Supplemental Information

Supplemental Data

Figure S1

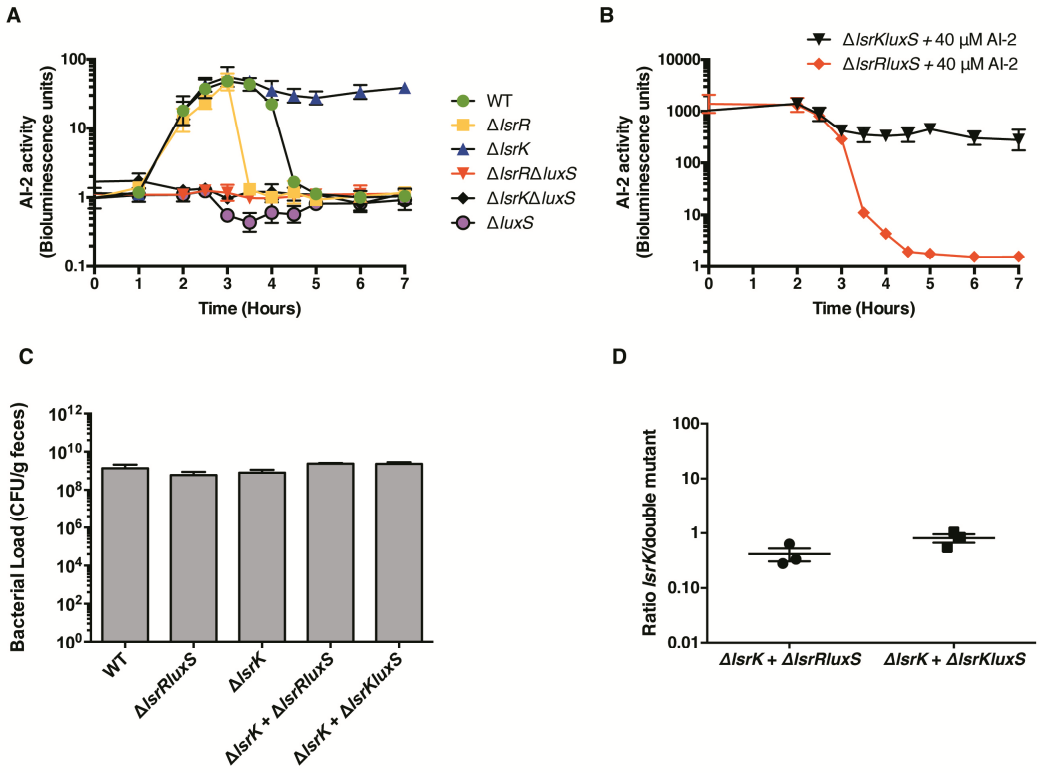

Figure S1. *In vitro* and *in vivo* validation of engineered *Escherichia coli* mutant strains, related to Figure 1. A. *In vitro* extracellular AI-2 activity in cell-free supernatants from cultures of wild-type (WT), *ΔlsrK, ΔlsrR, ΔluxS, ΔlsrRΔluxS* and *ΔlsrKΔluxS E. coli*. **B.** Extracellular AI-2 activity in cell-free supernatants from cultures of *ΔlsrRΔluxS* and *ΔlsrKΔluxS E. coli* grown in the presence of exogenously supplied chemically synthesized AI-2 (40 μM). Data shown is the mean and standard deviation from 3 independent experiments. **C.** Fecal bacterial load as *E. coli* CFU/g feces on day 5 of colonization of germ-free mice colonized with 10^5 CFU of WT, *ΔlsrK*, *ΔlsrRΔluxS* or a 1:1 mix of *ΔlsrK* and *ΔlsrRΔluxS*, or *ΔlsrK* and *ΔlsrKΔluxS* mutant *E. coli*. No bacteria were recovered from mice gavaged with PBS. Data shown are the mean and SEM, where n =3. **D.** Ratios between the mutant *E. coli* strains from C, recovered in the feces 5 days after gavage in competition with each other. Data shown are the mean and SD, where n =3.

Figure S2

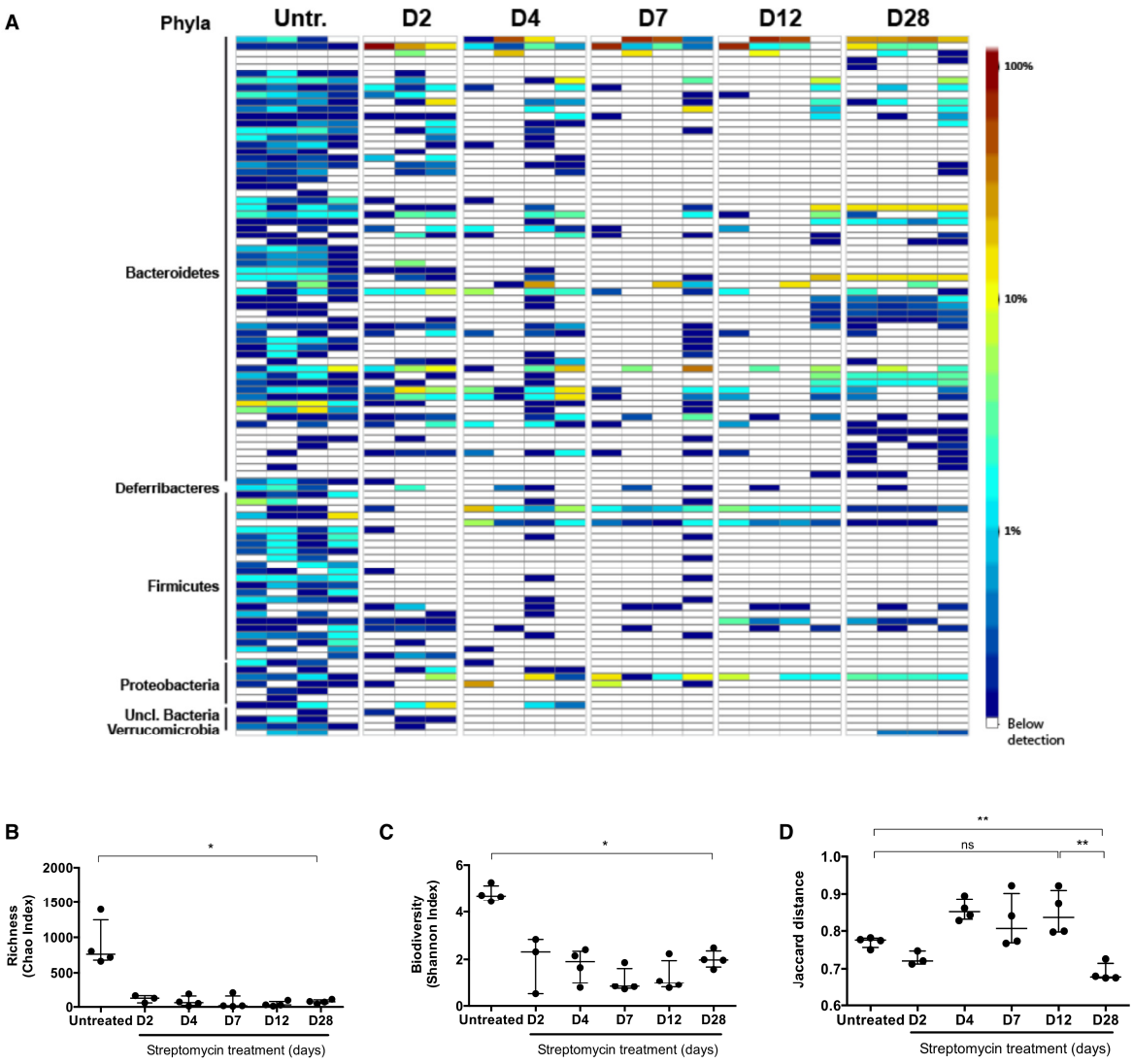

Figure S2. Effect of streptomycin treatment upon the gut microbiota, related to Figure 2. Analysis of the microbiota from fecal samples collected from 4 mice (except on day 2 where data for 3 mice is shown) prior to and 2, 4, 7, 12 and 28 days into streptomycin treatment. **A.** Heat map showing the relative abundance of the 100 most abundant phylotypes (OTUs defined with 97% identity in 16S rRNA gene sequence). Each column shows the OTUs for each mouse at the time point indicated. Below detection = 0 counts. **B.** Chao and **C.** Shannon indices of the richness and diversity of the gut microbiota, respectively. **D.** The mean Jaccard distance of the bacterial communities of each mouse versus all other mice. Data shown in **B.**, **C.** and **D.** are the median and interquartile range. Data were analyzed with the paired Students t-test (*p<0.05; ** p<0.01; ns, not significant).

Figure S3

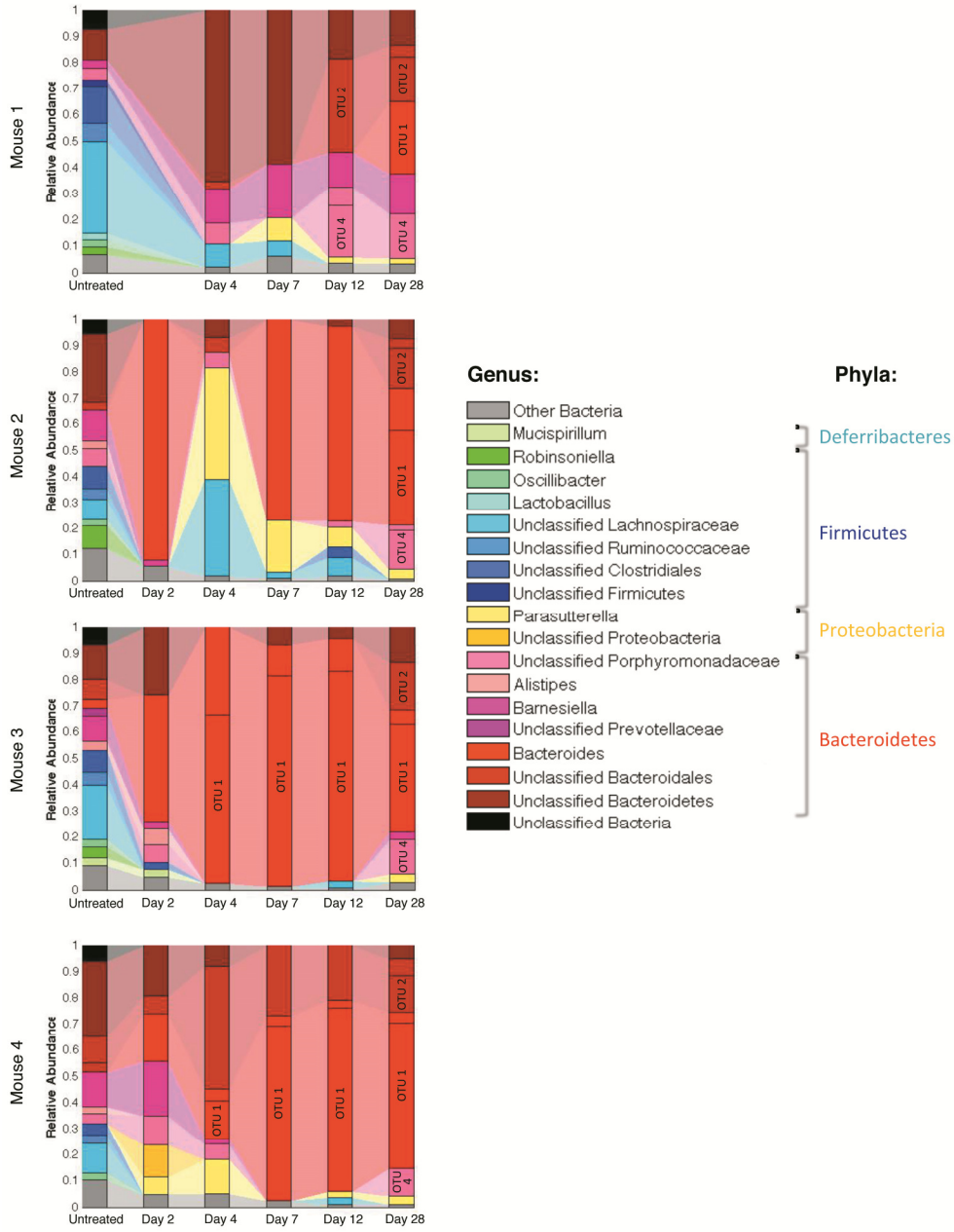

Figure S3. Analysis of the microbiota composition in each of the 4 individually housed mice, related to Figure 2. Intestinal microbiota composition for mice 1-4 at the time points indicated. Each stacked bar represents the microbiota composition of the most abundant bacterial taxa in each mouse. The colored segments represent the relative fraction of each bacterial taxon. The proportion of the 3 most abundant OTUs is also shown with the color corresponding of the taxa to which it belongs.

Figure S4

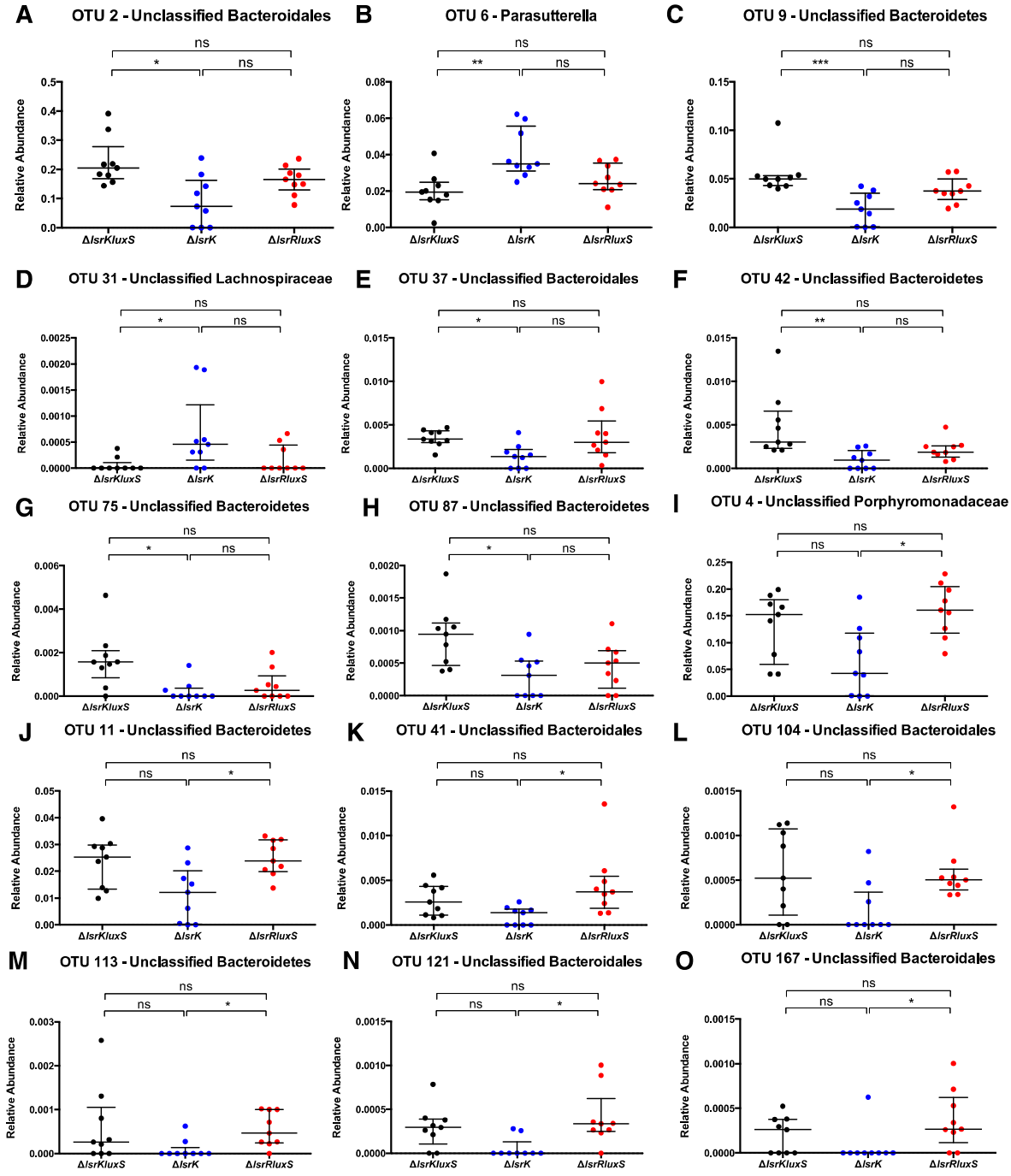

Figure S4. *ΔlsrK* mutant *E. coli* influences the relative abundance of individual OTUs, related to Figure 4. Relative abundance of OTUs in the flora of mice colonized with either *ΔlsrKΔluxS, ΔlsrK or ΔlsrRΔluxS* mutant *E. coli*. **A-H** relative abundance of individual OTUs that show differences between the group of mice colonized with *ΔlsrK* in comparison with *ΔlsrKΔluxS* while **I-O** show OTUs with differences between *ΔlsrK* in comparison with *ΔlsrRΔluxS*. OTUs that differ between *ΔlsrK* and both other groups are shown in the main

text (Fig. 4). No OTU with significant differences between *ΔlsrKΔluxS* and *ΔlsrRΔluxS* was identified. Data shown is the median with error bars indicating the interquartile range for each group where n=9. Data were analyzed with the Wilcoxon test using the Benjamini-Hochberg correction (*q<0.1; **q<0.05; ***q<0.01; ns, not significant).

Supplemental Experimental Procedures

Bacterial strains and culture conditions

All *E. coli* strains used in this study are listed in the table below. *E. coli* was routinely cultured in Luria-Bertani (LB) broth at 37˚C with aeration except where otherwise indicated. *Vibrio harveyi* strain TL26 (Long et al., 2009) was grown in Autoinducer Bioassay (AB) medium (Greenberg et al., 1979) or Luria Marine (LM) medium (Bassler et al., 1994) at 30˚C with aeration. Where appropriate, antibiotics were supplemented at the following concentrations: streptomycin, 100 µg/ml; ampicillin, 100 µg/ml; kanamycin, 50 µg/ml, chloramphenicol, 50 µg/ml; gentamycin, 10 µg/ml.

Table 1. *E. coli* strains used in this study

***E. coli* Strain**	**Relevant Genotype**	**Parental Strain**	**Plasmid**	**Source**
MG1655	WT			(Blattner et al., 1997)
KX1001	*rpsL-1(K43N),* sm^R	MG1655		This study
pKD46	*γ, β, exo* amp^R, T^s		pKD46	(Datsenko and Wanner, 2000)
pCP20	FLP recombinase, amp^R, cm^R, T^s		pCP20	(Datsenko and Wanner, 2000)
pTL17	IPTG inducible FLP recombinase, Cm^r		pTL17	(Long et al., 2009)
KX1200	Δ*luxS*::*cm*	MG1655		(Xavier and Bassler, 2005)
KX1326	Δ*lsrR::kan*	MG1655		This study
KX1440	Δ*lsrK::cm*	MG1655		This study
MC4100-YFP	yfp, sm^R	MC4100		(Hegreness et al., 2006)
MC4100-CFP	cfp, sm^R	MC4100		(Hegreness et al., 2006)
ARO043	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp*	MG1655		This study
ARO045	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp*	MG1655		This study
ARO071	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp, ΔlsrK::frt*	ARO043		This study
ARO073	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp, ΔlsrK::frt*	ARO045		This study
ARO085	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp, ΔluxS::frt*	ARO043		This study
ARO089	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp, ΔluxS::frt*	ARO045		This study
JAT154	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp, ΔlsrR::frt*	ARO043		This study
JAT156	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp, ΔlsrR::frt*	ARO045		This study
ARO093	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp, ΔlsrK::frt, ΔluxS::frt*	ARO071		This study
ARO097	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp, ΔlsrK::frt, ΔluxS::frt*	ARO073		This study
ARO081	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*yfp*::*amp, ΔlsrR::frt, ΔluxS::frt*	JAT154		This study
ARO0103	sm^R, Δ*lacIZYA*::frt, Δ*galk*:P*lac*::*cfp*::*amp, ΔlsrR::frt, ΔluxS::frt*	JAT156		This study

Genetics and molecular techniques

CFP- or YFP-expressing *E. coli* mutant strains affected in AI-2 production were constructed from KX1001, an *E. coli* K-12 MG1655 strain carrying the *rpsL-1(K43N)* mutation which confers streptomycin resistance. The *lacIZYA* operon was deleted using the λ red recombinase system encoded on the ampicillin resistant plasmid pKD46 (Datsenko and Wanner, 2000). The same method was used to introduce the yellow (yfp) or cyan (cfp) fluorescent protein-encoding genes linked to an ampicillin resistance-encoding gene in the *galK* locus under the control of the *lac* promoter (for constitutive expression), in this case using the gentamicin resistant pKD46 plasmid. The *yfp* and *cfp* gene fragments were amplified by PCR from the previously constructed MC4100_YFP/CFP strains. Deletions in *luxS*, *lsrR* and *lsrK* were introduced by bacteriophage P1-mediated transduction as described previously (Silhavy et al., 1984) using lysates from strains KX1200 (Xavier and Bassler, 2005), KX1326 and KX1440, respectively. KX1326 and KX1440 were also constructed using the λ red recombinase system to delete *lsrR* and *lsrK* using the primers described in the table below. The double mutants, *ΔlsrRΔluxS* and *ΔlsrKΔluxS,* were constructed by a second P1 transduction of the *luxS* gene deletion (from KX1200) into the *ΔlsrR* and *ΔlsrK* backgrounds, respectively. Antibiotic resistance cassettes were removed from the mutants using the FRT/FLP recombinases in either pCP20 or pTL17 (Datsenko and Wanner, 2000; Long et al., 2009). Details of all primers used in this study are in the following table.

Table 2. Primers used in this study

Primer name	Sequence (5'-3')	Reference
ΔlacIZYA del F-	CCTTTGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGTAGGCTGGAGCTGCTTC	This study
ΔlacIZYA del R-	GCTGAACTTGTAGGCCTGATAAGCGCAGCGTATCAGGCAATTTTTATAATCATATGAATATCCTCCTTAGT	This study
CFP_Δgalk-F	TTCATATTGTTCAGCGACAGCTTGCTGTACGGCAGGCACCAGCTCTTCCGCACGTTAAGGGATTTTGGTCA	This study
CFP_Δgalk-R	GTTTGCGCGCAGTCAGCGATATCCATTTTCGCGAATCCGGAGTGTAAGAACGCCTTTGAGTGAGCTGATA	This study
YFP_Δgalk-F	TTCATATTGTTCAGCGACAGCTTGCTGTACGGCAGGCACCAGCTCTTCCGTGAAGTCAGCCCCATACGAT	This study
YFP_Δgalk-R	GTTTGCGCGCAGTCAGCGATATCCATTTTCGCGAATCCGGAGTGTAAGAAGAGTCAGTGAGCGAGGAAGC	This study
lsrR1-F	GTGAAGAATGAATTATGACAATCAACGATTCGGCAATTTCAGAACAGGGAGTGTAGGCTGGAGCTGCTTC	
lsrR2-R	CTCTATACGTTCTCCATCATTCCCGGTAATAAGGTCATGCAAATTTAACTCATATGAATATCCTCCTTAGT	
dellsrK1	GCTCGACTCTTTACCCTTTCAGAATCAAAGTACTACCTGATGGCGCTGGATGTGTAGGCTGGAGCTGCTTC	
dellsrK2	TAACCCAGGCGCTTTCCATAACGACGTCGTCAGTCCATGATCAACCAGCATATGAATATCCTCCTTAGT	
Univ Bact_qPCR F-	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
Univ Bact_qPCR R-	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
Y(C)FP_qPCR_F-	TTGTGTCCAAGAATGTTTCCATCT	This study
Y(C)FP_qPCR_R-	GCCATGCCCGAAGGTTATGT	This study
SeqV1_V3-F	CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNTCAGAGTTTGATCMTGGCTCAG	This study
SeqV1_V3-R	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGCTTACCGCGGCKGCTGGCAC	This study

Animal Studies

Germ-free C57BL/6J mice were bred and maintained in the gnotobiology facility of the Instituto Gulbenkian de Ciência, before individual transfer to ISOcages (Tecniplast). To demonstrate the production of AI-2 in the gut, mice were gavaged with 100 µL of sterile PBS, or 10^5 CFUs of either ARO043 (WT), ARO071 (*ΔlsrK*), or ARO081 (*ΔlsrRΔluxS*). Fecal samples were collected daily during the experiment to determine bacterial load as above, and stored at -80˚C for subsequent DNA extraction and verification of monocolonisation status by qPCR and DGGE analysis (Carvalho et al., 2011). Microbiological analysis of the mice gavaged with PBS also confirmed successful maintenance of gnotobiological status throughout the experiment. Mice were sacrificed 5 days post-colonization and cecal contents harvested for analysis of AI-2 levels. To demonstrate removal of AI-2 from the gut

by the *E. coli ΔlsrRΔluxS* mutant strain, germ-free mice were gavaged with a 1:1 mix of 10^5 CFUs of *ΔlsrK* and *ΔlsrKΔluxS*, or *ΔlsrK* and *ΔlsrRΔluxS,* either CFP- or YFP-labelled mutant bacteria. 5 days after initial colonization, fecal pellets were collected, diluted and plated to determine the total bacterial load as CFU/g feces and the ratio between the two mutant strains. Mice were then sacrificed and the cecal contents recovered for analysis of AI-2 levels.

6-8-week old male C57BL/6J mice bred under SPF conditions in the animal house facility at the Instituto Gulbenkian de Ciência were used to analyze the effects of streptomycin and colonization by *E. coli* mutant strains upon the fecal microbiota. To determine the effects of streptomycin treatment on the gut microbiota composition, 4 non-litter mate mice were housed individually and maintained under 5 g/L streptomycin *ad libitum* in the drinking water. Fresh fecal samples were collected prior to antibiotic administration (day 0), and 2, 4, 7, 12 and 28 days during treatment. Samples were weighed and stored at -80˚C for subsequent DNA extraction. To assess the effects of different *E. coli* mutants upon the gastrointestinal flora, 5 groups of mice (n=6) were treated and maintained under streptomycin, as described above. On day 2 of treatment, 2 mice per group were gavaged with 100 µL PBS containing 10^8 colony-forming units (CFU) of either ARO071 (*ΔlsrK*), ARO093 (*ΔlsrKΔluxS*) or ARO081 (*ΔlsrRΔluxS*) and individually caged. Bacteria were prepared for gavage by growth to late stationary phase followed by sub-culture to an OD_{600}=2, corresponding to approximately 10^9 CFU/ml. Bacteria were pelleted and re-suspended in sterile PBS at this same concentration. Following gavage, fecal samples were collected from each mouse at different time points during streptomycin treatment. A fraction of the fecal material was weighed, homogenized in 1 ml sterile PBS, diluted and plated on LB agar to

determine colonization levels. Fluorescently labelled colonies were counted with a stereoscope (SteREO Lumar, Carl Zeiss) to calculate the number of *E. coli* CFU/g feces. The remainder of each sample was stored at -80˚C for subsequent DNA extraction.

Ethics statement

All experiments involving mice were approved by the Institutional Ethics Committee at the Instituto Gulbenkian de Ciência and the Portuguese National Entity (Direção Geral de Alimentação e Veterinária; Ref. number 008957, approval date 19/03/2013) following the Portuguese legislation (PORT 1005/92), which complies with the European Directive 86/609/EEC of the European Council.

Detection of AI-2 activity

To measure extracellular AI-2 activity, overnight *E. coli* cultures were diluted 1:100 into buffered LB (LB + 0.1M MOPS pH7) medium with or without 40µM of chemically synthesized AI-2 (Ascenso et al., 2011). AI-2 activity was measured as previously described (Taga and Xavier, 2011) using the *V. harveyi* biosensor strain TL26 (*ΔluxN ΔluxS ΔcqsS)* which produces light in response to exogenous AI-2 (Long et al., 2009). To determine AI-2 activity in mouse cecal extracts, the cecal contents were weighed then homogenized at a 10% weight/volume concentration in 0.1M MOPS pH7. Samples were centrifuged twice at 13,000 rpm, 4˚C for 10 minutes to pellet debris, and then filtered (0.22 µm). Filtrates were mixed in a 1:1 volume ratio with methanol and centrifuged at 13,000 rpm, 4˚C for 10 minutes to remove further debris. Supernatants were dried under vacuum and stored at -80˚C for future use. Before use samples were resuspended at 50% weight/volume in sterile water. Activity was measured by mixing 10 µL of sample in 90 µL of 1:5,000 dilutions of overnight cultures of

TL26 in AB. Solutions of chemically synthetized AI-2 (Ascenso et al., 2011) diluted in cecal extracts from PBS mice were used as standards. Samples were incubated at 30˚C and luminescence measured (Victor^3, Wallac) across time. To confirm that differences in light production were due to differences in AI-2 and not bacterial growth, TL26 cultures were plated on LM agar and grown at 30˚C. Enumeration of CFUs demonstrated no significant differences in growth of the reporter strain across the samples.

Sample collection and DNA extraction

Fresh stool samples collected for each time point were immediately frozen and stored at -80°C. DNA was extracted from stool samples using a combination of the QIAamp DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions and a mechanical disruption, where bacterial membrane disruption was enhanced using 0.1mm glass beads in ASF buffer (QIAGEN) and high-speed shaking in a TissueLyzer device (2 minutes, 30Hz; QIAGEN). Samples were stored at -20°C. Total DNA obtained was quantified with Qubit® dsDNA BR Assay Kit (Invitrogen). Only samples with more than 10 ng/µL were sequenced and further analyzed (corresponding to n=9 per group for the *E. coli* experiments).

Quantification of bacterial load by qPCR

Quantitative PCR (qPCR) was performed with the iTaq Universal SYBR Green Supermix (BioRad) on DNA extracted from fecal samples to determine bacterial density as 16S rRNA or YFP (CFP) gene copy number. Universal Bacterial primers (Muyzer et al., 1993) were used at a concentration of 0.5 µM and the YFP (CFP) specific primers were used at a concentration of 10 µM (Table 2). Standard curves were generated using standards prepared by PCR amplification of the 16S rRNA and YFP gene of MG1655, normalization of the PCR products

to 1ng/µL and serially dilution. The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 5 s, 55°C for 20 s, and 60°C for 30 s.

16S rRNA gene amplification and 454 pyrosequencing

For each sample, 25µl PCRs were performed in duplicate, with each containing 20 ng of purified DNA, 2.5µl 10x PCR buffer, 0.25 mM of deoxynucleoside triphosphates (dNTPs), 0.6 U of Taq DNA polymerase and 0.2 µM of primers designed to amplify the V1-V3 region: a forward primer (5'- CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNN**TC***AGAGTTTGATCM TGGCTCAG*-3') composed of 454 Lib-L primer A (underline), a unique 8-base barcode (Ns), linker nucleotides (bold) and the universal bacterial primer 8F primer (italics); and a reverse primer (5'- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG**GC***TTACCGCGGCKGCTGGCAC*-3') composed of 454 Lib-L primer B (underline), linker nucleotides (bold) and the broad-range bacterial primer 515R (italics). Cycling conditions were 94˚C for 5 min, and 22 cycles of 94˚C for 30 sec, 52˚C for 30 sec and 68˚C for 30 sec, and a final elongation cycle at 68˚C for 5 min. PCRs were purified using QIAquick PCR purification kit (QIAGEN) and pooled. Samples were sequenced using a 454 GS FLX Titanium platform following Roche recommendations.

Sequence analysis

Sequences were processed using mothur (Schloss et al., 2009) as previously described (Ubeda et al., 2013), with some modifications. All sequences were converted to standard FASTA format. Sequences shorter than 200 bp that contained homopolymers longer than 8 bp or undetermined bases, with no exact match with the forward primer and barcode or that did not align with the appropriate 16S rRNA variable region were not included in the analysis. Using the 454 base quality scores, which range from 0 to 40 (0 being ambiguous base), sequences were trimmed using a sliding-window technique, such that the minimum

mean quality score over a window of 50 bases never dropped below 30. Sequences were trimmed from the 3' end until this criterion was met. Sequences were aligned to the 16S rRNA gene using as a template SILVA reference alignment (Pruesse et al., 2007). Potential chimeric sequences were removed using ChimeraSlayer program (Haas et al., 2011). 3077±762 sequences were obtained per sample after applying quality thresholds and removing chimeras. To minimize the effect of pyrosequencing errors in overestimating microbial diversity (Huse et al., 2008), rare abundance sequences that differ in one or two nucleotides from a high abundant sequence were merged to the high abundant sequence using the pre.cluster option in mothur. Sequences with distance-based similarity of 97% or higher were grouped into the same taxonomic operational unit (OTU) using the average-neighbour algorithm. OTU-based microbial diversity and richness were estimated by calculating the Shannon diversity and Chao index respectively. Each sequence was classified using the Bayesian classifier algorithm with the bootstrap cutoff of 60% (Wang et al., 2007). Classification was assigned to the genus level where possible otherwise the closest level of classification to the genus level was given, preceded by “unclassified”.

16s rRNA sequences have been deposited in the Sequence Read Archive of NCBI under submission number SRP051373.

To identify orthologues of LuxS in members of Bacteroidetes and Firmicutes, the protein sequences of LuxS from *Bacteroides vulgatus* and *Lactobacillus reuteri* were used as references for each phylum, respectively, to search for orthologues within the fully sequence genomes of Bacteroidetes and Firmicutes available in the KEGG database (as of September 2014). Selected gene pairs had to meet the criteria of being the best bidirectional hit.

Principal Coordinate Analysis

A phylogenetic tree was inferred using clearcut (Sheneman et al., 2006) on the 16S rRNA sequence alignment generated by mothur. Unweighted UniFrac was run using the resulting tree to quantify UniFrac distances between each pair of samples. Jaccard measure of dissimilarity was calculated with mothur using the OTU abundance information. PCoA were performed on the resulting distance matrices with mothur.

Statistical analysis

In order to determine statistically significant differences in the relative abundance of different taxa and OTUs between the different groups of mice, the non-parametric Wilcoxon test was applied using wilcox.test function in the 'stats' R package. Taxa and OTUs with less than 10 counts in both groups were not included in the analysis. To adjust for multiple hypothesis testing, we used the FDR approach by Benjamini and Hochberg (Benjamini and Hochberg, 1995) and used the fdr.R package. Results with a p-value < 0.05 and q-value < 0.1 were considered statistically significant. To compare bacterial load, Chao and Shannon index, Jaccard and UniFrac distances between the different time points analyzed during streptomycin treatment normal distribution was assumed and paired Student t-test was used. Analysis of Molecular Variance (AMOVA) was performed on the UniFrac and Jaccard distance matrices.

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