an important reservoir for outbreaks of infectious *S. aureus* disease[23,24]. Several studies have reported levels of *S. aureus* in the faeces of human adults of around 10^3–10^4 CFU per gram[25–27]. Possibly, intestinal *S. aureus* colonization explains the failure of previous topical decolonization efforts aimed solely at the nose[16,22,28].

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

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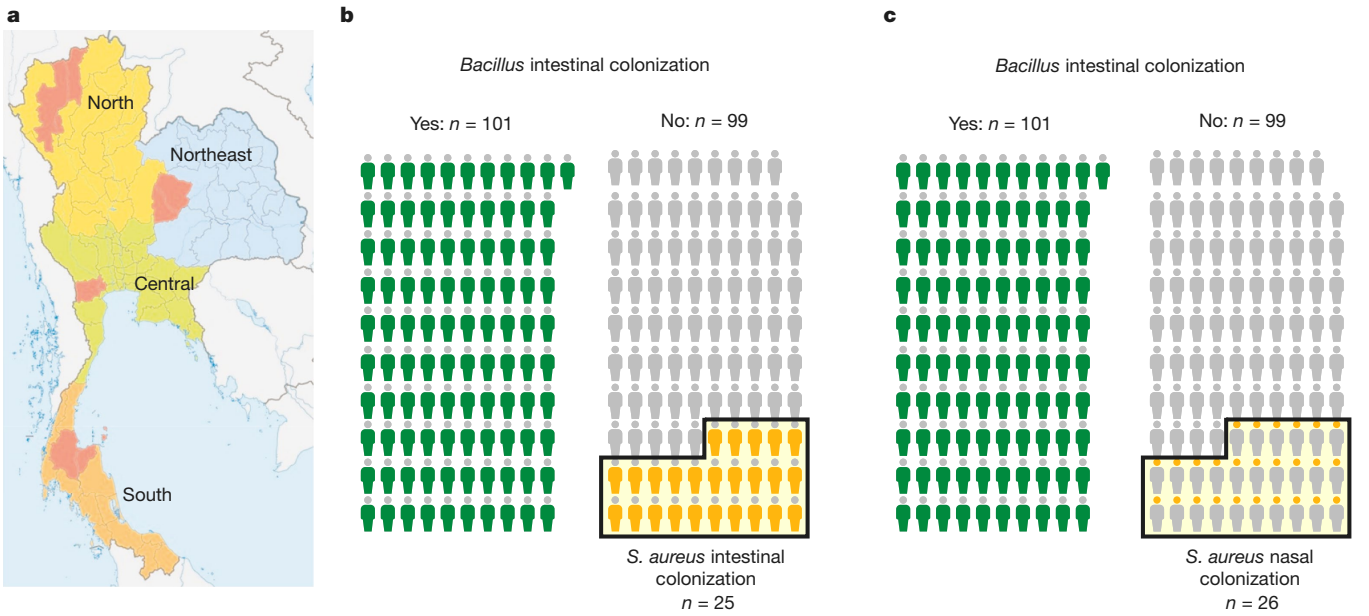

Fig. 1 | Exclusion of *S. aureus* colonization by dietary *Bacillus* in a human population. a, Areas (in red) from which faecal samples were collected in rural populations and analysed for the presence of *Bacillus* and *S. aureus*. **b**, **c**, Intestinal (**b**) and nasal (**c**) colonization with *S. aureus* (yellow) in individuals that showed (green) or did not show (grey) intestinal colonization with *Bacillus*.

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[22]. 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 *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 *S. aureus* carriers and non-carriers (Extended Data Fig. 1).

By contrast, we found a striking correlation between the presence of *Bacillus* bacteria and the absence of *S. aureus*. *Bacillus* species (mostly *B. subtilis*; Extended Data Table 1) were found in 101/200 (50.5%) of subject samples. *S. aureus* was never detected in faecal samples when *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 *S. aureus* colonization in a general fashion. While *Bacillus* was generally absent from nasal samples, *S. aureus* nasal colonization was never detected when intestinal *Bacillus* was present ($P < 0.0001$, Fisher's exact test; Fig. 1c). Notably, the levels of *S. aureus* colonization that we found in non-*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 *Bacillus* species that comprehensively inhibits colonization with *S. aureus*. Moreover, they suggest that *S. aureus* colonization is increased in urban populations because of the lack of a probiotic, *Bacillus*-containing diet. Of particular note, the results also indicate that the intestinal site has a previously underappreciated role in determining general *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 *S. aureus* and the presence of *B. subtilis*: studies that reported considerable *B. subtilis* or *S. aureus* numbers (samples with more than 10% colonization by either species) did not reveal exclusion phenomena (average $14.89 \pm 15.69\%$ 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 *S. aureus* carriers and non-carriers, exclude an indirect effect of *Bacillus* on the microbiome composition. Rather, we hypothesized that the *Bacillus* isolates produce a substance that directly and specifically inhibits intestinal colonization by *S. aureus*. We first analysed whether there is a growth-inhibitory effect of the *Bacillus* isolates on *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 *Bacillus* and the absence of *S. aureus*, and rules out a bacteriocin-mediated phenomenon.

The factors that are important for *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 *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 *S. aureus* intestinal colonization is unknown, we first used a mouse model of *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),

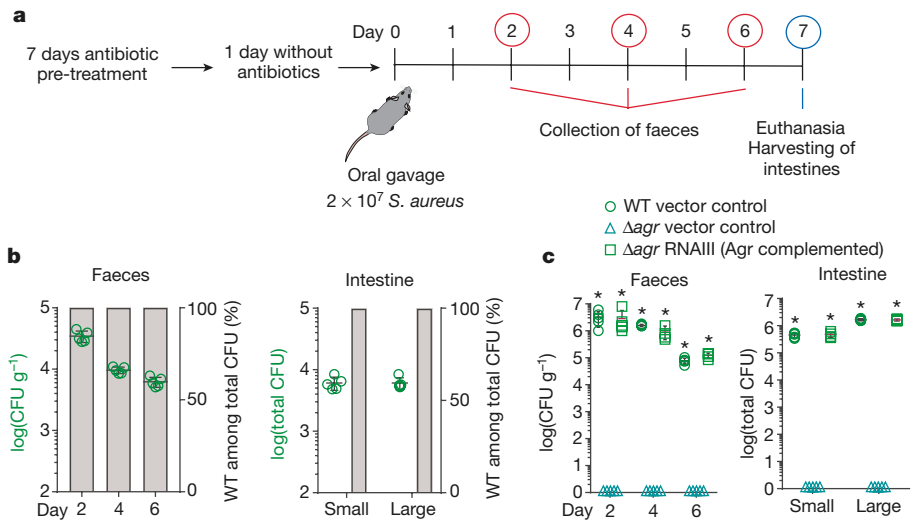

Fig. 2 | Quorum-sensing dependence of *S. aureus* intestinal colonization. a, Experimental set-up of the mouse intestinal colonization model. Mice received, by oral gavage, either 100 µl containing 10^8 CFU ml^{-1} of wild-type (WT) *S. aureus* strain ST2196 F12 and another 100 µl of 10^8 CFU ml^{-1} of the corresponding isogenic *agr* mutant ($n = 5$ per group; competitive experiment, shown in **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. **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 (which is present only in the *agr* mutant). No *agr* mutants were detected in any experiment; therefore, all bars show 100% wild type. Given that 100 isolates were tested, the competitive index of 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 pKX$_\Delta$16 control plasmid; Agr-complemented strains harboured pKX$_\Delta$RNAIII and constitutively expressed RNAIII, which is the intracellular effector of Agr. During the experiment, mice received 200 µg ml^{-1} kanamycin in their drinking water to maintain plasmids. Statistical analysis was performed using Poisson regression versus values obtained with the *agr* mutant strains. *$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* Δ*agr* with vector control. The corresponding zero values are plotted on the *x* axis of the logarithmic scale. See Extended Data Fig. 2 for corresponding data obtained using strains USA300 LAC and ST88 JSNZ.

according to multi-locus sequence typing (MLST) that we performed (Supplementary Table 1); second, a mouse infection isolate (ST88)[32]; and third, a human infection isolate of the highly virulent MRSA type USA300[33]. In competition experiments with equal amounts of wild-type and isogenic *agr* mutant strains, only wild-type *S. aureus* was detected in the faeces and colonized the large and small intestines at the end of the experiment (competition index ≥ 100) (Fig. 2b and Extended Data Fig. 2a, b). Furthermore, in a non-competitive experimental set-up, only those bacteria expressing the intracellular Agr effector RNAIII[34] achieved colonization; *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[30,35], the Agr quorum-sensing system is absolutely indispensable for intestinal colonization.

Fengycin quorum quenchers

Having established that the Agr quorum-sensing regulatory system is essential for *S. aureus* intestinal colonization, we next analysed whether culture filtrates of the *Bacillus* isolates collected from human faeces can inhibit Agr. To that end, we used an *S. aureus* reporter strain, into the genome of which we had transferred the luminescence-conferring *luxABCDE* operon under the control of the Agr P3 promoter[34], which controls production of RNAIII. Remarkably, culture filtrates from all 105 isolates reduced Agr activity in the *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 *B. subtilis* strain suppressed the production of key Agr-regulated virulence factors (phenol-soluble modulins, α-toxin and Panton–Valentine leucocidin; Fig. 3b, c and Supplementary Fig. 1). These results indicate that the inhibitory effect of the *Bacillus* isolates on *S. aureus* colonization is due to a secreted substance that inhibits Agr signalling.

To characterize the Agr-inhibitory substance(s), we performed experiments with culture filtrate of the reference *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 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[36], allowed us to identify the 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 β-hydroxylated (β-OH), and because different *Bacillus* strains produce different fengycin species[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 β-OH-C17-fengycin A and β-OH-C16-fengycin B. The second peak consisted of one fengycin species, β-OH-C17-fengycin B. According to RP-HPLC/ESI-MS analysis, smaller, adjacent peaks also contained fengycin species, which we tentatively identified as β-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 β-OH-C17-fengycin B to homogeneity from culture filtrate and verified the dose-dependent 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 β-OH-C17-fengycin B was the most strongly produced type. Notably, almost complete inhibition of Agr was detected at a concentration of about 1.4 µM total fengycin (Fig. 3d). This corresponds to the median concentration of total fengycin (1.5 µM) produced by stationary-phase cultures of the *Bacillus* isolates (Fig. 3e).

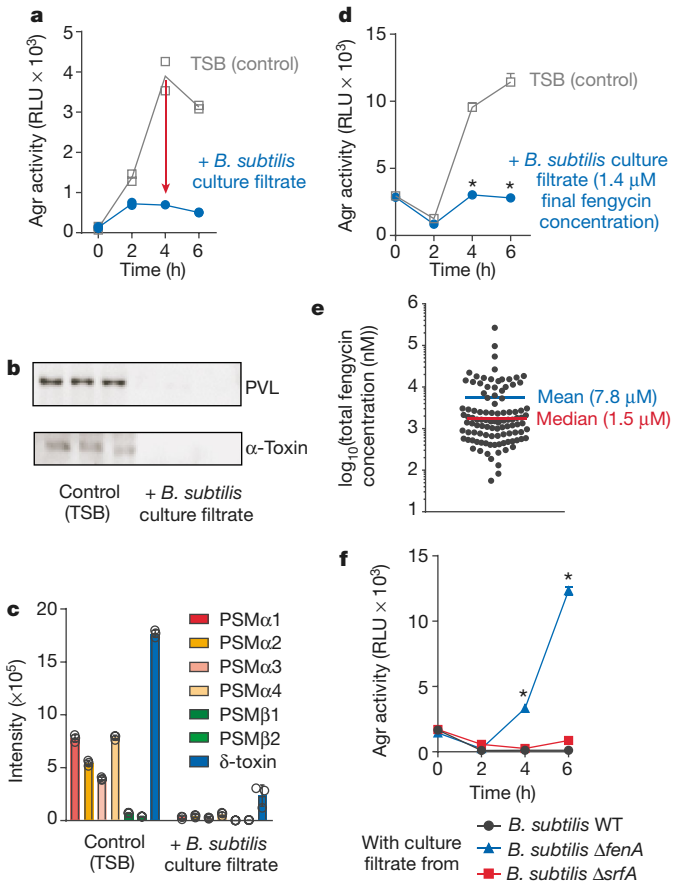

Fig. 3 | Inhibition of *S. aureus* quorum sensing by *Bacillus* fengycin lipopeptides. a, Example of an Agr-inhibition experiment. The *Bacillus* isolate was considered inhibitory if luminescence after 4-h growth of *S. aureus* was less than or equal to half that of the control value (red arrow). RLU, relative light units; TSB, tryptic soy broth (control conditions). The experiment was performed with $n = 2$ biologically independent samples. The lines connect the means. **b**, Inhibition of expression of Panton–Valentine leucocidin (PVL) and α-toxin, using culture filtrate from the *B. subtilis* reference strain. Western blot analysis of $n = 3$ biologically independent samples was performed with filtrates from *S. aureus* cultures that had been grown for 4 h. See Supplementary Fig. 1 for the entire blots. **c**, Inhibition of expression of phenol-soluble modulins (PSMs) using culture filtrate from the *B. subtilis* standard strain. PSM expression was determined by RP-HPLC/ESI-MS after 4 h of *S. aureus* growth. **d**, Test for Agr-inhibitory capacity of *Bacillus* culture filtrate applied at a final concentration that represents the median concentration of total fengycin in the tested 106 *Bacillus* isolates. $^*P < 0.0001$ (two-way analysis of variance (ANOVA) with Tukey's post-test versus control). **e**, Total fengycin concentrations in stationary-phase culture filtrates of the 106 *Bacillus* isolates (see Extended Data Table 1 for details). **f**, Agr-inhibiting activities of *B. subtilis* wild-type (WT) in comparison to Δ*fenA* (fengycin-deficient) and Δ*srfA* (surfactin-deficient) strains. $^*P < 0.0001$ (two-way ANOVA with Tukey's post-test versus wild type). The experiments shown in **c**, **d**, **f** were performed with $n = 3$ biologically independent samples. Data are mean ± s.d.

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[38]. 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.

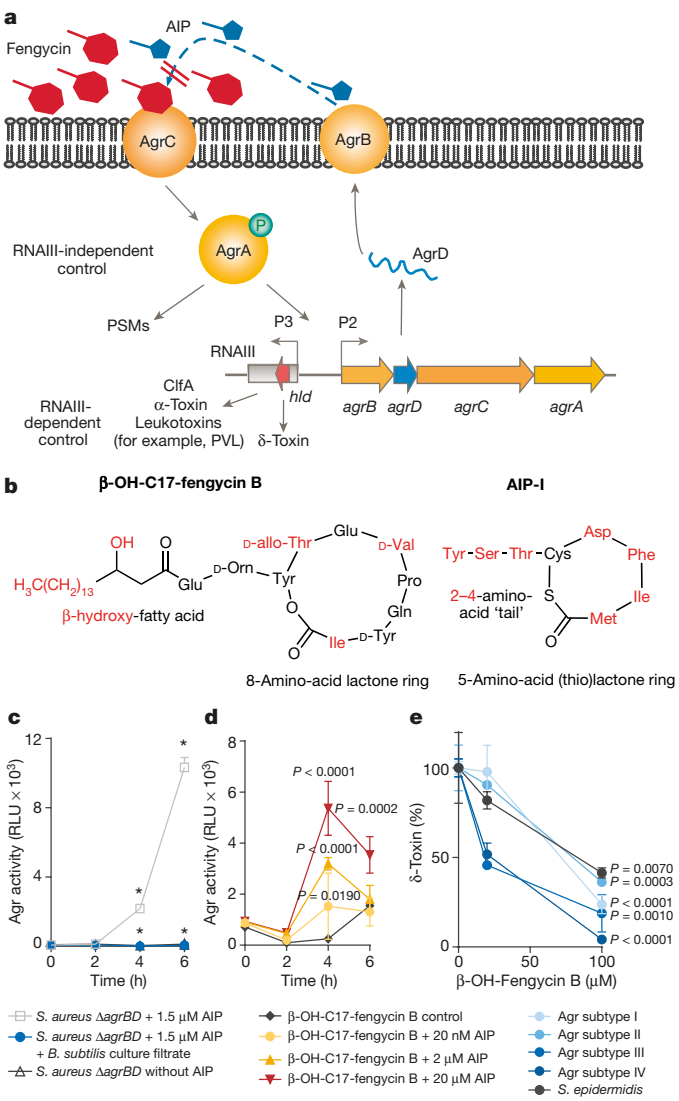

Fig. 4 | Competitive inhibition of *S. aureus* AIP activity by fengycins. a, Model of competitive Agr inhibition by fengycins. The *agrBDCA* operon (bottom right), whose expression is driven by the P2 promoter, encodes the AgrD precursor of the autoinducing-peptide (AIP), which is modified and secreted by AgrB. AIP binds to membrane-located AgrC, which, upon autophosphorylation, triggers phosphorylation and activation of the DNA-binding protein AgrA. In addition to stimulating transcription from the P2 promoter (autoinduction), AgrA drives expression of RNAIII, which in turn regulates the expression of target genes such as those encoding ClfA, α-toxin and leukotoxins. RNAIII also encodes the δ-toxin. Furthermore, AgrA drives the expression of phenol-soluble modulins (PSMs) in an RNAIII-independent fashion. **b**, Structural similarity of fengycins with AIPs. The structures of β-OH-C17-fengycin B and AIP-I are shown as examples. In red are structures and/or amino acids that may differ in different subtypes. **c**, Fengycins work by inhibiting AgrC. Shown is the inhibition of Agr by fengycin-containing *Bacillus* culture filtrate, using an *agrBD*-deleted *S. aureus* strain in which AgrC was stimulated by exogenously added AIP. $^*P < 0.0001$ (two-way ANOVA with Tukey's post-test; values obtained in Δ*agrBD*/AIP versus Δ*agrBD*/control (no AIP), and Δ*agrBD*/AIP/culture filtrate versus Δ*agrBD*/AIP). **d**, Competitive titration of fengycin-mediated Agr inhibition by increasing amounts of AIP, as assayed by the Agr luminescence assay. RLU, relative light units. Statistical analysis is by two-way ANOVA with Tukey's post-test versus control. **e**, Inhibition of Agr in different Agr-subtype *S. aureus* and *S. epidermidis* (strain 1457) by β-OH-C17-fengycin B, as measured by relative expression of δ-toxin using RP-HPLC/ESI-MS. Statistical analysis is by two-way ANOVA with Tukey's post-test versus intensity values obtained without addition of fengycin. Values were calculated as percentages relative to intensity values obtained without addition of fengycin, owing to different δ-toxin expression levels in the different strains. **c–e**, Experiments were performed with $n = 3$ biologically independent samples. Data are mean ± s.d.

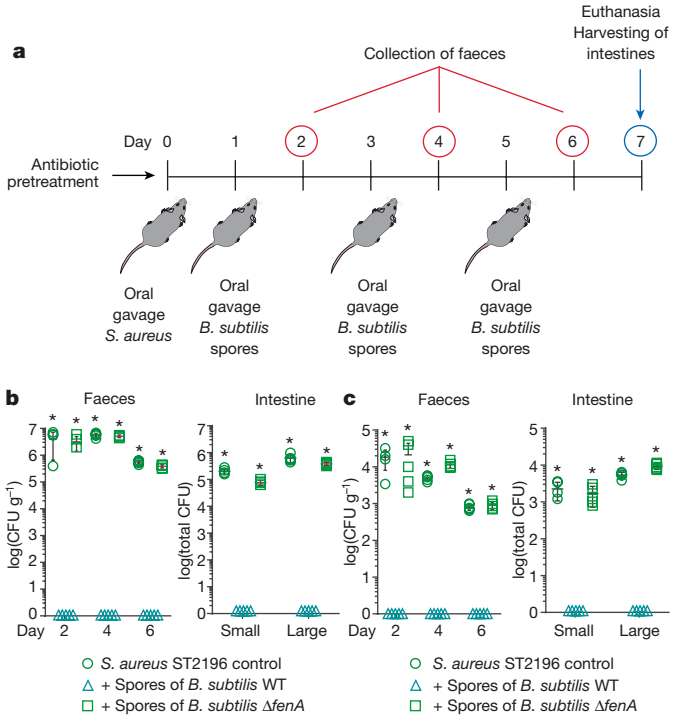

Fig. 5 | Inhibition of *S. aureus* colonization by dietary fengycin-producing *Bacillus* spores in a mouse model. a, Experimental set-up. $n = 5$ mice per group received 200 µl of 10^8 CFU ml^{-1} *S. aureus* strain ST2196 F12 by oral gavage. On the next day and every following second day, they received 200 µl of 10^8 CFU ml^{-1} spores of the *B. subtilis* wild-type (WT) 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 (**b**) or without (**c**) antibiotic pretreatment. **b**, **c**, Experimental results. Statistical analysis was performed using Poisson regression versus values obtained with the *B. subtilis* WT 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 *S. aureus* and receiving *Bacillus* wild-type spores. The corresponding zero values are plotted on the *x* axis of the logarithmic scale. See Extended Data Fig. 5 for corresponding data obtained using strains USA300 LAC and ST88 JSNZ.

Mechanism of fengycin-mediated inhibition

In the *S. aureus* Agr quorum-sensing regulatory circuit, the secreted Agr autoinducing peptide (AIP) interacts with an extracellular domain of AgrC, the histidine kinase part of a two-component signal-transduction system, to signal the cell-density status[39] (Fig. 4a). Different Agr subgroups of *S. aureus*, as well as different staphylococcal species, produce distinct cyclic heptapeptide to nonapeptide AIPs[35]. AIPs from other subgroups or species frequently inhibit Agr signal transduction by competitive inhibition at the AgrC-binding site[39–41]. Given that fengycins, being cyclic lipopeptides, show structural similarity to AIPs (Fig. 4b), it appears likely that fengycins compete with the natural AIP for AgrC binding. The only other theoretically possible site of interference from the extracellular space would be the membrane-located AIP production/secretion enzyme AgrB. Using an *S. aureus agrBD* deletion strain and stimulation of AgrC by synthetic AIP, which led to complete Agr activation, we ruled out that the target of Agr inhibition by *Bacillus* is AgrB (Fig. 4c). In further support of a mechanism that works through competition with AIP for binding to the AgrC receptor, we found that fengycin inhibition could be reversed in a dose-dependent fashion by adding AIP (Fig. 4d). Finally, we determined the AIP concentration in early stationary growth phase (at 6–8 hours) to be about 1 µM (Extended Data Fig. 5a), which is approximately equal to the concentration of fengycin for which we 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* Agr 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 eradicate *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. aureus*[7]—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[10,42] 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[43]—could potentially be used as quorum-sensing blockers in eagerly sought antivirulence-based efforts to treat staphylococcal infections[15,44]. 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 nose[45].

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.

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Author contributions P.P., S.P. and S.K. collected human samples and analysed bacterial isolates by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS). Y.Z., H.-S.J. and M.O. performed analytical and preparative chromatography. P.P., T.H.N., E.L.F., R.L.H., J.C. and G.Y.C.C. performed animal studies. S.W.D. constructed the *S. aureus agrBD* mutant and A.E.V. constructed all other *agr* mutants and complemented strains. K.A.G., A.E.V. and B.L. performed MLST. P.P. performed reporter assays, the microbiome study, and all further analyses not specifically mentioned. P.K. supervised the human analyses and M.O. all other parts of the study.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment, except for when noted.

Sample collection and bacterial screening. Nasal swabs and faecal samples were obtained from 200 Thai healthy volunteers from four different locations in southern, central, northeastern and northern Thailand. One sterile nasal swab, a sample collection tube, a sterile container and tissue paper were given to each participant. All participants provided informed written consent. The study was performed in compliance with all relevant ethical regulations and approved by the Siriraj Institutional Review Board (approval no. Si 733/2015). All participants were at least 20 years old (age range 20–87 years; median age 57 ± 14.5 years; 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 before the study.

Nasal swabs and faecal samples were streaked on mannitol salt agar (MSA) and then incubated at 37 °C for 24 h. Positive or negative *S. aureus* or *Bacillus* colonization could easily be distinguished by either strong growth on the entire plate, or the absence of any colonies, respectively. At the time of this analysis, the purpose was to obtain and archive colonizing *S. aureus* strains. As the hypothesis regarding *Bacillus*/*S. aureus* exclusion was developed only after we obtained the results of this analysis, the staff performing the analysis were blinded as to the exclusion hypothesis. Isolates were easily recognized as *S. aureus* or *Bacillus* by colony morphology and colour; however, every isolate was confirmed for species identity using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS; see below), and *Bacillus* species were further distinguished by 16S rRNA sequencing (Extended Data Table 1). To that end, 16S rRNA genes were amplified by the polymerase chain reaction (PCR) using primers 27FB and 1492RAB[46] and similarity analysis with the basic local alignment search tool (BLAST) was used to identify the species. Subjects were considered as permanently colonized by *S. aureus* if two positive samples were obtained, tested after a four-week interval. All individuals tested either negative or positive for *S. aureus* at both times. In total, 105 *Bacillus* isolates from 101 individuals were analysed. In the samples from four individuals, two isolates each were taken owing to their apparent phenotypic differences.

Bacterial identification using MALDI–TOF MS. Isolates were inoculated onto sheep blood agar and incubated for 24 h at 37 °C. Bacterial colonies were applied onto a 96-spot target plate and allowed to dry at room temperature. Subsequently, 2 μl of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied onto the colonies and allowed to dry before testing. Then the target plate was loaded into the MALDI–TOF MS instrument (MicroFlex LT mass spectrometer, Bruker Daltonics). Spectra were analysed using MALDI Biotyper automation control and the Bruker Biotyper 2.0 software and library (version 2.0, 3,740 entries; Bruker Daltonics). Identification score criteria used were those recommended by the manufacturer: a score of ≥2.000 indicated species-level identification; a score of 1.700–1.999 indicated identification to the genus level; and a score of <1.700 was interpreted as no identification. Isolates that failed to produce a score of <1.700 with direct colony or extraction methods were retested. *S. aureus* ATCC25923, *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as controls.

Bacterial strains and growth conditions. The reference *B. subtilis* strain and parent of the *fenA* and *srfA* mutants used in this study was strain ZK3814 (genotype NCIB3610). The *S. aureus* strains used in all experiments (except the experiment in which we analysed different Agr-subtype *S. aureus*) were: first, the human faecal isolate F12 of ST2196 (Supplementary Table 1); second, strain JSNZ of ST88, a mouse isolate previously described as mouse adapted[32]; and third, strain LAC of pulsed-field type USA300, an MRSA lineage predominantly involved in community-associated infections, but now generally representing the major lineage responsible for *S. aureus* infections in the United States[47].

Isogenic mutants in *agr* were previously described (for strain LAC)[48] or produced in this study (for strains JSNZ and F12) by phage transduction of the *agr* deletion from strain RN6911. The *agr* system is entirely deleted in these strains, except for a 3′ part of RNAIII, which is not transcribed owing to the absence of the corresponding promoter. All mutants were verified by analytical PCR.

Owing to the tetracycline resistance introduced in the *agr* deletion strains, kanamycin derivatives (pKX$_\Delta$) of the pTX$_\Delta$ expression plasmid series were constructed and used for complementation of Agr. (This was not possible in strain LAC, which harbours resistance to multiple antibiotics, including kanamycin.) To that end, we treated plasmid pKX15[49]—provided by B. Krismer, University of Tübingen—as described[48] to delete the *xylR* repressor gene, in order to make expression of any fragment cloned under control of the *xyl* promoter constitutive. To obtain plasmid pKX$_\Delta$RNAIII, the RNAIII BamH1–MluI fragment was transferred from pTX$_\Delta$RNAIII[50]. Plasmid pKX$_\Delta$16 is the corresponding empty control plasmid, derived from pKX16 by analogous deletion of the *xylR* repressor gene.

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 SmaI site of plasmid pIMAY[51] and used inverse PCR to delete *agrBD*. Allelic 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.

To construct the tetracycline-resistant derivatives of *S. aureus* ST88 and ST2196, we carried out φ11-phage-mediated transduction as described in order to transfer the tetracycline cassette in the donor strain (*S. aureus* RN4220 with integrated pLL29) to *S. aureus* strains ST88 and ST2196[52].

To construct the *B. subtilis* fengycin mutant strain, SPP1-phage-mediated transduction[53] was performed to transfer the *fenA* deletion present in the donor strain (BKE18340, a *fenA*(*ppsA*)::*erm* mutant 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 described[54]. PCR amplicons of seven *S. aureus* housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* 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–ST4638) were deposited to the website. The Agr subtype of *S. aureus* isolates was determined using a modified multiplex quantitative reverse transcription PCR (qRT–PCR) protocol[55]. 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 *m*/*z* 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 rRNA paired-end sequencing of the V4 region of 16S rRNA was performed by Illumina using an Illumina MiSeq system as described[56].

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 1.9.1)[57]. This study used the Nephele (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 uclust-based[58] open-reference OUT-picking protocol[59] and the Greengenes 13_8 reference sequence set[60] at 99% similarity. Alpha diversity was calculated using Chao1 and Shannon analyses[61] 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 at 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 β-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 3,700*g* 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 and 25% buffer B for five column volumes, after which 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 redissolved 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 Zorbax SB-C18 9.4 mm × 25 cm reversed-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 described[62], 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–*luxABCDE* reporter fusion construct that was inserted into the genome of *S. aureus* strain LAC[34]. Strain LAC P3–*luxABCDE* 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 described[30].

To determine the Agr-inhibiting activity with target strains other than LAC (Agr subtype I), we measured the production of δ-toxin, for which the gene is embedded in the Agr intracellular effector RNA, RNAIII, in most staphylococci. Production of δ-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 in a table-top centrifuge at maximum speed for 5 min; the supernatants were collected and loaded onto 12% SDS–polyacrylamide gel electrophoresis (PAGE) gels, which were run at 160 V for 1 h. Proteins were transferred to nitrocellulose membranes using an iBlot western blotting system. Membranes were incubated with Odyssey blocking buffer for 1 h at room temperature. Anti-α-toxin antibodies (polyclonal rabbit serum; Sigma S7531; dilution 1:5,000) or anti-LukF-PV antibodies (affinity-purified rabbit IgG specific for a peptide region of LukF-PV, produced by GenScript USA and provided by F. DeLeo, NIAID; dilution 1:500) were added to the blocking buffer and membranes were incubated overnight at 4 °C. Then, membranes were washed five times with Tris-buffered saline containing 0.1% Tween-20, pH 7.4, and incubated with Cy5-labelled goat anti-rabbit IgG (diluted 1:10,000 in blocking buffer) in the dark for 1 h at room temperature. Membranes were washed five times with the washing buffer and scanned with a Typhoon TRIO+ variable mode imager.

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 medium[63] and allowed to sporulate for 96 h. Cells were pelleted, washed with water, and resuspended in 20% metrizoic acid (Sigma). Five different concentrations (w/v) of metrizoic 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 previously[64]). 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 determined 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 grown to mid-exponential growth phase, washed, and resuspended in sterile phosphate-buffered saline (PBS) at 10^8 CFU ml^{-1}. Mice were inoculated by oral gavage with 200 μl of a 10^8 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 pKX_{Δ} type, mice received kanamycin (0.2 g l^{-1}) in the drinking water during the experiment to maintain plasmids. For the *B. subtilis* spore competition experiment, oral gavage with 200 μl of spores of wild-type *Bacillus* or its isogenic Δ*fenA* fengycin mutant (10^8 CFU ml^{-1} in sterile PBS) was performed on the day following the *S. aureus* gavage, and repeated every second day thereafter for a total of three times (days 2, 4 and 6). Intestinal colonization was evaluated by quantitative cultures of mouse stool samples and samples from the small and large intestines of mice. In detail, stool was collected and suspended to a final volume of 1 ml of PBS, diluted and plated on TSB agar. Plates were incubated for 24 h at 37 °C, and colonies were enumerated. Moreover, after mice were euthanized seven days after infection, the small and large intestines were collected, resuspended each in 1 ml PBS, and homogenized. Serial dilutions of the homogenates were plated on TSB agar and incubated at 37 °C. Bacterial colonies were enumerated on the following day. In the experiments without antibiotic pretreatment, extracts were plated on MSA 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 correct for 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.

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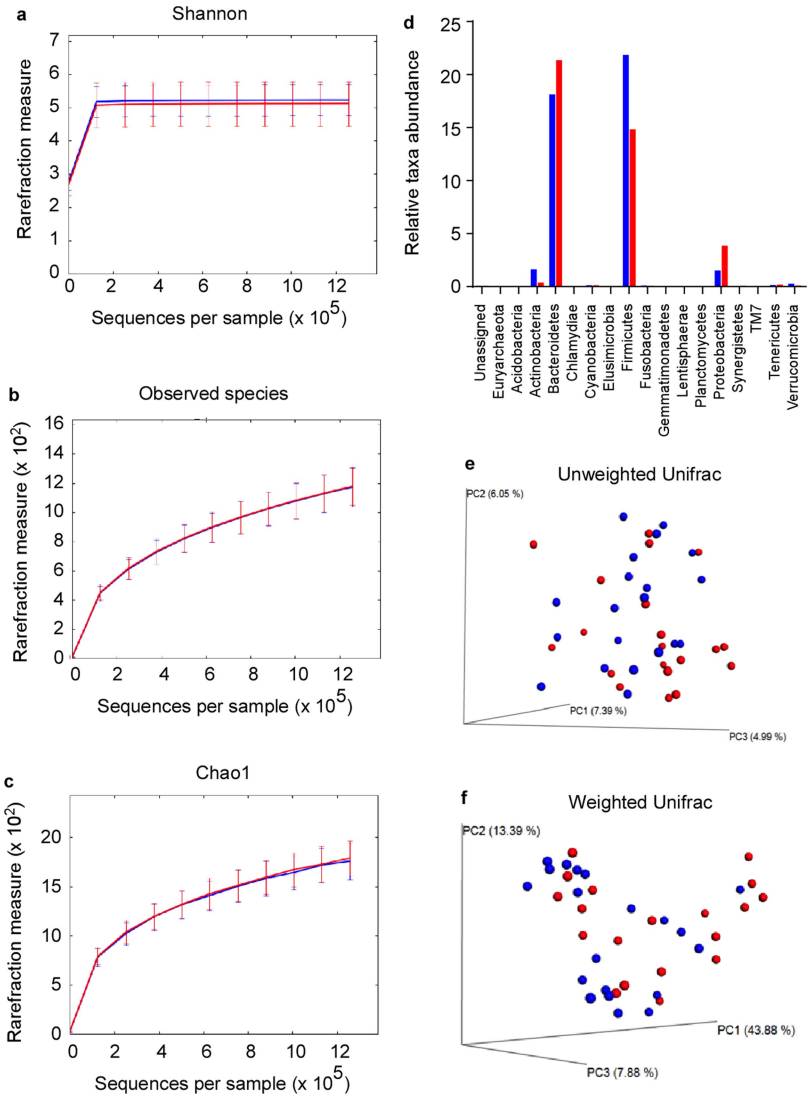
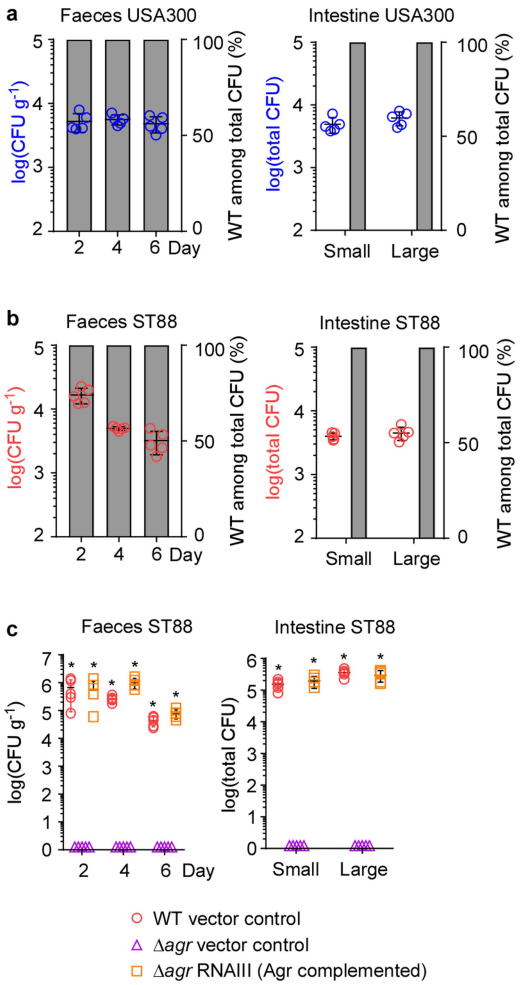
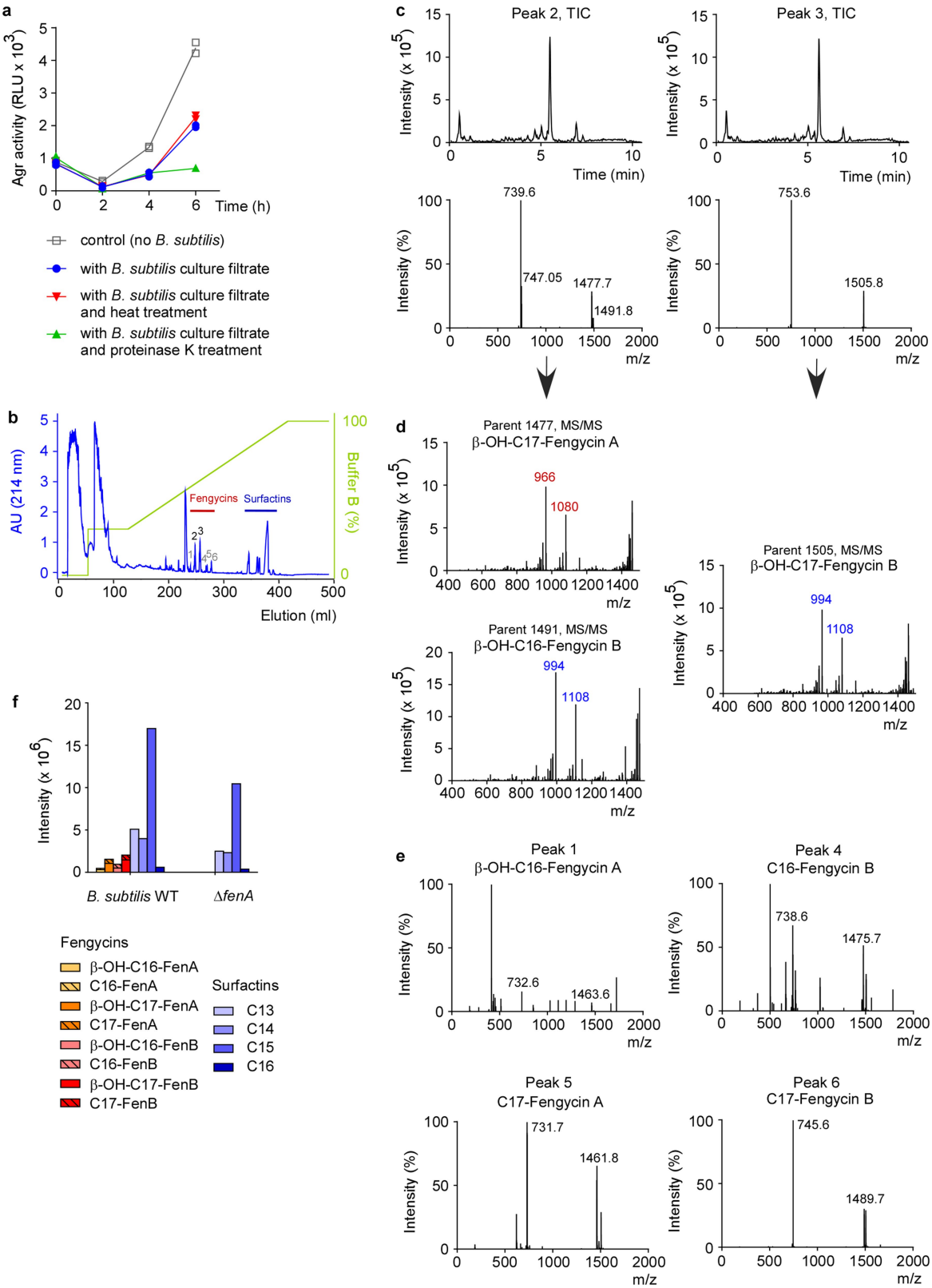


Extended Data Fig. 1 | Microbiome analysis of *S. aureus* carriers versus non-carriers. The microbiota of $n = 20$ randomly selected *S. aureus* carriers (red) and $n = 20$ non-carriers (blue) were analysed in faecal samples. **a–c**, Rarefaction (species-richness) curves based on 16S rRNA gene sequences. 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).

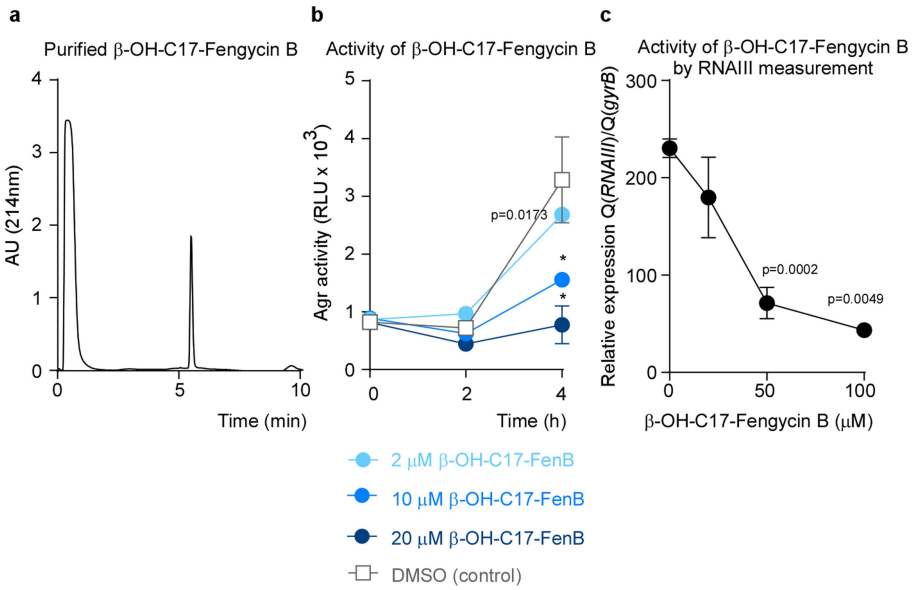


Extended Data Fig. 2 | Quorum-sensing dependence of *S. aureus* intestinal colonization. Data from strains USA300 LAC and ST88 JSNZ. The experimental set-up is the same as in Fig. 2: mice received by oral gavage either 100 µl containing 10^8 CFU ml^{-1} of wild-type *S. aureus* strain USA300 LAC or ST88 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 $pKX_{\Delta}16$ control plasmid; Agr-complemented strains harboured pKX_{Δ}RNAIII 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* Δ*agr* with vector control. The corresponding zero values are plotted on the *x* axis of the logarithmic scale.

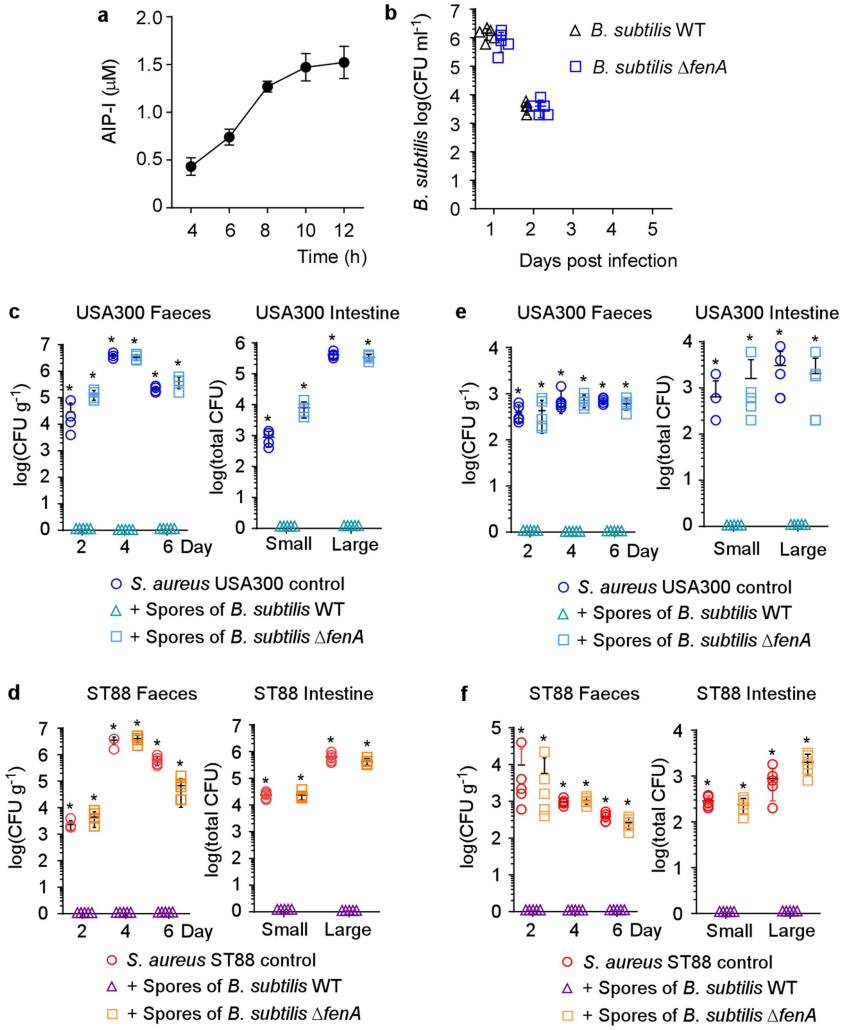


Extended Data Fig. 3 | See next page for caption.

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 Δ*fenA* 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 0 μM 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 10^8 CFU ml^{-1} suspension of wild-type *B. subtilis* or Δ*fenA* 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 10^8 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 10^8 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

Isolate Code	*Bacillus* species*	β - OH-C16-FenA	C16-FenA	β - OH-C17-FenB	C17-FenB	β - OH-C17-FenA	C17-FenA	β - OH-C16-FenB	C16-FenB	% Agr inhibition†	Total Fengycin Concentration
10	*licheniformis*	100	48	65	80	33	33	51	32	98	442
14	*subtilis*	82	27	104	138	52	111	186	0	97	700
15	*amyloliquefaciens*	7	212	212	83	152	56	274	81	92	1076
16	*sonorensis*	5,833	437	984	288	2,751	1,248	1,760	1,090	98	14390
18	*subtilis*	107	0	58	118	0	0	53	23	97	359
19	*licheniformis*	0	61	0	75	13	0	149	31	97	329
21	*sonorensis*	0	85	159	48	0	104	18	33	95	447
26	*megaterium*	47	0	112	8	33	134	48	23	98	405
30	*subtilis*	0	0	63	28	109	77	87	43	96	407
31	*sonorensis*	23	0	52	0	157	15	0	0	97	246
32	*sonorensis*	23	118	145	0	0	0	74	59	94	418
33	*licheniformis*	89	119	70	99	98	102	150	0	96	727
35	*licheniformis*	0	0	98	0	149	72	28	132	96	479
36	*pumilus*	0	0	10	0	0	136	144	0	98	290
37	*subtilis*	116	232	67	0	15	45	0	114	96	589
38	*subtilis*	59	167	215	0	79	114	50	212	93	896
39	*licheniformis*	150	117	249	0	241	271	226	230	96	1484
40	*subtilis*	753	174	1,260	1,124	1,548	538	841	821	80	7058
41	*subtilis*	2,298	860	1,777	0	4,524	816	1,563	1,957	91	13796
42	*sonorensis*	34	43	0	0	41	57	88	0	96	263
43	*sonorensis*	477	0	2,488	816	1,667	604	1,216	858	96	8126
45	*sonorensis*	98	0	24	25	0	0	0	39	99	187
47	*pumilus*	342	14	237	0	0	108	105	0	97	806
48	*subtilis*	0	0	49	0	7	0	0	0	94	56
49	*subtilis*	5,007	828	979	0	3,147	0	2,027	1,210	97	13200
50	*subtilis*	429	0	933	0	1,065	354	614	526	90	3922
51	*subtilis*	712	185	911	208	1,187	665	863	586	88	5316
52	*subtilis*	9,630	1,571	923	0	2,365	1,775	4,690	1,099	95	22052
53	*licheniformis*	0	0	0	0	0	0	58	25	95	83
55	*subtilis*	45	43	113	12	94	0	0	216	96	523
56	*sonorensis*	167	53	0	0	83	201	109	58	98	671
57	*subtilis*	127	104	166	301	61	96	98	246	96	1200
58	*sonorensis*	498	510	841	0	967	222	0	0	94	3039
59	*subtilis*	0	0	1,008	0	0	297	715	488	82	2508
61	*subtilis*	124	84	21	0	160	39	42	335	97	805
62	*licheniformis*	93	128	7	48	40	188	0	153	97	657
63	*amyloliquefaciens*	4,153	380	975	0	2,584	1,047	1,975	1,084	95	12197
64	*sonorensis*	133	91	187	230	29	387	62	34	95	1154
65	*subtilis*	9	126	0	0	35	90	239	36	97	535
66	*sonorensis*	254	0	0	0	377	0	131	0	97	762
67	*subtilis*	215	84	200	0	0	0	155	168	97	821
68	*subtilis*	41	12	144	0	0	0	115	182	93	494
69	*subtilis*	266	27	236	290	132	0	54	195	98	1200
70	*subtilis*	54	55	390	0	196	0	187	112	94	994
71	*subtilis*	14,881	4,578	88,106	34,879	39,939	16,967	42,502	23,859	97	265710
74	*pumilus*	157	56	54	14	0	62	177	41	95	560
75	*licheniformis*	281	22	40	0	0	292	125	204	95	964
76	*subtilis*	124	0	74	0	0	101	82	192	97	573
77	*sonorensis*	0	0	0	25	0	0	93	13	97	131
78	*amyloliquefaciens*	73	99	0	0	79	17	176	13	94	458
79	*amyloliquefaciens*	10	0	0	0	0	180	51	63	97	304
80	*subtilis*	1,741	322	4,222	1,105	3,327	910	2,207	1,529	91	15363
81	*subtilis*	1,739	426	5,073	1,579	3,371	998	3,241	1,933	87	18361
82	*subtilis*	1,002	0	3,413	921	1,998	0	1,710	992	91	10037
83	*licheniformis*	356	0	536	4	83	201	107	52	97	1338
85	*subtilis*	52	0	49	0	84	161	0	132	95	479
87	*subtilis*	1,327	312	3,931	0	2,763	667	2,624	1,167	98	12790
88	*pumilus*	101	0	215	156	313	367	276	216	96	1643
89	*subtilis*	105	59	266	0	0	0	51	38	93	519
91	*subtilis*	325	91	493	17	120	290	302	186	98	1825
92	*subtilis*	254	0	234	156	275	0	154	66	96	1140
93	*subtilis*	493	273	91	342	204	287	277	435	96	2402
94	*subtilis*	876	115	134	37	384	190	445	529	91	2712
95	*amyloliquefaciens*	351	175	157	146	110	225	196	197	97	1557
97	*subtilis*	1,845	912	4,714	1,310	3,686	1,492	2,484	1,557	86	18000
98	*subtilis*	1,367	804	3,572	1,512	2,803	1,511	2,005	1,626	93	15200
99	*subtilis*	77	375	705	0	28	170	115	77	91	1547
100	*amyloliquefaciens*	81	117	337	267	105	166	237	502	81	1811
103	*pumilus*	249	45	350	207	162	249	536	279	98	2077
104	*subtilis*	293	77	105	269	75	509	286	17	99	1632
106	*subtilis*	978	796	4,415	3,419	1,874	1,378	2,478	1,527	97	16866
107	*subtilis*	423	199	322	150	520	517	384	160	98	2675
108	*pumilus*	224	114	397	0	229	211	413	43	99	1631
110	*subtilis*	140	77	317	286	404	127	117	139	95	1607
111	*pumilus*	184	104	319	67	96	212	294	120	93	1395
112	*subtilis*	470	183	1,412	637	1,211	732	950	655	96	6251
113	*pumilus*	463	202	276	204	211	0	156	137	95	1650
115	*subtilis*	268	232	297	62	313	410	713	88	97	2382
116	*subtilis*	352	205	369	0	172	298	561	350	96	2306
117	*subtilis*	143	104	716	328	0	149	97	266	98	1803
118	*subtilis*	163	34	1,788	0	0	155	306	42	93	2488
119	*amyloliquefaciens*	604	256	947	258	0	350	361	104	93	2880
121	*subtilis*	503	151	0	364	63	84	174	162	98	1502
122	*subtilis*	152	311	24	165	86	213	296	83	91	1329
123	*subtilis*	8,801	3,540	23,045	10,778	16,465	6,428	16,077	10,706	98	95839
124	*subtilis*	106	139	316	168	64	175	195	103	86	1266
125	*subtilis*	0	0	0	158	0	318	290	0	95	765
126	*subtilis*	288	157	211	110	428	421	185	112	87	1913
127	*subtilis*	478	193	103	435	240	303	551	132	96	2434
128	*subtilis*	177	156	228	118	96	276	426	48	97	1525
129	*pumilus*	249	37	0	162	0	224	0	144	97	816
130	*subtilis*	1,267	0	4,668	1,266	2,940	1,168	2,158	1,863	88	15332
131	*subtilis*	4,164	496	513	307	1,226	671	1,948	662	83	9986
132	*subtilis*	441	291	491	391	200	562	381	279	95	3036
134	*subtilis*	6,647	750	1,011	0	3,143	1,166	2,829	953	97	16498
136	*subtilis*	751	233	2,569	773	1,813	890	1,701	1,010	89	9740
137	*subtilis*	1,572	328	3,297	1,036	2,221	627	2,266	0	82	11347
138	*pumilus*	288	311	232	11	415	236	236	403	85	2133
139	*subtilis*	1,898	709	7,830	2,453	5,328	2,073	3,880	2,210	98	26381
140	*sonorensis*	0	106	217	815	225	0	103	3,987	91	5454
141	*thuringiensis*	258	26	325	230	0	264	0	0	87	1102
142	*pumilus*	422	159	262	0	402	335	639	251	87	2471
143	*subtilis*	250	37	210	16	64	68	293	89	83	1027
144	*sonorensis*	110	208	134	191	0	304	361	91	87	1399
	B. subtilis ZK3814	2,563	1,781	17,078	5,725	11,963	4,448	5,444	4,902	94	53904

The table shows intensity values from the integration of *m/z* peaks associated with the specific fengycin species, as obtained by RP-HPLC/ESI-MS. The two most abundant peaks, corresponding to double- and triple-charged ions, were used for the integration. Values are in nM, obtained by calibration using weighed and diluted aliquots of the *Bacillus* lipopeptide surfactin.

**Bacillus* species were determined by sequencing 16S RNA 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	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	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 analyse 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.

nature research

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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

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).

n/a	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.

Stata Release 15 (for Poisson regression), Graph Pad Prism 6.05 (all other statistical analyses). Bruker Biotyper 2.0 version 2.0, Bruker Daltonics (MALDI-TOF species identification). Nephele (release 1.6) (Microbiome analysis). Agilent Mass Hunter Quantitative Analysis Version B.07.00 (HPLC/MS)

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.

no restrictions on availability.

9. Antibodies

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

anti-alpha-toxin: Sigma S7531. Dilution for Western Blot: 1:5,000
Papers cited on manufacturer's website:S Forti and G Menestrina Eur J Biochem 181(3); M Thelestam and L Blomqvist Toxicon 26(1); Jon Oscherwitz and Kemp B Cease, PloS one, 10(1); Jon Oscherwitz et. al Mol Immunol 60(1)
Anti-LukF-PV: Synthesized by GenScript against a peptide region of LukF-PV. Dilution for Western Blot: 1: 500. Citation: Graves et al. Microbes and Infection 12 (2010) 446-456.

10. Eukaryotic cell lines

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

No eukaryotic cell lines were used.

b. Describe the method of cell line authentication used.

Describe the authentication procedures for each cell line used OR declare that none of the cell lines used have been authenticated OR state that no eukaryotic cell lines were used.

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

Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination OR state that no eukaryotic cell lines were used.

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.

Provide a rationale for the use of commonly misidentified cell lines OR state that no commonly misidentified cell lines were used.

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.