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Pathogen elimination by probiotic *Bacillus* via signaling interference

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Abstract

Probiotic nutrition is frequently claimed to improve human health. In particular, live probiotic bacteria obtained with food are believed to reduce pathogen colonization and thus, susceptibility to infection. However, the underlying mechanisms remain poorly understood. Here, we report that the consumption of probiotic *Bacillus* bacteria comprehensively abolishes colonization with the dangerous pathogen, *Staphylococcus aureus*. We discovered that the widespread fengycin class of *Bacillus* lipopeptides achieves colonization resistance by inhibiting the *S. aureus* Agr quorum-sensing signaling system. Our study presents a detailed molecular mechanism underlining the importance of probiotic nutrition in reducing infectious disease. Notably, we provide human evidence supporting the biological significance of probiotic bacterial interference and show for the first time that such interference can be achieved by blocking a pathogen's signaling system. Furthermore, our findings suggest a probiotic-based method for *S. aureus* decolonization and new ways to fight *S. aureus* infections.

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Contributions

P.P., S.P. and S.K. collected human samples and analyzed bacterial isolates by MALDI-TOF. Y.Z., H.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 *agrBD* and A.E.V. all other *S. aureus agr* mutant 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, and M.O. all other parts of the study.</author_notes>

The authors declare that there are no conflicts of interest.

There is increasing appreciation of the key role that the intestinal microbiome plays 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 bacteriocins, which can kill phylogenetically related pathogenic bacteria[2], and it has been shown that a bacteriocin-producing *Escherichia coli* strain inhibited colonization with 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 ways of 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 dependence on dietary customs, the concentration of *Bacillus* spores in human feces is highly variable. It has been reported to be ~ 10^5 CFU/g in average, occasionally reaching up to 10^8 CFU/g[7]. Several probiotic formulae contain *Bacillus* species[10], which are believed 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 have traditionally been considered the primary *S. aureus* colonization site[19], there is increasing evidence indicating that the intestinal tract is also commonly colonized by *S. aureus*[20–22] and forms an important reservoir for *S. aureus* infectious disease outbreaks[23,24]. Several studies have reported levels of *S. aureus* in the feces of human adults of ~ 10^3 - 10^4 CFU/g[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 fecal samples from 200 healthy individuals from rural populations in Thailand (Fig. 1a). This exemplary population was selected to rule out, as much as possible, food sterilization and antibiotic presence as used frequently in highly developed urban areas, which potentially could diminish the abundance of probiotic bacteria in the food and the intestinal tract 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* intestinal colonization and discovering that secreted *Bacillus* fengycin lipopeptides function as quorum-sensing blockers to achieve complete eradication of intestinal *S. aureus*, we provide evidence strongly suggesting that this pathogen exclusion effect in humans is due to a widespread and efficient probiotic-mediated mechanism that inhibits pathogen quorum-sensing signaling.

S. aureus exclusion by *Bacillus*

S. aureus intestinal carriage as determined by growth from fecal samples was found in 25/200 (12.5%) of human subjects. 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[22]. These rates are considerably lower than those commonly found in adult populations during cross-sectional culture-based surveys that were mainly performed 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 analyzed the composition of the gut microbiome by 16S rRNA sequencing. However, we did not detect significant differences in the composition of the microbiome between *S. aureus* carriers and non-carriers (Extended Data Fig. 1).

In contrast, we found a striking correlation between the presence of *Bacillus* bacteria and absence of *S. aureus*. *Bacillus* species (mostly *B. subtilis*, Extended Data Fig. 1) were found in 101/200 (50.5%) of subject samples. *S. aureus* was never detected in fecal 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 in the presence of intestinal *Bacillus* ($p<0.0001$, Fisher's exact test) (Fig. 1c). Interestingly, the levels of *S. aureus* colonization 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 due to the lack of a probiotic, *Bacillus*-containing diet. Intriguingly, they also imply that the intestinal site plays a previously underappreciated role in determining general *S. aureus* colonization, a notion in accordance with findings attributing a key role to fecal transmission in MRSA re-colonization[28].

When we analyzed 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 colonization by either species > 10%) did not reveal exclusion phenomena (average 14.89 ± 15.69% colonization by both species) (Extended Data Fig. 2). While we did not find a correlation, we feel this may 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 showing no significant 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 *S. aureus* intestinal colonization. We first analyzed whether there is a growth-inhibitory effect of the *Bacillus* isolates on *S. aureus*. However, only a minor growth inhibition occurred in 6 out of 105 isolates (maximally 1 mm inhibition zone when analyzed using an agar diffusion test with five-times concentrated culture filtrate). This fails to explain the observed complete correlation between the presence of *Bacillus* and absence of *S. aureus*, and rules out a bacteriocin-mediated phenomenon.

It is poorly understood which factors are important for *S. aureus* intestinal colonization. Only one mouse study implicated wall teichoic acids and the surface protein, clumping factor A (ClfA)[29]. Prompted by our previous finding that ClfA is positively regulated by the Agr quorum-sensing system[30], we hypothesized that the *Bacillus* isolates secrete a substance that interferes with quorum-sensing signaling. Quorum-sensing is responsible for sensing the density of the bacterial population ("quorum") and controlling the concomitant alteration of cell physiology[31]. As quorum-sensing signals and sensors differ between different types of bacteria[31], an underlying quorum-quenching mechanism would 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 quorum-sensing plays a role (Fig. 2a). In all mouse models in our study, we included (i) a human fecal isolate belonging to a sequence type (ST) frequently detected in the obtained fecal isolates (ST2196), according to multi-locus sequence typing (MLST) we performed (Supplementary Tab. 1), (ii) a mouse infection isolate (ST88)[32], and (iii) 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* were detected in the feces and colonized the large and small intestines at the end of the experiment (competition index ≥100) (Fig. 2b, Extended Data Fig. 2 a,b). Furthermore, in a non-competitive experimental setup, only those bacteria expressing the intracellular Agr effector RNAIII[34] achieved colonization, while *agr*-negative control strains never did (Fig. 2c, 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 analyzed whether culture filtrates of the *Bacillus* isolates collected from human feces can inhibit Agr. To that end, we used an *S. aureus* reporter

strain, in which we had transferred the luminescence-conferring *luxABCDE* operon into the genome under control of the Agr P3 promoter[34], which controls production of RNAIII. Remarkably, culture filtrates from all 105 isolates reduced Agr activity in that assay by at least 80% (Fig. 3a, Extended Data Tab. 1). No growth effects were observed, substantiating that growth inhibition does not underlie the inhibitory phenotype. Furthermore, 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 leukocidin) (Fig. 3b,c, Supplementary Fig. 1). These results indicated that the inhibitory effect of the *Bacillus* isolates on *S. aureus* colonization is due to a secreted substance that inhibits Agr signaling.

To characterize the Agr-inhibitory substance, we performed experiments with culture filtrate of the reference *B. subtilis* strain. The substance in question was thermostable and resistant to protease digestion (Extended Data Fig. 3 a). In reversed-phase high performance chromatography (RP-HPLC) (Extended Data Fig. 3b), significant Agr-inhibiting activity was associated with two peaks, which we then analyzed by RP-HPLC/electrospray ionization mass spectrometry (ESI-MS) (Extended Data Fig. 3c). This analysis, together with the elution behavior and published literature[36], allowed us to identify the substances as members of the fengycin cyclic lipopeptide family. Because fengycins can differ in specific amino acids in addition to the length of the attached fatty acid, which usually is β-hydroxylated (β-OH), and different *Bacillus* strains produce somewhat 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. Smaller, adjacent peaks also contained fengycin species according to RP-HPLC/ESI-MS analysis, which we tentatively identified as β-OH-C17-fengycin A and the de-hydroxylated versions of the identified three major fengycins (Extended Data Fig. 3e). For further analyses, higher amounts of β-OH-C17-fengycin B were purified to homogeneity from culture filtrate and dose-dependent Agr-inhibiting activity of this pure substance was verified (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 Tab. 1). While the production pattern of different fengycins varied in the analyzed 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 ~ 1.4 μM total fengycin (Fig. 3d). This corresponds to the median concentration of total fengycin produced by stationary phase-cultures of the *Bacillus* isolates (1.5 μM) (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[38]. RP-HPLC/ESI-MS showed specific absence of fengycins in that mutant strain, while 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 the wild-type strain (Fig. 3f). These results confirmed that fengycin production is the source of the observed Agr inhibition.

Mechanism of fengycin 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 (Fig. 4a)[39]. Different Agr subgroups of *S. aureus*, as well as different staphylococcal species, produce cyclic hepta- to nonapeptide AIPs[35]. AIPs from different subgroups or species frequently inhibit Agr signal transduction by competitive inhibition at the AgrC binding site[39–41]. Given that fengycins as 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 inhibition, we ruled out that the target of Agr inhibition by *Bacillus* is AgrB (Fig. 4c). In further support of a mechanism that works by competition with AIP for binding to the AgrC receptor, fengycin inhibition could be reversed in a dose-dependent fashion by addition of AIP (Fig. 4d). Finally, we determined the AIP concentration in early stationary growth phase (6–8 h) to be ~ 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 by Agr subtype raises the question whether fengycins have 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 the mouse experiments belong to different Agr subtypes (USA300, type I, ST88, type III, 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 analyzing the colonization kinetics of *B. subtilis* when given as spores, which corresponds to the form *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 *fenA* mutant was not different from that by the *B. subtilis* wild-type strain, ruling out that fengycin production per se impacts *B. subtilis* colonization.

Feeding mice *B. subtilis* spores completely abrogated colonization of all tested *S. aureus* strains in the feces and intestines, in experimental setups with or without antibiotic pre-

treatment to eliminate the pre-existing microbiota. (Fig. 5b,c, Extended Data Fig. 5c-f). In contrast, spores of the *fenA* mutant had no significant 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 highly likely to be even more pronounced in the tested rural population, our mouse data obtained with *S. aureus* numbers approximately equaling or exceeding those of applied *Bacillus* spores suggest that fengycin-mediated quorum-sensing interference contributes to the *S. aureus* colonization exclusion we observed in humans.

Conclusions

Scientific evidence to support the frequent claims that probiotic nutrients improve human health is scarce. This study provides evidence for a molecular mechanism of probiotic food bacteria-mediated direct interference that limits pathogen colonization. In particular, our data underscore the often-debated[10,42] probiotic value of *B. subtilis*. Interestingly, we found the responsible agents to work by quorum-quenching, demonstrating that pathogen exclusion in the gut may work by inhibition of a pathogen signaling system. Furthermore, our findings emphasize the importance of quorum-sensing for pathogen colonization.

Our study suggests several highly valuable translational applications regarding alternative strategies to combat antibiotic-resistant *S. aureus*. First, the quorum-quenching fengycins, which previously had only been known for their anti-fungal activities[43], may be used as quorum-sensing blockers for eagerly sought antivirulence-based efforts to treat staphylococcal infections[15,44]. Second, *Bacillus*-containing probiotics may 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 multiple advantages over the current standard topical strategy with antibiotics, which is aimed exclusively at decolonizing the nose[45].

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 fecal 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 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. Nasal swabs and fecal samples were streaked on mannitol

salt agar (MSA) and then incubated at 37 °C for 24 hours. Positive or negative *S. aureus* or *Bacillus* colonization could easily be distinguished by either strong growth on the entire plate, or absence of any colonies, respectively. At the time of this analysis, the purpose of the analysis was to obtain and archive colonizing *S. aureus* strains. As the hypothesis on *Bacillus*-S. *aureus* exclusion was only developed from the results of this analysis, the staff performing the analysis were blinded as for the exclusion hypothesis. Isolates were easily recognized as *S. aureus* or *Bacillus* by colony morphology and color; 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 also further distinguished by 16S rRNA sequencing (Extended Data Tab. 1). To that end, 16S rRNA genes were PCR-amplified using primers 27FB and 1492RAB[46] and BLAST similarity analysis was used to identify the species. Subjects were considered as permanently colonized by *S. aureus* if two positive samples were obtained, tested in a 4-week interval. All individuals tested either negative or positive for *S. aureus* at both times. In total, 105 *Bacillus* isolates from 101 individuals were analyzed. In the samples from four individuals, two isolates each were taken due to 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 analyzed 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 to 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 analyzing different Agr-subtype *S. aureus*) were (i) the human fecal isolate F12 of ST2196 (Supplementary Tab. 1), (ii) strain JSNZ of ST88, a mouse isolate previously described as mouse-adapted[32], (iii) 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.

Due 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 harbors multiple antibiotic resistance including to kanamycin). To that end, plasmid pKX15[49] kindly provided by B. Krismer, University of Tübingen, was treated as described[48] to delete the *xylR* promoter 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 pTX$_\Delta$16 is the corresponding empty control plasmid.

To construct the *agrBD* deletion mutant of strain LAC P3-*lux*, a 4.8 kb-PCR product from USA300 genomic DNA that included the *agrBDCA* operon, 1 kb upstream, and 1 kb downstream, was cloned into the SmaI site of pIMAY[51] and inverse PCR was used 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 the PCR product. See Supplementary Table 2 for oligonucleotides used.

To construct the tetracycline–resistant derivatives of *S. aureus* ST88 and ST2196, φ11 phage-mediated transduction was performed as described to transfer the tetracycline cassette in the donor strain (*S. aureus* RN4220 with integrated pLL29) to *S. aureus* 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* 168 obtained from the *Bacillus* Genetic Stock Center) to *B. subtilis* ZK3814. This was necessary as *B. subtilis* 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 multi-locus sequence typing (MLST) as previously 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 at the PubMLST database (https://pubmlst.org/saureus/). Not previously described alleles (*arcC* 520–521 and *gmk* 337) and STs (STs 4630 – 4638) were deposited to the website. The Agr subtype of *S. aureus* isolates was determined using a modified multiplex qRT-PCR protocol[55]. Two duplex qRT-PCR protocols using the respective described primer sets and two colored probes each were set up for Agr types I and II, and III and IV, respectively. Isolates whose Agr type could not be determined by that method were analyzed for the type of AIP production using HPLC/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 fecal 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 (San Diego, California) using an Illumina MiSeq system as described[56].

For all obtained paired-end sequences, the abundance of Operational Taxonomic Units (OTU) 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. The sequences were assigned to OTUs with the QIIME's uclust-based[58] open-reference OTU 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 nonparametric *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 of each isolate was spotted on sterile filter disks. The filters were let 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 culture filtrate of the *B. subtilis* reference strain grown for 48 h in TSB were applied to a Zorbax SB-C18 9.4 mm x 25 cm reversed-phase column (Agilent) using an AKTA Purifier 100 system (GE Healthcare). After washing with three column volumes with 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. 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 test for Agr inhibition (see below).

To purify larger amounts of the main active peak containing β-OH-C17-fengycin B, acetonitrile was added 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 3700 x *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. 10-ml fractions were collected and positive fractions (as determined by RP-HPLC/MS) were lyophilized. The lyophilisate was redissolved in 2 ml acetonitrile. Six ml of water was added and the precipitated material was removed by 5 min centrifugation in a tabletop centrifuge at maximum speed. The cleared supernatant was then further purified on a Zorbax SB-C18 9.4 mm x 25 cm reversed-phase column as described above.

PSM and lipopeptide detection by RP-HPLC/MS

PSMs were analyzed 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 × 5 mm Perkin-Elmer SPP C8 (2.7 μm) guard column was used at a flow rate of 0.5 ml/min. After sample injection, the column was washed for 0.5 min with 90% buffer A /10% buffer B, then for 3 min with 25% buffer B. Then, 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 lipopeptide (fengycins, surfactins) containing fractions were analyzed using the same column, system, and elution conditions. To quantify production of different fengycins, the two most abundant peaks, corresponding to double and triple charged ions, were used 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 strain *S. aureus* LAC[34]. Strain LAC P3-*luxABCDE* was diluted 100-fold from a pre-culture grown overnight in TSB before distribution into a 96-well microtiter plate. To 100 μl of that dilution, 100 μl of sterilized culture filtrate sample was added, 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 2. 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. Agr-inhibiting activity of purified fengycin was also measured using quantitative real-time PCR of RNAIII as described previously[30].

To determine the Agr-inhibiting activity on target strains other than LAC (Agr subtype I), the impact on the production of δ-toxin, whose gene is embedded in the Agr intracellular effector RNA, RNAIII, in most staphylococci, was used. 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 pre-culture grown in TSB. β-OH-C17-fengycin B dissolved in DMSO was added to each sample to a final

concentration of 20 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/MS detection.

Analysis of PVL and α–toxin expression

S. aureus strain LAC was diluted 100-fold from a pre-culture grown in TSB and inoculated into 500 μl TSB. Two hundred and fifty 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 tabletop centrifuge at maximum speed for 5 min; the supernatants were collected and loaded onto 12% SDS-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 (polyclonal rabbit serum; Sigma S7531; dilution 1:5,000) or anti-LukF-PV (affinity-purified rabbit IgG specific for a peptide region of LukF-PV, produced by GenScript USA Inc. and kindly provided by F. DeLeo, NIAID; dilution 1:500) antibodies 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-labeled 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 pre-culture (1:100) into 1 l of 2 x SG medium[63] and allowed to sporulate for 96 hours. Cells were pelleted, washed with water, and resuspended in 20% metrizoic acid (Sigma). Five different concentrations (w/v) of metrizoic acid (60% - 20%) were added stepwise to a 50-ml centrifuge tube to obtain a density gradient. Then, a cell suspension was added to the top of the gradient, followed by centrifugation at 40,000 × *g* for 60 min at 4 °C[64]. Spores were found in the middle layers and were collected. They were washed with 10 ml water for three times. The total obtained number of viable spores per ml was determined by serial dilution, plating on TSA, and counting CFUs. The total number of heat-resistant spores per ml was determined by submerging the spores in water bath at 80 °C for 20 min followed by serial dilution and quantification of CFU/ml as described above.

Murine intestinal colonization model

In-vivo studies were approved by the Institutional Animal Care and Use Committee of the NIAID. Animal work was conducted adhering to the institution's guidelines for animal use, and followed the guidelines and basic principles in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited facility.

All C57BL/6J mice were female and six to eight weeks of age at the time of use. In one setup, before *S. aureus* was given by oral gavage, mice were pre-treated to eradicate the pre-existing intestinal microbiota using an antibiotic mix consisting of ampicillin (1 g/l), metronidazole (1 g/l), neomycin trisulfate (1 g/l), and vancomycin (1 g/l), in the drinking

water. The last day before gavage, antibiotics were omitted from the drinking water. No bacteria could be found in the feces or intestines of mice for 7 days after this treatment in a control experiment. In another setup, antibiotic pre-treatment was omitted. In all setups, *S. aureus* strains were grown to mid-exponential growth phase, washed, and resuspended in sterile PBS at 10^8 CFU/ml. Mice were inoculated by oral gavage with 200 µl of a 10^8 CFU/ml 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 pKX_Δ plasmid-containing strains, mice received kanamycin (0.2 g/l) 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 the *Bacillus* wild-type or its isogenic Δ*fenA* fengycin mutant (10^8 CFU/ml in sterile PBS) was performed on the day following the *S. aureus* gavage, and repeated every second day thereafter for a total of 3 times (days 2, 4, and 6). Intestinal colonization was evaluated by quantitative cultures of mice stool samples and samples from the small and large intestine. 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 pre-treatment, extracts were plated on MSA plates containing 4 µg/ml oxacillin (for strain USA300 LAC) or 3 µg/ml tetracycline (for tetracycline-resistant derivatives of ST88 and ST2196), incubated for 48 h at 37 °C, and enumerated.

Statistics

Statistical analysis was performed using GraphPad Prism Version 6.05 using 1-way or 2-way ANOVA, or Fisher's exact test, as appropriate, except for the experiments shown in Figures 2c, 5b,c, and Extended Data Figs. 2 b,cand 5c-f, for which Stata Release 15 and Poisson regression was used, due to the exclusive presence of 0 values in one group (no variance). In ANOVAs, Tukey post-tests were used, which correct for multiple comparisons using statistical hypothesis testing. All error bars show the mean and standard deviation (SD). All replicates are biological.

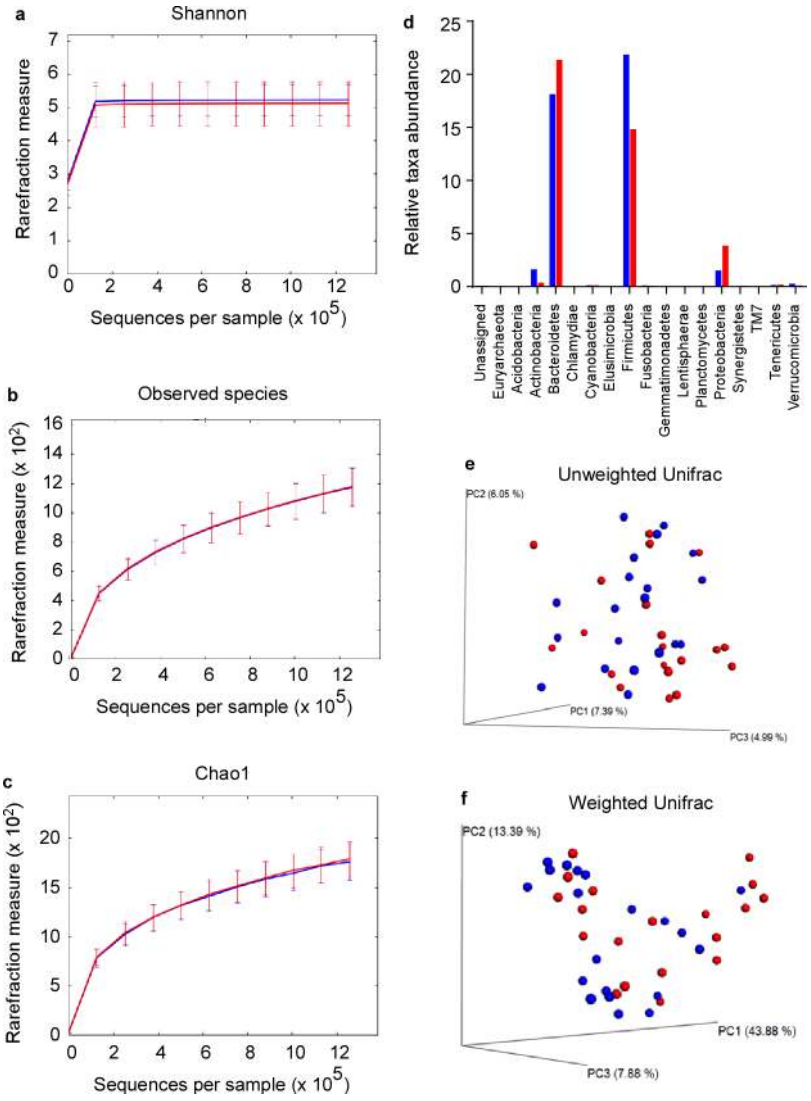
Data availability

Microbiome sequencing data are available at http://www.ncbi.nlm.nih.gov/bioproject/483343. All other data generated or analyzed during this study are included in this published article or in the supplementary information files.

Supplementary Material

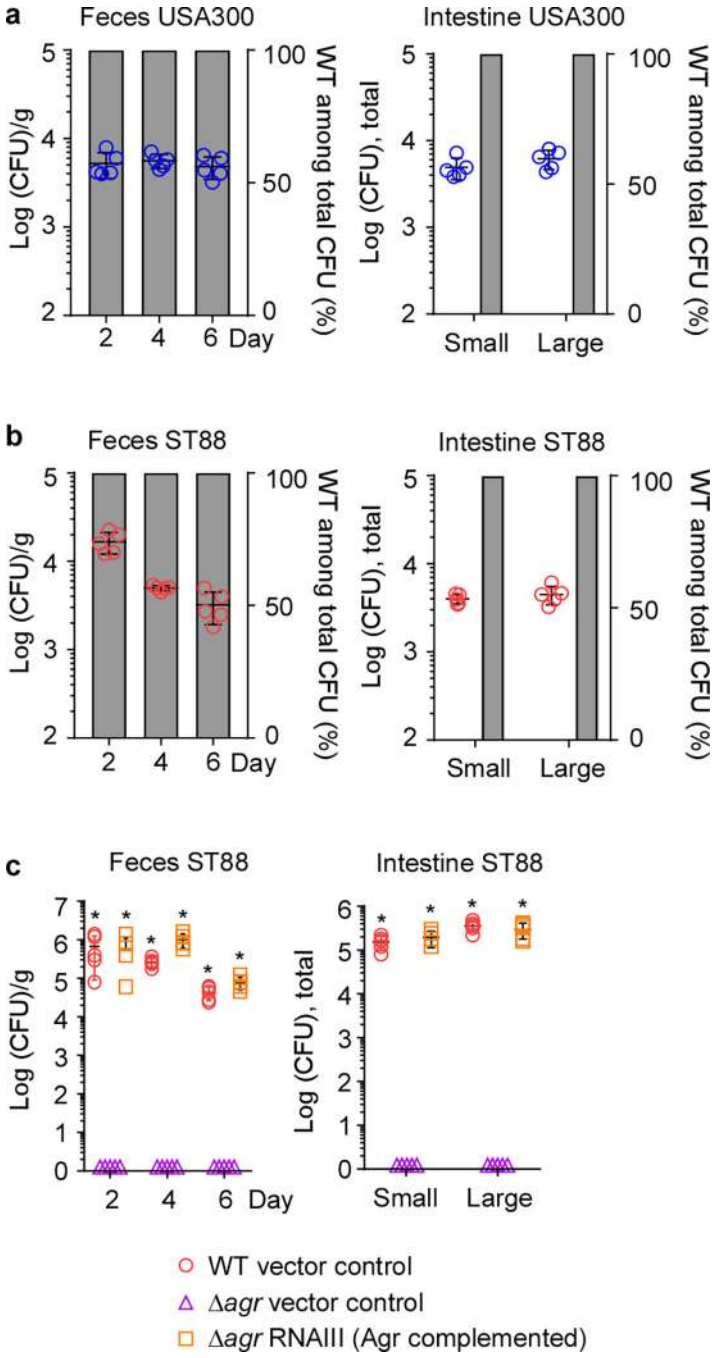
Refer to Web version on PubMed Central for supplementary material.

Extended Data



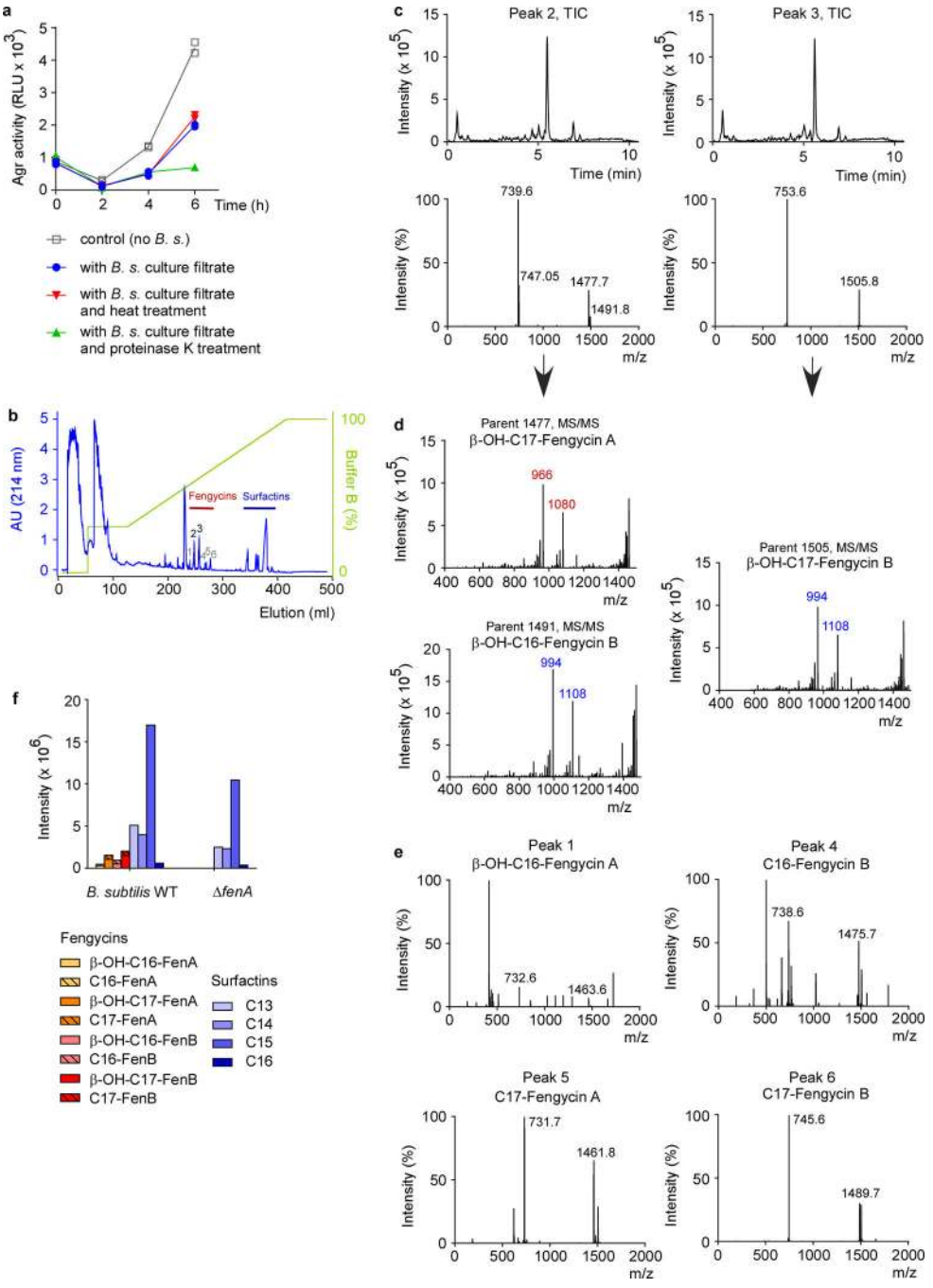
Extended Data Figure 1 |. Microbiome analysis of *S. aureus* carriers versus non-carriers.

The microbiota of n=20 randomly selected *S. aureus* carriers (red) and n=20 non-carriers (blue) were analyzed in fecal samples. **a-c**, Rarefaction curves of 16S rRNA gene sequences. Error bars shown the means ±SD. **a**, Shannon index. **b**, Observed species against the number of sequences per sample. **c**, Chao1 index. **d**, Relative taxa abundance comparison between *S. aureus* carriers (red) and non-carriers (blue), **e-f,** beta diversity, represented by a Principal Coordinate Analysis (PCoA) plot based on unweighted UniFrac **(e)** and weighted UniFrac metrics **(f)** for samples from *S. aureus* carriers (red) and non-carriers (blue).



Extended Data Figure 2 |. Quorum-sensing dependence of *S. aureus* intestinal colonization.
Data from strains USA300 LAC and ST88 JSNZ. The experimental setup is the same as in **Fig. 2** of the main manuscript: Mice received by oral gavage 100 µl containing 10^8 CFU/ml of wild-type (WT) *S. aureus* strains USA300 LAC or ST88 JSNZ and another 100 µl of 10^8 CFU/ml of the corresponding isogenic *agr* mutant (n=5/group, competitive experiment shown in **a,b**), or 200 µl containing 10^8 CFU/ml wild-type, isogenic *agr* mutant, or Agr (RNAIII)-complemented *agr* mutant (n=5/group, non-competitive experiment shown in **c**). CFU in the feces were determined two, four, and six days after infection. At the end of the

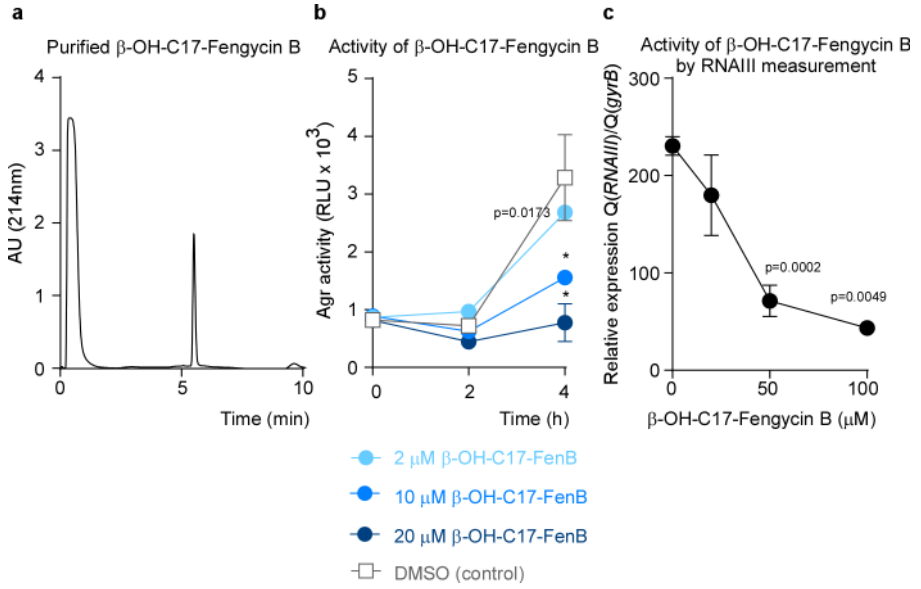
experiment (day seven), CFU in the small and large intestines were determined. **a**,**b** Competitive experiment. Total obtained CFU are shown as dot plots, also showing the means ±SD. Bars show the percentage of wild-type among total determined CFU, of which 100 were analyzed for tetracycline resistance 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 harbored the $pKX_{\Delta}16$ control plasmid; Agr-complemented strains harbored pKX_{Δ}RNAIII, constitutively expressing RNAIII, which is the intracellular effector of Agr. During the experiment, mice received 200 μg/ml kanamycin in the drinking water to maintain plasmids. Statistical analysis was performed using Poisson regression versus values obtained with the *agr* mutant strains. *, $p<0.0001$. Error bars show the means ±SD. Note no bacteria were found in the feces 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 Figure 3 |. Analysis of Agr-inhibitory substances.

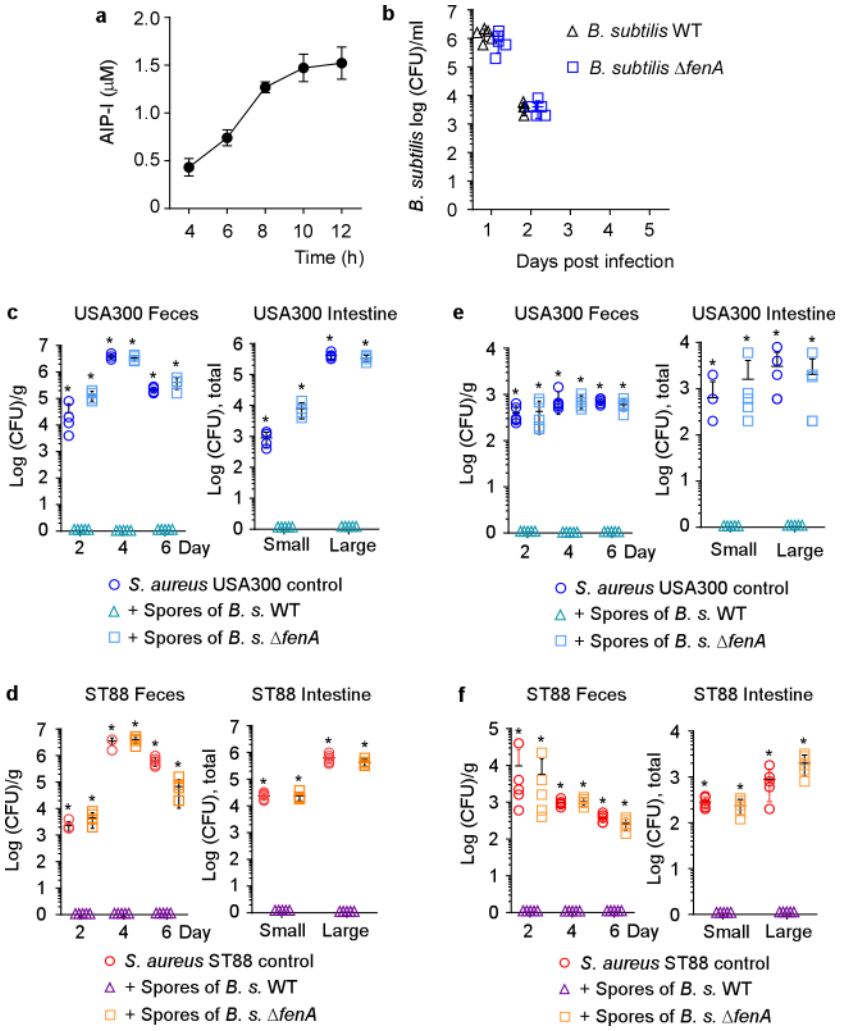
a, Influence of heat and protease on Agr inhibition. *B. subtilis* (*B. s.*) culture filtrate was subjected to heat (95 °C, 20 min) or proteinase K digestion (50 μg/ml, 37 °C, 1 h) and the impact on inhibition of Agr activity was measured using the luminescence assay with the USA300 P3-*luxABCDE* reporter 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, as compared to the *B.s.* culture filtrate sample, is expected due to proteolytic

inactivation of intrinsic AIP.) **b**, Preparative RP chromatography of *B. subtilis* culture filtrate to determine the Agr-inhibiting substance. The peaks labeled 2 and 3 showed significant 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 labeled 1 and 4–6 also contained fengycin species (see **e**). **c**, Fractions corresponding to the 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 for a given fengycin subtype (A or B in this case) are marked in color. **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 HPLC/MS analyses (as shown in **b** and **d**) were repeated multiple (> 10) times for fengycin purification with similar results. MS/MS analyses were not repeated. **f**, Analysis of fengycin and surfactin lipopeptide expression of the *B. subtilis* wild-type strain and its isogenic Δ*fenA* mutant.



Extended Data Figure 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 2-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 (1-way ANOVA with Tukey's post-test. Comparisons shown are those versus 0 μM value). **b**,**c**, The experiments were performed with n=3 independent biological samples. Error bars show the means ±SD.



Extended Data Figure 5 |. Inhibition of *S. aureus* colonization by dietary fengycin-producing *Bacillus* spores in a mouse model.

a, AIP concentration during *S. aureus* growth. Strain LAC (USA300) was grown in TSB and AIP concentrations were measured by HPLC/MS. Calibration was performed using synthetic AIP. The detection limit of this assay is ~ 0.3 μM. The experiment was performed with n=3 independent biological samples. Error bars show the means ±SD. **b**, *B. subtilis* colonization kinetics in the mouse intestinal colonization experiment. Mice (n=5) received 200 μl of a 10^8 CFU/ml suspension of *B. subtilis* wild-type or Δ*fenA* mutant spores by oral gavage and CFU in the feces were analyzed up to 5 days afterwards. Error bars show the means ±SD. **c-f**, Inhibition mouse model with strains USA300 LAC and ST88 JSNZ. The experimental setup was the same as shown in Fig. 5a. n=4 or 5 mice/group received 200 μl of 10^8 CFU/ml *S. aureus* strains USA300 LAC or ST88 JSNZ by oral gavage. On the next and every following second day, they received 200 μl of 10^8 CFU/ml spores of the *B. subtilis* wild-type (WT) or its isogenic *fenA* mutant, also by oral gavage. CFU in the feces 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 pre-treatment. Statistical analysis was performed using

Poisson regression versus values obtained with the *B. subtilis* WT spore samples. *, $p<0.0001$. Error bars shown the means ±SD. Note no *S. aureus* were found in the feces or intestines of any mouse challenged with any *S. aureus* strain receiving *Bacillus* wild-type spores. The corresponding zero values are plotted on the x-axis of the logarithmic scale.

Extended Data Tab. 1 |

Fengycin production and Agr inhibitory potency of *Bacillus* fecal 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*	5833	437	984	288	2751	1248	1760	1090	98	14390
18	*subtilis*	107	0	58	118	0	0	53	23	97	359
19	*ticheniformis*	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	*ticheniformis*	89	119	70	99	98	102	150	0	96	727
35	*ticheniformis*	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	*ticheniformis*	150	117	249	0	241	271	226	230	96	1484
40	*subtilis*	753	174	1260	1124	1548	538	841	821	80	7058
41	*subtilis*	2298	860	1777	0	4524	816	1563	1957	91	13796
42	*sonorensis*	34	43	0	0	41	57	88	0	96	263
43	*sonorensis*	477	0	2488	816	1667	604	1216	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*	5007	828	979	0	3147	0	2027	1210	97	13200
50	*subtilis*	429	0	933	0	1065	354	614	526	90	3922
51	*subtilis*	712	185	911	208	1187	665	863	586	88	5316

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
52	*subtilis*	9630	1571	923	0	2365	1775	4690	1099	95	22052
53	*ticheniformis*	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	1008	0	0	297	715	488	82	2508
61	*subtilis*	124	84	21	0	160	39	42	335	97	805
62	*ticheniformis*	93	128	7	48	40	188	0	153	97	657
63	*amyloliquefaciens*	4153	380	975	0	2584	1047	1975	1084	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*	14881	4578	88106	34879	39939	16967	42502	23859	97	265710
74	*pumilus*	157	56	54	14	0	62	177	41	95	560
75	*ticheniformis*	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*	1741	322	4222	1105	3327	910	2207	1529	91	15363
81	*subtilis*	1739	426	5073	1579	3371	998	3241	1933	87	18361
82	*subtilis*	1002	0	3413	921	1998	0	1710	992	91	10037
83	*ticheniformis*	356	0	536	4	83	201	107	52	97	1338
85	*subtilis*	52	0	49	0	84	161	0	132	95	479
87	*subtilis*	1327	312	3931	0	2763	667	2624	1167	98	12790

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
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*	1845	912	4714	1310	3686	1492	2484	1557	86	18000
98	*subtilis*	1367	804	3572	1512	2803	1511	2005	1626	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	4415	3419	1874	1378	2478	1527	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	1412	637	1211	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	1788	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*	8801	3540	23045	10778	16465	6428	16077	10706	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

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
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*	1267	0	4668	1266	2940	1168	2158	1863	88	15332
131	*subtilis*	4164	496	513	307	1226	671	1948	662	83	9986
132	*subtilis*	441	291	491	391	200	562	381	279	95	3036
134	*subtilis*	6647	750	1011	0	3143	1166	2829	953	97	16498
136	*subtilis*	751	233	2569	773	1813	890	1701	1010	89	9740
137	*subtilis*	1572	328	3297	1036	2221	627	2266	0	82	11347
138	*pumilus*	288	311	232	11	415	236	236	403	85	2133
139	*subtilis*	1898	709	7830	2453	5328	2073	3880	2210	98	26381
140	*sonorensis*	0	106	217	815	225	0	103	3987	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	2563	1781	17078	5725	11963	4448	5444	4902	94	53904

[*] The table shows intensity values of the integration of m/z peaks associated with the specific fengycin species as obtained by HPLC/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 Methods.

[‡] Percentage of Agr inhibition was determined by dividing the 4-h value in the luminescence assay (using 100 µl of culture filtrate) obtained for the sample by that obtained for the control and multiplying by 100.

Extended Data Tab. 2 |

Analysis of previous microbiome studies* for correlation between *S. aureus* and *B. subtilis* presence 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%)

* Inclusion criteria: All studies found on the EBI Metagenomics website (https://www.ebi.ac.uk/metagenomics/) with > 200 participants (independent samples) using Illumina Miseq instruments were included in the analysis.

Analysis: Raw 16S rRNA sequencing data were pooled from the EBI Metagenomics website. Taxonomic assignment (TSV) files were used for analysis. The number of sequence reads was used to analyze how many samples contain *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 non-colonized.

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Tübingen, for plasmid pKX15, Frank DeLeo, NIAID, for anti-LukF-PV, and Nana A. Amissah for technical assistance. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH) (project ZIA AI000904–16, M.O.) and the Thailand Research Fund through the Royal Golden Jubilee PhD Program (grant no. PHD/0072/2557 to P.P. and P.K.). P.K. was also supported by the Faculty of Medicine Siriraj Hospital, Mahidol University, Grant Number (IO) R015833012, P.P. by the Graduate Partnership Program of the NIH, and S.W.D. by the Postdoctoral Research Associate Program of the National Institute of General Medical Sciences (1FI2GM11999101).

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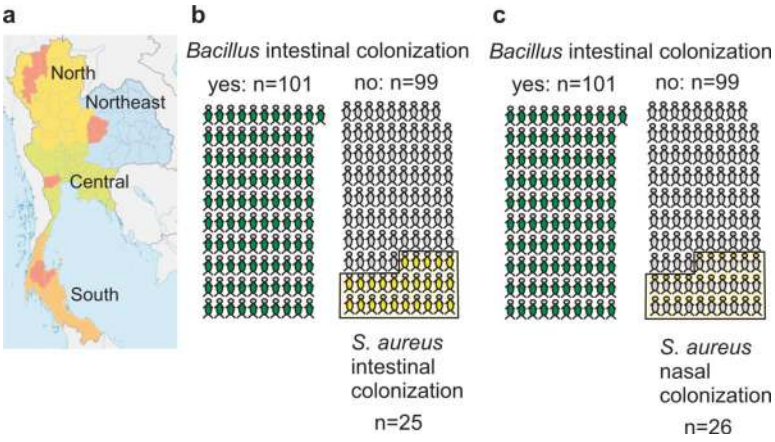

Figure 1 |. *S. aureus* colonization exclusion by dietary *Bacillus* in a human population.

a, Areas (in red) from which fecal samples were collected in rural populations and analyzed for 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*.

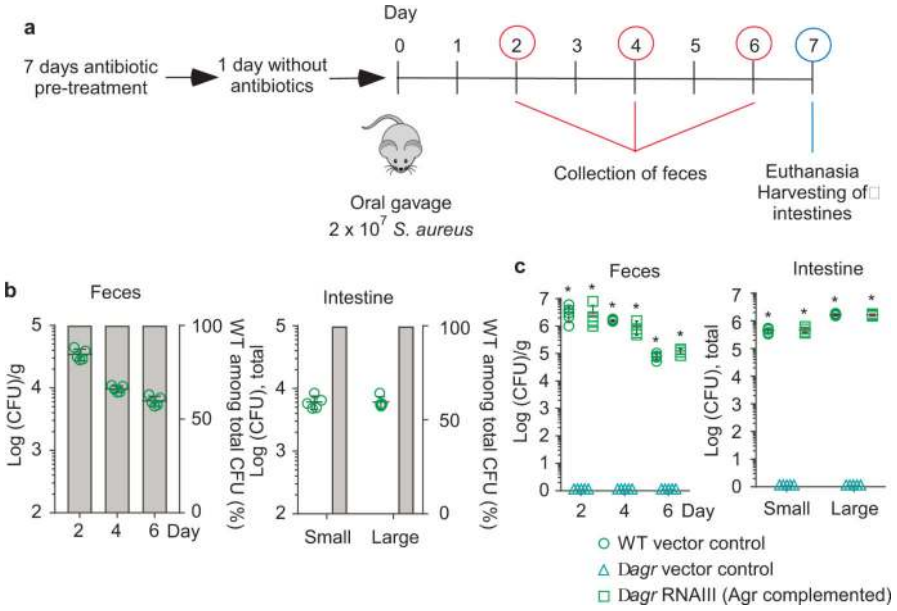

Figure 2 |. Quorum-sensing dependence of *S. aureus* intestinal colonization.

a, Experimental setup of the murine intestinal colonization model. Mice received by oral gavage 100 μl containing 10^8 CFU/ml of wild-type (WT) *S. aureus* strain ST2196 F12 and another 100 μl of 10^8 CFU/ml of the corresponding isogenic *agr* mutant (n=5/group, competitive experiment shown in **b**), or 200 μl containing 10^8 CFU/ml wild-type, isogenic *agr* mutant, or Agr (RNAIII)-complemented *agr* mutant (n=5/group, non-competitive experiment shown in **c**). CFU in the feces 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 showing the means ±SD. Bars show the percentage of wild-type among total determined CFU, of which 100 were analyzed 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 harbored the $pKX_{\Delta}16$ control plasmid; Agr-complemented strains harbored pKX_{Δ}RNAIII, constitutively expressing RNAIII, which is the intracellular effector of Agr. During the experiment, mice received 200 μg/ml kanamycin in the drinking water to maintain plasmids. Statistical analysis was performed using Poisson regression versus values obtained with the *agr* mutant strains. *, $p<0.0001$. Error bars shown the means ±SD. Note no bacteria were found in the feces 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 the corresponding data using strains USA300 LAC and ST88 JSNZ.

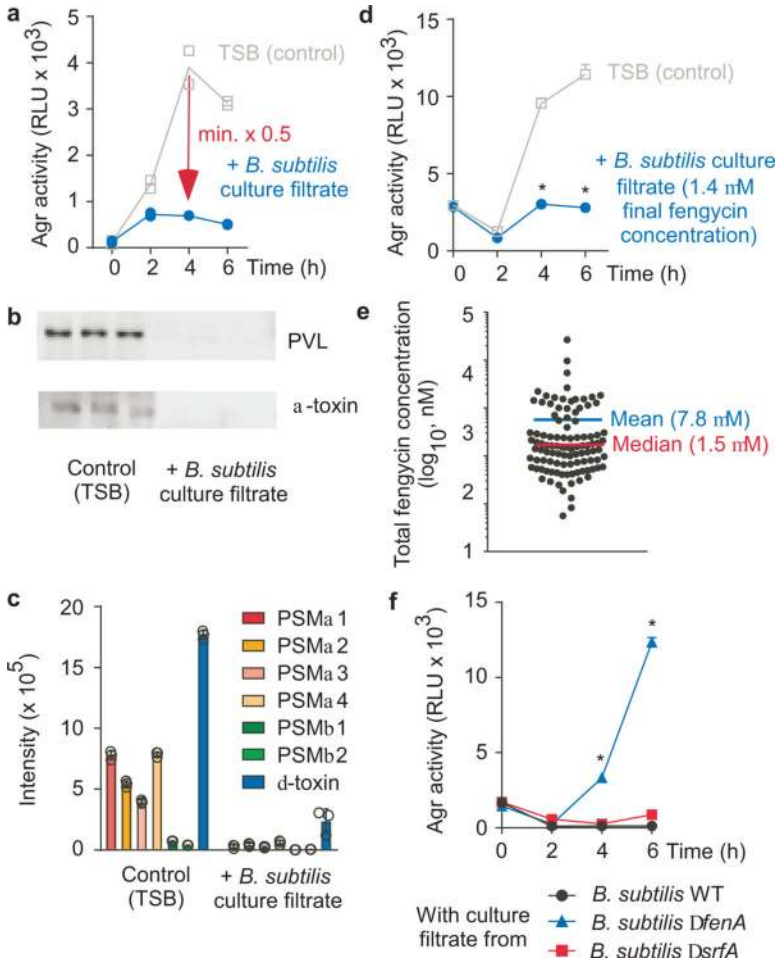

Figure 3 |. *S. aureus* quorum-sensing inhibition by *Bacillus* fengycin lipopeptides.

a, Example of an Agr inhibition experiment. The *Bacillus* isolate was considered inhibitory if luminescence after 4 h growth of *S. aureus* was ≤0.5 times that of the control value. RLU, relative light units. The experiment was performed with n=2 biologically independent samples. The lines connect the means. **b**, Inhibition of expression of PVL and α-toxin, using culture filtrate of the *B. subtilis* reference strain. Western blot analysis of n=3 biologically independent samples was performed with *S. aureus* culture filtrates grown for 4 h. See Supplementary Fig. 1 **f**or the entire blots. **c**, Inhibition of expression of PSM toxins using culture filtrate of the *B. subtilis* standard strain. PSM expression was determined by RP-HPLC/ESI-MS after 4 h of growth. **d**, Test for inhibitory capacity of *Bacillus* culture filtrate applied to a final concentration representing the median concentration of total fengycin in the tested 106 *Bacillus* isolates. *, p<0.0001 (2-way 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 (2-way ANOVA with Tukey's post-test versus WT). **c,d,f**, The experiments were performed with n=3 biologically independent samples. Error bars shown the means ±SD.

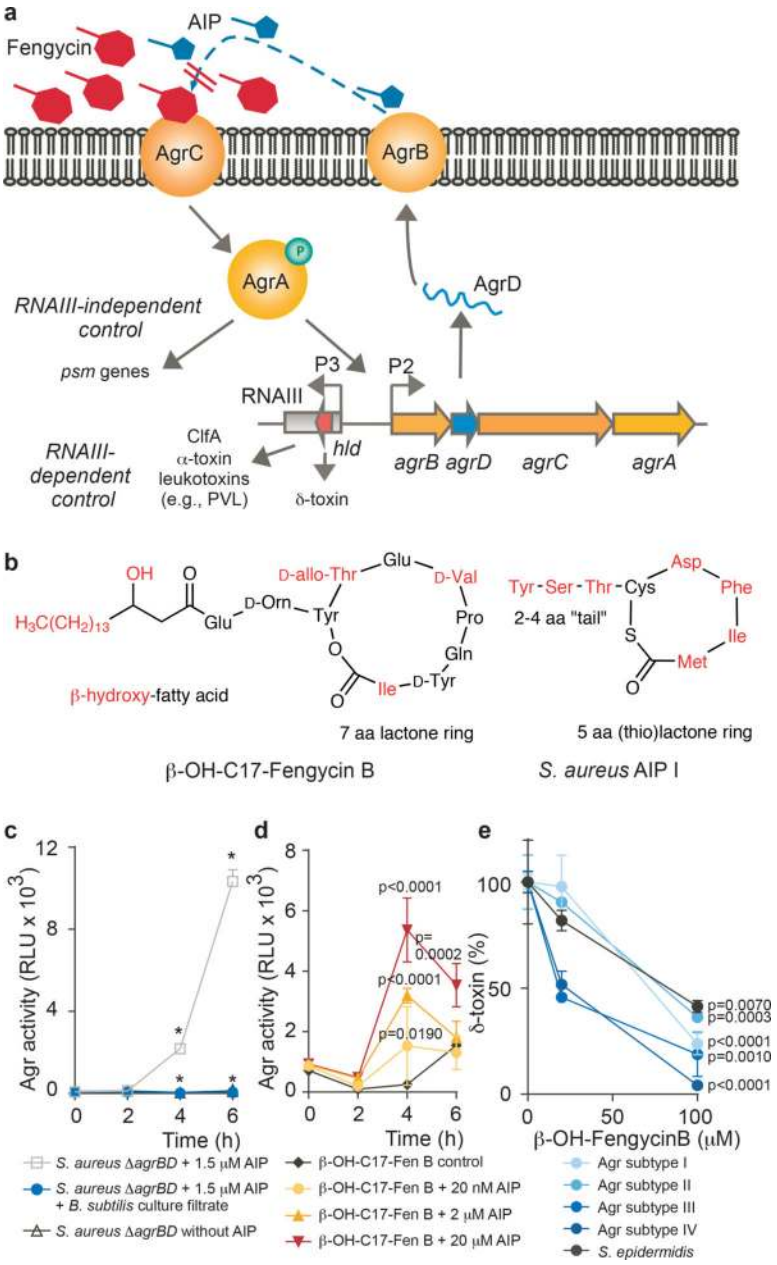

Figure 4 |. Competitive inhibition of *S. aureus* AIP activity by fengycins.

a, Model of competitive Agr inhibition by fengycins. The *agrBDCA* operon, 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 auto-phosphorylation triggers phosphorylation and activation of the DNA-binding protein, AgrA. In addition to stimulating transcription from the P2 promoter (auto-induction), AgrA drives expression of RNAIII, which in turn regulates expression of target genes. RNAIII also encodes the δ-toxin. Furthermore, AgrA drives PSM expression 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/amino acids that may differ in different subtypes. **c**, Fengycins work by inhibition of AgrC.

Shown is the Agr inhibition by fengycin-containing *Bacillus* culture filtrate using an *agrBD*-deleted strain in which AgrC was stimulated by exogenously added AIP. * $p<0.0001$ [2-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 2-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 via RP/HPLC-MS. Statistical analysis is by 2-way ANOVA with Tukey's post-test versus intensity values obtained without addition of fengycin. Values were calculated as percentage relative to intensity values obtained without addition of fengycin, due to different δ-toxin expression levels in the different strains. **c-e**, The experiments were performed with n=3 biologically independent samples. Error bars shown the means ±SD.

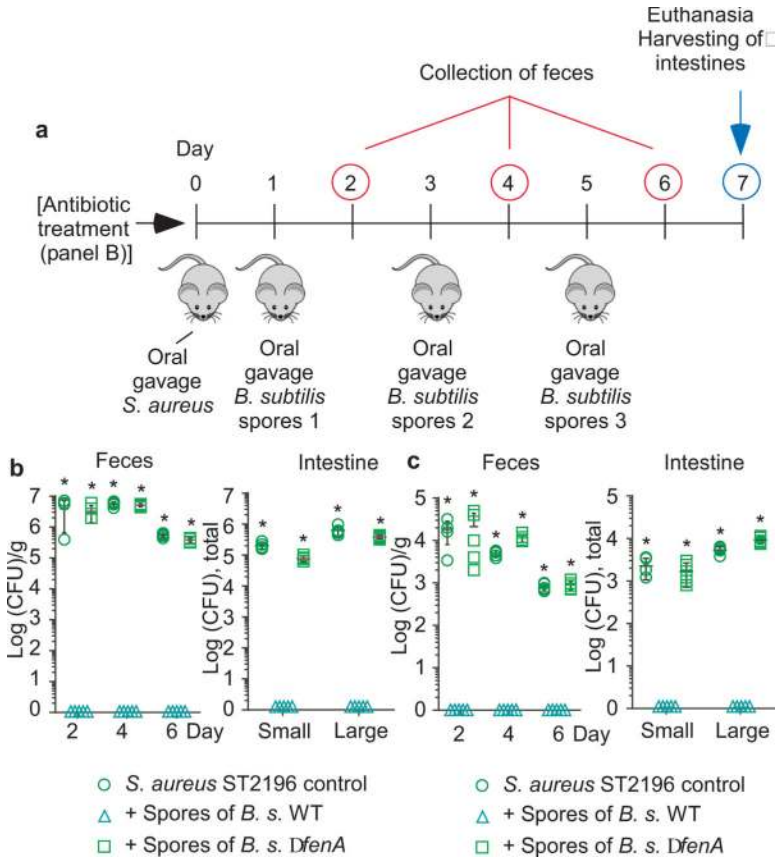

Figure 5 |. Inhibition of *S. aureus* colonization by dietary fengycin-producing *Bacillus* spores in a mouse model.

a, Experimental setup. n=5 mice/group received 200 μl of 10^8 CFU/ml *S. aureus* strain ST2196 F12 by oral gavage. On the next and every following second day, they received 200 μl of 10^8 CFU/ml spores of the *B. subtilis* wild-type (WT) or its isogenic *fenA* mutant, also by oral gavage. CFU in the feces 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 pre-treatment. **b**,**c** Results. Statistical analysis was performed using Poisson regression versus values obtained with the *B. subtilis* WT spore samples. *, p<0.0001. Error bars shown the means ±SD. Note no *S. aureus* were found in the feces or intestines of any mouse challenged with any *S. aureus* strain 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 the corresponding data using strains USA300 LAC and ST88 JSNZ.