Supplementary Discussion

Functional metagenomic selections.

Although human fecal microbiota represented 67% of screened libraries and 52% of all encoded AR proteins identified from the selections, only 14% of novel proteins originated from them (Pearson’s Chi-squared test, p < 2x10^{-13}; Extended Data Figure 1C). Of the unique AR proteins identified in our functional selections, 156 (14%) were encoded in more than one library (2 to 26), with the five most prevalent (the class A β-lactamases TEM and CblA, the tetracycline efflux pump TetA, the AraC transcriptional regulator Rob, and the multidrug efflux pump MdfA) present in >15 libraries. 86 (8%) unique AR proteins were encoded in more than one microbial habitat (e.g., human feces, soil).

Whole metagenome shotgun sequencing depth.

As AR genes are at low abundance even in human fecal metagenomes, we did not rarefy shotgun reads before quantification of translated AR genes using ShortBRED, although all analyses are performed on abundances normalized to reads per kilobase per million reads (RPKM). Shotgun sequencing depth accounted for 1.8% of the variation in resistome in all samples and 2.4% of variation with only a single sample per human individual (adonis; Bray-Curtis; p < 0.001), and the variation by sample habitat attributable to sequencing depth was very small (variation explained by sample habitat = 17.8% vs.16.6% when considering shotgun sequencing depth for all samples, 22.4% vs. 21.0% with single sample per human, adonis, p < 0.001). However, the amount of variation explained by sequencing depth was higher in some subsets.

The number of sequences per sample was significantly different by sample habitat (Kruskal-Wallis, p < 1x10^{-12}, all samples and with a single sample per human), and human, pre-treatment sewage, and post-treatment sewage all had higher sequencing depth than latrine and soil samples (pairwise Wilcox test with Bonferroni correction, p < 0.05). Human samples also had greater sequencing depth than water.

Demographics of human populations.

The distribution of sexes was significantly different between RES, PST, and the seven published 16S cohorts included in human fecal phylogenetic composition comparisons (Pearson’s Chi-squared test, p < 0.0001; Supplementary Table 14), and there was a significant deviation from expectation in the age distribution (Kruskal-Wallis rank sum test, p < 0.01). However, age and sex explained only 0.4% and 0.3% of the variation among all samples, compared to the 21.2% and 15.4% explained by cohort and lifestyle (unweighted UniFrac, adonis, excluding missing values, all p-values < 0.05). With weighted UniFrac, cohort and lifestyle explained 37.6% and 23.8% of the variation (p < 0.001), while age explained 0.7% (p < 0.01), and sex was not significant.

The distribution of sexes was slightly significantly different between CLN, PSJM, and the three published shotgun cohorts (Pearson’s Chi-squared test, p < 0.046; Supplementary Table 15), but there was not significant deviation from expectation in the age distribution (Kruskal-Wallis rank sum test, p > 0.05). However, age and sex both did not explain a significant amount of the variation between RES, PST, and the published human fecal shotgun cohorts (p > 0.05, including only markers not exclusive to RES and PST, Bray-Curtis, adonis, missing values excluded), but cohort explained 19.7% (p < 0.001).

Human fecal microbiota and resistomes.

When compared to environmental samples from RES and PST, RES and PST human fecal microbiota separated along the second principal coordinate axis in both resistome and
phylogenetic composition (PC2, 11.6 – 13.2% of variation) ([Extended Data Figure 2A-B]). Compared alone, RES and PST human fecal microbiota separated by cohort in phylogenetic composition, with cohort explaining 29.7% of the variation (weighted UniFrac, adonis, p < 0.001; [Extended Data Figure 3A]). The amount of variation explained between RES and PST fecal microbiota in phylogenetic composition was lower using unweighted UniFrac (adonis $R^2 = 6.9\%$, p < 0.001).

We performed linear discriminant analysis with LEfSe [55] to identify the taxa that best discriminate RES and PST and the host lifestyles from each other. Among the four most abundant phyla across RES and PST (Extended Data Figure 3E), Actinobacteria, Firmicutes, and Proteobacteria were discriminative for PST, while Bacteroidetes was discriminative for RES (effect size threshold 4.0; [Extended Data Figure 3B]). The family Ruminococcaceae had the highest overall abundance across RES and PST human fecal microbiota but was not significantly different between them ([Extended Data Figure 3F]), as was also seen across cultures and degrees of urbanization in Asia [58]. The families Bifidobacteriaceae, Lachnospiraceae, Lactobacillaceae, and Enterobacteriaceae were discriminative for PST ([Extended Data Figure 3B]). Within the Bacteroidetes, Prevotellaceae was a marker for RES, while Bacteroidaceae was a marker for PST. Prevotella was significantly enriched in RES vs. PST (27.5 vs. 3.9%), while Bacteroides exhibited the opposite trend (3.4 vs. 0.5%).

RES and PST human fecal resistomes also separated by cohort (Bray-Curtis; [Extended Data Figure 4E]), differing primarily in resistome membership, as only slightly more variation was explained when AR protein abundance was considered (Bray-Curtis vs. Sorensen-Dice, adonis $R^2 = 25.0\%$ vs. 22.6\%, p < 0.001). Less stringent (90% identity) AR protein clustering increased the variation between RES and PST (Bray-Curtis, adonis $R^2 = 26.0\%$, p < 0.001), indicating that different resistance mechanisms rather than small sequence divergence in shared AR proteins drove resistome separation. PST carried more AR proteins per individual than RES (95 vs. 63, Wilcox rank sum test, p < 0.001; [Extended Data Figure 4D]).

To identify which AR categories best discriminated RES and PST, we performed supervised learning classification with the Random Forests classifier ([Extended Data Figure 4E]). The model estimated error for the Random Forests supervised learning classification was 0.06861 +/- 0.05976, a ~7X improvement over baseline. PST resistomes were enriched in general for drug efflux AR proteins, while RES resistomes were enriched for target protection mechanisms ([Extended Data Figure 4A]; p < 0.05, Wilcoxon test with Bonferroni correction). RES and PST did not differ significantly in carriage of tetracycline resistance, which was highest overall ([Extended Data Figure 4B]) [11,22,24]. However, PST resistomes were enriched in resistance to seven of ten antibiotics and eight of fourteen AR categories abundant in either cohort (p < 0.05, Wilcoxon test with Bonferroni correction; [Extended Data Figure 4A-C]).

Across continents and cultures, human fecal microbiota studied to date from rural and non-industrialized areas typically harbor greater intra-individual diversity than those from urban industrialized areas [14,19,22,25,58]. However, fecal microbiota from RES did not have higher taxonomic or phylogenetic diversity than those from PST (observed species and Faith’s PD, p > 0.05). The distribution of OTUs was less even in RES (equitability 0.65 ± 0.07 vs. 0.71 ± 0.06, p < 0.05), likely because they were dominated by Prevotella. Also in contrast to earlier studies [14,59-61], RES was not more homogeneous than PST in fecal phylogenetic composition or resistome (distance to centroid, p > 0.05, weighted UniFrac, Bray-Curtis), despite our sampling half of RES inhabitants, but only some 0.1% of the estimated ~57,000 PST residents, who also occupied a broader geographic area (see Methods). Although some ~81% of RES residents leave
the community ≤5 times per month, their contact with people from larger communities may be enough to diversify their microbiota (see Methods).

Previous research in industrialized settings has shown that co-habiting family members, including unrelated partners, share more similar microbiota than strangers \(^{20,62}\), but living conditions could impact the degree of sharing within and between households. In RES and PST, members of the same household had more similar microbiota composition using unweighted but not weighted UniFrac (non-parametric Student’s t-tests, 999 permutations, Bonferroni correction), indicating that household members shared more low-abundance taxa but that the overall microbiota structure was not more similar within households. However, in neither country did members of the same household share greater resistome similarity, potentially due to the high heterogeneity of the resistomes (Bray-Curtis and Sorensen-Dice, non-parametric Student’s t-tests, 999 permutations, Bonferroni correction).

We used longitudinal sampling to interrogate human fecal microbiota and resistome stability in RES and PST. The microbiota were not significantly different in overall phylogenetic stability (average distance to median within an individual, weighted UniFrac, Wilcoxon test, \(p > 0.05\); RES vs. PST: 44 vs. 9 individuals, median samples (± IQR) per person, 3 ± 1.25 vs. 2 ± 0). However, PST resistomes were more variable than RES resistomes (average distance to median, 0.2904 vs. 0.4178; Bray-Curtis, Wilcoxon test, \(p\)-value = 6.4 x 10^{-6}; RES vs. PST: 38 vs. 16 individuals, median samples (± IQR) per person, 3 ± 1). As a caveat to the longitudinal analysis, the RES 16S-sequenced samples included both short- and long-term sampling, while PST included only long-term (1 year) separated samplings, potentially inflating the relative phylogenetic stability of RES microbiota (see Methods). However, resistome stability in both cohorts was more comparable, as approximately the same number of samples was shotgun-sequenced per individual in RES and PST, and the average distance from baseline sample was slightly lower in the PST cohort.

In the comparison to global public datasets by lifestyle, the amount of variation attributable to study was only 7.8% (adonis, unweighted UniFrac, \(p < 0.001\)) to 12.7% (weighted UniFrac, \(p < 0.001\)), roughly half of that attributable to lifestyle (15.4% for unweighted and 23.8% for weighted, \(p < 0.001\)).

Although PST microbiota were intermediate in composition between rural agriculturalist and urban industrialized microbiota, they were specifically distinguished by Actinobacteria, particularly Bifidobacteriaceae, from the other lifestyles (LEfSe, effect size threshold 3.0; Extended Data Figure 3D, G-H). The fecal microbiota from urban industrialized and peri-urban industrializing communities had a higher Firmicutes-to-Bacteroidetes (F/B) ratio than did those of rural agriculturalists and hunter-gatherers (3.4 and 10.0 vs. 2.0 and 1.7, respectively; Extended Data Figure 3G). This has been linked to fat vs. fiber consumption \(^{63,64}\) and could reflect different dietary patterns across the cohorts, although a low F/B ratio also was observed among rural Mongolians who consume primarily meat and dairy products \(^{65}\). Both rural agriculturalists and hunter-gatherers harbored on average higher Spirochaetes and Cyanobacteria than did more industrialized populations (\(p < 0.05\), pairwise Wilcoxon tests with Bonferroni correction) \(^{33,67,74,25,59,66}\). However, Prevotellaceae was a marker only for rural agriculturalists (Extended Data Figure 3D) \(^{14,19,25,58,60,63-65}\).

As visualized by PCoA ordination, the global fecal resistomes also clustered by cohort (Bray-Curtis; Extended Data Figure 4F). Despite originating from different geographic locations in Latin America, the resistomes from RES and PST were most closely related to each other and the rural agriculturalist cohort from the Peruvian Andes, and they were closer to the
USA cohort than to the hunter-gatherers (Bray-Curtis, non-parametric Student’s t-test, 999 permutations, Bonferroni correction). Interestingly, the industrialized resistomes were closer to the hunter-gatherer resistomes than were RES or PST, though they are often cited as the most disparate lifestyles.

**RES human fecal and environmental microbiota and resistomes.**

RES animal feces and latrines were equally close to human fecal microbiota in phylogenetic composition (weighted UniFrac, p < 0.05, non-parametric Student’s t-tests with Bonferroni correction; Figure 2A). In contrast, human feces was more similar in resistance content to animal feces than latrines, but they were still more similar to soil than water (Bray-Curtis, p<0.05, non-parametric Student’s t-tests with Bonferroni correction; Figure 2B). Human fecal microbiota were closer to the dog sample than to the cow samples in phylogenetic composition (p < 0.05, non-parametric Student’s t-tests with Bonferroni correction, weighted and unweighted UniFrac).

The four most abundant phyla across all habitats were those most abundant in human fecal microbiota, but Actinobacteria and Proteobacteria were significantly enriched in latrine and soil over human fecal microbiota, while the opposite was true for Firmicutes and Bacteroidetes (p < 0.05, pairwise Wilcoxon test with Bonferroni correction; Extended Data Figure 5A). Linear discriminant analysis on RES human fecal microbiota, latrines, soil, and water revealed that Bacteroidetes and Firmicutes, the two most abundant phyla in human fecal microbiota, were discriminative for those samples (LEfSe, effect size threshold 4.0, Supplementary Table 6), while Acidobacteria was discriminative for soil and Cyanobacteria and Proteobacteria for water. Actinomycetaceae, which includes many antibiotic producers, was discriminative for latrines vs. human fecal microbiota and soil (Supplementary Tables 7-8, Extended Data Figure 5B). Animal vs. human fecal microbiota were discriminated by Methanobacteriaceae, likely because most animal fecal samples were from cows (Supplementary Table 9).

The Random Forests model estimated error was 0.17 +/- 0.16, an improvement of ~3X over baseline. Human and soil resistomes had low Random Forests misclassification rates (2% and 10%, respectively), while latrine resistomes were misclassified often (43.8%), likely because they were intermediate between human feces and soil along the ecological gradient observed for RES resistomes (Figure 2B). Tetracycline resistance, which was highest in human fecal resistomes, was enriched in RES human fecal vs. latrine and soil resistomes, whereas latrine resistomes harbored higher aminoglycoside and sulfonamide resistance (pairwise Wilcoxon tests with Bonferroni correction, p < 0.05; Extended Data Figure 5D).

To identify RES habitats prone to exchange with human feces, we estimated the proportion of each latrine, soil, and water microbiota attributable to human fecal microbiota using SourceTracker (Figure 3E) 29. The average human fecal input for each latrine sample was only 32.5 ± 32.9%. In contrast, 83% of soil and 82% of water samples had no detected human fecal input, and the average microbiota proportion attributable to human fecal input was far lower (2.7 ± 11.6% and 0.5 ± 1.7%, respectively). However, six soil and water samples had >5% human fecal input: one storage barrel water sample (7.8%); soil from household washing place (38.5%); a latrine urine exit (77.8%); and three of four samples collected near chicken coops (20.0 – 53.0%). In contrast, latrine resistomes had an average proportion of 26.6% human fecal input (± 28.3%), while soil and water had 1.7 ± 6.5% and 0%, respectively (Figure 3F).

We examined the antibiotic-resistant ORFs and contigs identified in our functional metagenomic selections to determine if any originated from Halomonadaceae, the family discriminative for latrines. The top blastp hit in NCBI nr for five unique AR proteins identified
in thirteen selections was a *Halomonas* species (67.4 - 82.4 % amino acid identity to target). All were isolated from latrines. They included a chloramphenicol transporter of class *bcr/cflA*, two class A β-lactamases, a beta-lactam-resistant MFS transporter, and a class D β-lactamase. Additionally, one contig (PE_10F_Contig_17) encoding a CARB/PSE class A β-lactamase was classified as *Halomonas* by PhyloPythia \(^{56,57}\). However, none of the ORFs was co-localized with an MGE or part of a multidrug resistance cluster, so these encoded AR proteins may have been tightly linked to this species and not easily poised for HGT.

Soils collected from different locations in each RES household cluster by phylogenetic composition to a small but significant extent (weighted UniFrac, ANOSIM R statistic = 0.2312, \( p < 0.001 \)). Several taxa discriminated between the three most sampled soil locations (latrine compartment, urine exit, and washing place) (*Supplementary Table 10*). Washing areas (where soap Suds were frequently observed) were enriched for Cyanobacteria. Interestingly, soils collected from the urine exit but not elsewhere around the latrines were enriched for the Halomonadaceae family discriminative for latrines and for Trueperaceae, which also contains extremophile bacteria. None was enriched for human fecal taxa, despite their proximity to human waste disposal. The microbial composition of soils was more similar if they were from the same location type (e.g., washing place versus latrine) than if they were from the same house but different locations associated with that house (weighted UniFrac, \( p < 0.05 \), non-parametric Student’s t-tests with Bonferroni correction, same collection date), highlighting the relative importance of environmental selection pressures over local dissemination of microbes. Clustering was also observed for RES soil resistomes (Bray-Curtis, ANOSIM R statistic = 0.2474, \( p < 0.01 \)), but soils from the same location type were not closer than soils from the same house (Bray-Curtis, same collection date).

In phylogenetic composition, water samples also exhibited clustering by collection location (weighted UniFrac, ANOSIM R statistic = 0.4503, \( p < 0.001 \)). Human fecal microbiota were equally closest to drinking water, pond water, and pond inflow in phylogenetic composition (\( p < 0.05 \), non-parametric Student’s t-tests with Bonferroni correction, weighted UniFrac). Shotgun sequencing was not possible for most water samples, which had low DNA yield. Only eight ARGs were present in those that were sequenced (\( n = 4 \)), a far lower prevalence of AR than in any other sample type, and there was no clustering by water type (Bray-Curtis, ANOSIM, not significant). A lower abundance of AR in aquatic vs. terrestrial metagenomes was also observed in an earlier study \(^{67}\), and may be explained by the relative density of microbial communities. Five of the AR proteins found in water were also found in RES fecal microbiota.

We compared the distances between human fecal and environmental microbiota from the same house to pairs from different houses as a proxy for frequent contact, considering only samples collected on the same date. In phylogenetic composition, human fecal microbiota were not closer to latrine, soil, or water from their own house, nor were they closer to soil or latrine samples from the same house in resistome (water not tested) (non-parametric Student’s t-tests with Bonferroni correction).

**PST human fecal vs. sewage microbiota and resistomes.**

In terms of phylogenetic composition, influent was more homogenous than any other stage (distance to centroid, weighted UniFrac). However, there was little difference between the stages of sewage treatment in distance to centroid for resistome (Bray-Curtis): influent samples were more homogeneous than human fecal resistomes.

Linear discriminant analysis between PST human fecal microbiota, pre-treatment sewage (street-access and WWTP influent), and WWTP effluent confirmed that Firmicutes, which
decreased at each stage of sewage treatment, was a marker for feces, while pre-treatment sewage was discriminated by Fusobacteria and several Proteobacteria families (LEfSe, effect size threshold 4.0; Supplementary Table 11). Effluent had the highest number of discriminatory taxa, including Actinobacteria, Planctomycetes, and Proteobacteria. Wastewater treatment occurs in open-air pools, so the effluent composition was likely influenced by ambient environment microbes. Furthermore, potentially novel taxa not assigned to any kingdom were enriched in WWTP effluent.

The Random Forests model estimated error was 0.08444 +/- 0.09225, an improvement of ~5X over baseline. Influent was enriched in aminoglycoside, macrolide, macrolide/streptogramin, and trimethoprim resistance compared to human fecal microbiota (pairwise Wilcoxon tests with Bonferroni correction, p < 0.05; Extended Data Figure 6D). Although the most abundant mechanisms of action were more highly represented in human fecal microbiota than in sewage effluent, drug inactivation mechanisms were not significantly different, and they were enriched in sewage influent compared to fecal microbiota or street-access sewage (p < 0.05, pairwise Wilcox tests with Bonferroni correction).

Our results contrasted with those found recently in WWTP activated sludge (AS) in Denmark, in which few AR proteins were shared with other habitats, despite a highly stable AS resistome consistent between WWTPs 32. This may result from AS representing part of the WWTP process and resulting biomass distinct from influent or effluent, different design and operation of WWTPs in Peru and Denmark, and/or analytical differences in the AS study that utilized a much smaller AR database than that used here. These differences may have also arisen due to different antibiotic use practices in the two locations.

Influent and effluent pairs collected on the same date from the same half of the sewage treatment plant were no more similar to each other than random influent/effluent pairs in phylogenetic composition or resistome (non-parametric Student’s t-tests). Influent and effluent samples from the PST or VES halves of the treatment plant show no greater similarity to samples at the same treatment stage from their own half, nor do street-access point samples (all from the PST region) show any more similarity to influent in the PST plant half, even considering only samples collected on the same dates. Human fecal microbiota were also not more similar to the street access point samples collected nearest their house. However, human fecal microbiota were closer to PST influent samples than VES influent for weighted but not unweighted UniFrac, although not in resistome.

**Highly cosmopolitan proteins and HGT.**

Four AR proteins were encoded in all seven sampled habitats: the sulfonamide-resistant dihydropteroate synthetase (DHPS) Sul2, an efflux pump with high identity to the multidrug transporter MdfA, an MFS efflux pump from Bifidobacterium, and the tetracycline efflux pump TetA. 17 other AR proteins were encoded in six of seven habitats, and eleven of the 21 proteins were found in 10% of samples in each habitat in which they were present.

Five of the nine highly cosmopolitan proteins identified in our selections were adjacent to putative MGEs 13 – 88% of the time, including TetA, qacEA1, a ribosomal protection protein, and the chloramphenicol transporters FloR and CmxA/CmxB. However, four of the highly cosmopolitan AR proteins present in our functional selections were not ever associated with MGEs, including MdfA, which was found in all habitats at 10% prevalence, including in drinking water and a water reservoir. Its top hit was *E. coli* (99.1% amino acid identical), whose ability to survive in the environment may explain its prevalence.
The DHPSs in our functional metagenomic contigs were found in association with TEM and class D β-lactamases, aminoglycoside phosphotransferases and adenylyltransferases, dihydrofolate reductases, SMR transporters, chloramphenicol transporters, \textit{tet}A, and \textit{tet}X. The highly cosmopolitan ribosomal protection protein and qacE\Delta1 were also found in multidrug resistance clusters that resemble integrons, including combinations of the genes associated with DHPSs and Erm 23S rRNA methyltransferases.

AR proteins encoded by both RES and PST libraries were encoded by a significantly higher proportion of contigs with MGEs or multidrug resistance clusters than AR proteins found in only one country (\(p < 0.005\), Wilcox rank sum test). Interestingly, the AR proteins found only in PST were found in slightly more genetic contexts than those found only in RES (1.15 vs. 1.07; Wilcox rank sum test, \(p < 0.01\)), and they were also more likely to be associated with MGEs and multidrug resistance clusters (10.3 vs. 15.8\%, \(p < 0.005\), and 13.1 vs. 17.2\%, \(p < 0.03\), respectively). However, we interrogated different microbial habitats in both locations (e.g., latrines in RES, street-access sewage and WWTP in PST).

Four unique MGEs comprised 25.1\% of all MGEs identified in our selections. This result recapitulated previous reports\textsuperscript{13}, where 27\% of predicted transfers involved an MGE, but a few promiscuous elements were responsible for most of them. The most prevalent MGE was encoded 59 times and is 99.6\% amino acid identical to IS26 \textit{TnpA} transposase, often found in \textit{Enterobacteriaceae} (WP_001313567.1). It was also >99\% identical to three other unique MGEs encoded 18 times. It was frequently associated with TEM β-lactamases, and its prevalence in our selections may have been driven by the large number of β-lactams included in our screen. The other prevalent MGEs were an integrase whose top hit in NCBI is Selenomonadales, also associated with class A β-lactamases, the transposon-encoded protein \textit{TnpV}, which was co-localized with ribosomal protection proteins, and the integrase \textit{IntI1}, which was found in coordination with a variety of AR genes, presumably in integrons.

Mobilome analyses

To determine whether the resistome trends observed across habitats in RES and PST were true when considering only recently acquired AR, we restricted our analyses to only those AR proteins shown to be adjacent to an MGE in our study. With this subset, all RES and PST resistomes were still related along an ecological gradient in terms of human fecal input, with the amount of variation explained by habitat 24.1\% (Bray-Curtis, adonis, \(p < 0.001\)), higher than with all AR proteins (22.4\%, \(p < 0.001\); \textbf{Extended Data Figure 8A}; see \textbf{Extended Data Figure 2A}). Procrustes analysis still showed significant correlation between phylogenetic composition and resistome (Bray-Curtis, \(M^2 = 0.493\), \(p < 0.001\) vs. \(M^2 = 0.360\), \(p < 0.001\) with all AR proteins; \textbf{Extended Data Figure 8B}; see \textbf{Extended Data Figure 2C}).

Human gut resistomes from RES and PST still exhibited separation by cohort, which explained 31.0\% of variation (Bray-Curtis, adonis, \(p < 0.001\), higher than with all AR proteins (adonis \(R^2 = 25.0\%\), \(p < 0.001\); \textbf{Extended Data Figure 8C}; see \textbf{Extended Data Figure 4E}). However, the number of observed AR proteins per person was no longer significantly different between RES and PST (\(p > 0.05\), non-parametric Student’s t-test; see \textbf{Extended Data Figure 4D}). When Random Forests analysis was performed to identify the most discriminative AR categories in the mobilome between RES and PST, although ABC and MATE transporters are not present in this subset, MFS transporters are still the third most discriminative AR category (vs. second with full AR set) and are still significantly enriched in PST (Wilcoxon test with Bonferroni correction, \(p < 0.05\)).
Human gut and environmental resistomes from RES also exhibited an ecological gradient, with habitat explaining 32.0% of the variation (Bray-Curtis, adonis, p< 0.001), higher than with all AR proteins (adonis R² = 26.6%, p < 0.001; Extended Data Figure 8D; see Figure 2B). When considering only mobile AR, soil still harbored fewer AR proteins per sample than human and animal feces, but no longer had fewer than latrines (non-parametric Student’s t-tests with Bonferroni correction; Extended Data Figure 8E; see Figure 2D). Animal feces had higher AR than latrines only with mobile AR, but human feces had more AR proteins per sample than water with both the mobile and full AR sets. Human fecal resistomes were more homogeneous than water resistomes in addition to soil and latrine resistomes when considering only AR (distance to centroid). Animal fecal resistomes were still more homogeneous than latrine and water but no longer soil resistomes.

As before, SourceTracker analysis indicated that no water resistome had any human fecal input, and that the only soil resistome with any fecal input was collected from a chicken coop (58.4% attributable to human fecal resistomes; Extended Data Figure 8F; see Figure 2F). The other soil that had >1% human fecal resistome input when considering all AR proteins (chicken coop, 16% attributable) no longer had any human fecal input. However, the average resistome proportion attributable to human feces was higher for latrines when considering only mobile AR (38.1% ± 37.5% vs. 26.6% ± 28.3%). For both the full AR and mobile AR sets, latrines had significantly more human fecal input than soil (p < 0.05, pairwise Wilcoxon tests with Bonferroni correction). Human fecal resistomes were still closest to soils collected from chicken coops than any other location (p < 0.05, non-parametric Student’s t-tests, Bonferroni correction).

When considering only mobile AR, sewage resistomes still decreased in similarity to human feces at each stage, but street-access sewage was more similar to human feces than was influent (p < 0.05, non-parametric Student’s t-tests with Bonferroni correction), rather than equally similar. Sewage stage explained 34.8% of the variation between resistomes (Bray-Curtis, adonis, p < 0.001), higher than with all AR proteins (adonis R² = 32.3%, p < 0.001; Extended Data Figure 8G; see Figure 3B). Human feces and effluent still harbored fewer AR proteins per sample than pre-treatment sewage, but human feces no longer had more than effluent (non-parametric Student’s t-tests with Bonferroni correction; Extended Data Figure 8H; see Figure 3D).

Based on SourceTracker analysis of mobile resistomes, the difference in human fecal input to the three sewage stages was still not significantly different (p > 0.05, pairwise Wilcox tests with Bonferroni correction; Extended Data Figure 8I; see Figure 3F). AR identified in assembled metagenomes of humans < 3 years old.

Of the humans included in our analysis, eight were less than three years old over the course of the study. We assembled metagenomes from nine of their samples, which produced 48156 contigs total. We assigned taxonomy to the contigs with PhyloPythia. 77% of the contigs were assigned to Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, with only 3% assigned to other phyla (87% of the functionally selected, antibiotic-resistant contigs are classified as those four phyla). 132 antibiotic resistance ORFs were identified from the contigs. In contrast to all contigs, 76% of the contigs containing AR were assigned to Actinobacteria, with 59% assigned to Bifidobacterium. Infant guts were both dominated by this genus and are low complexity ecosystems, potentially facilitating the assembly of longer contigs (which would be more likely to encode full-length AR genes). Alternatively, Bifidobacterium may encode more AR genes than other genera. ABC transporters and MFS transporters comprised 48% (63) and 28% (37) of the AR genes, respectively. 89% of the MFS transporters were assigned to
*Bifidobacterium*. Members of glycopeptide resistance cassettes were assigned only to Actinobacteria and Firmicutes.

The 132 proteins formed 106 ShortBRED marker clusters with 100% identity clustering, 90 of which did not include any proteins from the functional metagenomic selections or CARD and Lahey databases. However, all but one marker was found in older human fecal metagenomes, demonstrating that they were not exclusive to these assemblies. **Comparison of animal and human fecal microbiota.**

Animal fecal microbiota are specific to the host species, reflecting both evolutionary relationships between hosts and diet \(^{68,69}\). In both RES and PST, domestic animals live in close proximity to humans as pets and food sources. Contact with animals and their feces, combined with the unavailability of water for hand washing, may promote the transfer of microbes and AR genes between human and animal fecal microbiota, further facilitated by their phylogenetic similarity. In this study, we collected fecal samples from domestic cows and dogs in RES and from chickens, ducks, dogs, guinea pigs, and turtles in PST.

The animal fecal samples exhibited strong clustering in phylogenetic composition by host species only when taxa abundance was not considered (unweighted UniFrac ANOSIM R statistic = 0.9596, \(p < 0.001\), weighted UniFrac ANOSIM R statistic not significant), indicating that low-abundance taxa drove the separation between host species. Similarly, there was only clustering by higher-order classification (i.e., mammal, reptile, bird) with unweighted UniFrac (ANOSIM R statistic = 0.4052, \(p < 0.05\)). The low degree of clustering by host species observed in this study may reflect our small sampling size; in addition, all of the fecal samples were collected at unknown times after defecation and were exposed to the aerobic environment, which may drive convergence of the bacterial communities. AR genes are often chromosomally encoded, so more similar microbiota may encode more similar resistomes. However, although there was clustering by host species among the animal resistomes (Bray-Curtis, ANOSIM R statistic = 0.7635, \(p < 0.005\)), there was no clustering by higher-order classification (Bray-Curtis ANOSIM \(p > 0.05\)). This may reflect the fact that some samples came from the same animal.

Human fecal microbiota may be closer to those of animals more evolutionarily related to humans, those with a more similar diet, or even those that are in closer contact with people. We compared the human fecal microbiota from RES and PST to all animal samples. The animals represented a broad range of species, and human samples were more homogeneous in both phylogenetic composition and resistome (distance to centroid, \(p < 0.009\)). Despite this, human and animal fecal microbiota clustered apart with both measures (ANOSIM, 16S weighted UniFrac R statistic = 0.5744, AR Bray-Curtis R statistic = 0.5063, \(p < 0.001\)), with this classification explaining 11.6% and 3.6% of the variation (adonis, weighted UniFrac and Bray-Curtis, respectively, \(p < 0.002\)). Human fecal microbiota were most similar phylogenetically to dog microbiota, followed by turtle and cow, and were equally close to mammals and reptiles, which were represented only by the turtle samples (non-parametric Student’s t-tests with Bonferroni correction, weighted UniFrac). However, humans were closest to cow, then dog, fecal microbiota in resistome and were furthest from turtles, and they were closest to mammals (Bray-Curtis). Humans shared on average 37 AR proteins with dog resistomes and 25-28 with chickens and cows, significantly more than the 5 shared with guinea pigs and 0.4 shared with turtles (non-parametric Student’s t-tests with Bonferroni correction). Most AR proteins present in animal fecal microbiota were also present in human fecal microbiota, although the only protein shared with turtles was the highly cosmopolitan TetA. Human fecal microbiota were also closer
to animal fecal microbiota in the same house in phylogenetic composition (weighted UniFrac) but not resistome (Bray-Curtis).

Although human and animal resistomes separated in composition, few AR characteristics distinguished between them. No mechanism of action and only one antibiotic target with average overall RPKM > 1 (“Unknown”) was significantly different in abundance (p< 0.05, Wilcoxon rank sum test with Bonferroni correction). Ribosomal protection proteins were the only significantly different AR category and were enriched in humans, including the only AR protein with RPKM > 1 that was significantly different (TE_01G_005_Contig_3). This protein was the most abundant AR protein across RES and PST human fecal resistomes, but was not enriched in either cohort relative to the other (p< 0.05, Wilcoxon rank sum test with Bonferroni correction).

Animal fecal microbiota had higher observed species and phylogenetic diversity than human fecal microbiota, potentially because they were exposed to the environment for much longer (p < 0.05, non-parametric Student’s t-tests; 350.4 ± 170.0 vs. 239.8 ± 58.3, observed species; 20.2 ± 8.7 vs. 13.9 ± 3.0, Faith’s phylogenetic diversity). However, animals did not have significantly more AR proteins per sample than humans (p = 0.27, non-parametric Student’s t-tests; 65.3 ± 44.9 for animals vs. 79.3 vs. 36.2 for humans).
References