Metagenomic insights into the human gut resistome and the forces that shape it

Kristoffer Forslund, Shinichi Sunagawa, Luis P. Coelho and Peer Bork*

We show how metagenomic analysis of the human gut antibiotic resistome, compared across large populations and against environmental or agricultural resistomes, suggests a strong anthropogenic cause behind increasing antibiotic resistance in bacteria. This area has been the subject of intense and polarized debate driven by economic and political concerns; therefore such recently available insights address an important need. We derive and compare antibiotic resistomes of human gut microbes from 832 individuals from ten different countries. We observe and describe significant differences between samples from these countries in the gut resistance potential, in line with expectations from antibiotic usage and exposure in medical and food production contexts. Our results imply roles for both of these sources in increased resistance among pathogens in recent history. In contrast, other available metadata such as age, body mass index, sex, or health status have little effect on the antibiotic resistance potential of human gut microbes.

Keywords:
agricultural growth promoters; antibiotic resistance; antibiotic stewardship; antibiotics; gut microbiome; metagenomics; resistome

Additional supporting information may be found in the online version of this article at the publisher's web-site.

DOI 10.1002/bies.201300143

Structural and Computational Biology Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

***Corresponding author:**
Kristoffer Forslund
E-mail: forslund@embl.de

Introduction

Certain microbes produce metabolites that can eliminate or retard growth of other microbes, and harnessing these "antibiotics" [1] has revolutionized medicine. Strains soon emerged that were able to resist antibiotic exposure. Many often horizontally transmissible [2] genetic elements that allow this have since been characterized [3, 4]. The "resistome" concept was introduced by D'Costa et al. [5] and refers to the set of resistance determinants in a particular context (a genome, a community, an ecosystem, and so on up to the biosphere as a whole), emphasizing environmental resistomes as "reservoirs" within which antibiotic resistance capacities could evolve before moving into human pathogens.

It was suggested early on that producers of antibiotics should be resistant to their own weaponry, and might therefore be the source of transmissible resistance genes (the "producer hypothesis") [6–8]. Therefore, bacteria-rich environments such as soils should contain resistomes from which resistance genes in pathogens could have originated. In many cases, this is validated as more bacteria are characterized [5, 9]. However, unexpected diversity and spread of resistance determinants has also been found in environmental samples [10–13], including in remote environments such as Alaskan soil [14], 30,000 year old Beringian permafrost [12] and a cave system isolated for four million years [15]. Furthermore, antibiotic concentrations in nature are often too low to be viable for microbial warfare, hence suggesting alternative roles for antibiotics "in the wild", so to speak [16]. According to the revised model, "antibiotics" are in fact primarily signaling molecules for bacterial cooperation, and only become antimicrobial at concentrations much higher than those for which their function originally evolved [16–19]. Transmission of antibiotic gene systems into new hosts may break down the careful regulation of gene expression and turn plowshares (utilitarian tools) into swords or shields [19]. For at least some communities of co-occurring bacteria, antibiotics are used to compete against "outside" microbes, whereas the consortium members are all resistant, hence re-

contextualizing antibiotic weaponry to a community rather than species level [20].

These findings complicate analysis and interpretation of the antibiotic resistome. "Silent" or potential resistance genes [19, 21] causing little phenotypic resistance in hosts where they are found cannot be recovered through conventional susceptibility testing. Conversely, resistance genes may provide no benefit in their current host, thus leading sequence-based methods to overestimate the host's potential for antibiotic resistance. Despite this, the presence of resistance genes overwhelmingly predicts lower antibiotic susceptibility in pathogens, making them clinically relevant to screen for [22–24]. Antibiotic resistance in human commensals and pathogens [25–27] and in environmental samples [28] has risen dramatically during the last century, implying widespread adaptation of bacteria to antibiotic exposure. As treatments start to fail as a result of resistant pathogens [29–31], the phenomenon has become a matter of public concern. Because of limited profit margins, development of novel antibiotics is a minor priority for pharmaceutical companies, making the situation yet more alarming and possibly highlighting such development as an important area for public policy intervention [32].

Progress is ongoing within medicine to promote "responsible antibiotic stewardship" [8, 33]. However, human medical mis- and overuse may be only part of the cause of the problem. It was discovered in the 1940s that turkeys fed streptomycetes would grow faster and fatter, a phenomenon subsequently demonstrated in many food-producing animals [34–36]. Given also that prophylactic treatment of industrially housed food-producing animals prevents transmissible diseases, "medicated feed" and water quickly became standard practice globally [36, 37]. The risk of increased selection for antibiotic resistance in food production was recognized early on [38], but the practice was pervasive until recently, when – in particular – European legislation became more restrictive [36, 37, 39–41]. This legislative action was in response to studies indicating that "farm-to-fork" resistance transfer had occurred, and might continue [34, 42–47].

All in all, then, it becomes important to chart more clearly how different ways in which we use antibiotics have caused and continues to contribute to the present threat of widespread clinical resistance.

Potential influences on the human resistome

To what extent does antibiotic use in food production select for resistance?

Food production use of antibiotics is now a strongly loaded issue, with movements supporting the complete ban of the practice [40]. The scientific literature is divided. Some scientists express concerns that opposition to antibiotics in food production is not scientifically motivated and may even be detrimental to human or animal health ([40, 41, 48, 49] – see further Box 1A). On the other hand, others have argued that there is enough evidence to warrant caution both with regard to medical and food production use [34, 42–47, 51–54]. There are several paths along which food production antibiotic use could affect the human microbiome, involving different food categories, with experimental support for each ([43, 55] – see further Box 1B). A particularly broad path might exist between the resistomes of food producing animals and humans, especially where fermented foods are common, as in the "Mediterranean Diet" [55]; pasteurization and heat treatment, as well as stringent control of the food chain (e.g. HACCP protocols [48]), may reduce such transfer [57]. Overall, then, from what we know of modern food production, it is clear that antibiotic use there does contribute to resistance of our commensal and pathogenic microflora, but the scale of this contribution has not yet been quantified.

Medical antibiotic use selects for resistance also at a population level

The human microbiome should respond to antibiotic treatment administered to its host by evolving a larger, more capable resistome. This expectation is supported from a variety of studies, both experimental and meta-analyses, and which also implies treatment of individuals will affect the resistance potential of microbes hosted by other, untreated members of the community (see Box 1C for details). There is hence a clear, though sometimes complex, link between medical use of antibiotics and resistance in human-colonizing bacteria; however, as with food production use, the relative role of this link is not known.

New insights into resistome evolution

Metagenomics enables novel, comprehensive resistome analyses

In the last decade, metagenomic technologies [71–73], including functional metagenomics [3, 10], have been developed and widely adopted (see Box 2A for further details). These may reveal the complex background context in which antibiotic resistance evolves in both microbial and host communities. Clinical resistance can be studied through resistance in commensals, opportunists, and pathogens throughout human microbiomes, such as in the human gastrointestinal tract – the gut microbiome – by analyzing sequence data from fecal samples. This recent development can provide a complementary perspective to previous approaches, and possibly put many disparate results into context. We therefore bring together available metagenomic studies of the human gut metagenome and other relevant microbial communities in order to estimate the scale and scope of antibiotic resistance capacity. In addition to that, we have made a start on charting how antibiotic exposure in different ecosystems impacts the human gut microflora.

Resistance genes abound in environmental samples, and increase with human activity

Studies on environmental resistomes support a human-driven increase in resistance gene prevalence. Resistance genes are

Box 1

Factors influencing the resistome: a brief overview of relevant literature

A. Criticism of the "farm-to-fork" model

Casewell et al. [41] argue the European ban is not associated with reduction in vancomycin resistance in human-infecting enterococci, but instead with significant losses in productivity, poorer animal health, and with farm animals growing to less uniform sizes, increasing the risk of intestinal rupture during mechanized evisceration, with subsequent contamination of abattoirs and downstream food production infrastructure with resistant bacteria [50] as a result. Phillips et al. [48] argue there is insufficient evidence that resistance transfer to the food chain is relevant to human health, for a variety of reasons: safer cooking practices might provide a safe enough alternative to the cessation of agricultural antibiotic use, livestock gut colonizers might be too different from human equivalents to be able to become resident in the human gut or to transfer resistance elements horizontally to bacteria that are; and it is suggested that previous cases of antibiotic resistance transfer identified in "farm-to-fork" studies may rather be transmissions from human to animals. From a hazard analysis, the authors conclude that the cost in increased treatment failures from "farm-to-fork" resistance increases is too low to be worth the price of higher restrictions on food production antibiotic use. Oliver et al. [49] reviews research on mastitis pathogens in adult dairy cows, and concludes that these particular bacteria neither seem to have grown resistant from large-scale antibiotic use nor function as human pathogens.

B. Pathways of "farm-to-fork" transfer and their relative support

The most direct path would be contaminated meat. Fish and seafood from antibiotic-treated aquaculture operations may carry resistant bacteria [52]. More indirectly, eggs and milk can also do so, and consequently, raw milk consumption has been recognized as a risk factor [49]. The use of manure or water from wastewater lagoons for fertilization or irrigation, respectively, can contaminate vegetables; contrary to what consumers might think, resistant bacteria were equally common on "organic" and conventionally grown vegetables, directly implying a soil or manure origin, since carriage was much lower for above-grounds vegetables [56]. Increasing attention has also been given to fermented foods, both concerning "starter" culture strains, other bacteria, and the possibility of genetic interchange between them [57]. Recent studies have identified considerable amounts of resistance gene-carrying bacteria within vegetable, dairy, and meat fermented food products [58], especially lactic acid bacteria [42, 55].

C. Evidence of medical use impact on the resistome

Support for this includes susceptibility tests of gut isolates before and after treatment [59]. While some enrichment of resistance determinants appears to be temporary [60], other studies [61, 62] show persistence of acquired resistance genes for up to 2–4 years. On a population level, resistance in clinical isolates correlates with medical use [63–66]. Likewise, Walson et al. [67] showed relatively infrequent resistance capacity in bacteria from fecal samples from very isolated Nepalese villages. This observation is consistent with a connection on a demographic level between proximity to modern medicine and antibiotic resistance of the human commensal microbiome. Similar results were obtained from a remote Peruvian community [68]. Costelloe et al. [69] conducted a meta-analysis of studies on resistance of bacteria from patients receiving antibiotics, noting that resistance of isolates from different body sites followed treatment, persisting up to 12 months. Meyer et al. [70] surveyed intensive care units in Germany over time and likewise found correlations between antibiotic use and the prevalence of resistance.

found more often and in greater abundance in samples from environments where antibiotic exposure is higher, or which have influx from other environments where this is the case (see Box 3A for more details on these studies). Additionally, there is support for antibiotic exposure making bacterial gene transfer more likely, such that this influence is not limited merely to the most abundant species in those environments. Examples of these environments are waterways with influx from sewage treatment plants, animal feedlots, or antibiotic production industries, aquaculture operations, and soils treated with manure as fertilizer, where also an increase over time is visible.

High-throughput techniques allow investigation of the assumption that food production use of antibiotics actually causes higher resistant bacterial carriage in animal microbiomes. Such studies have demonstrated the infeasibility of a simplistic model whereby growth promotor usage is the only determinant of resistance gene carriage in food production animals: not only are resistant bacteria found also in wildlife such as feral pigs (though to a much lower extent than in domesticated ones) [87], but also in animals reared using "organic" methods [88]; however, there has been no comprehensive or quantitative analysis of whether higher relative resistance gene abundance in these animals is

Box 2

Method overview

A. Experimental techniques for analyzing the resistome

Phenotypic tests of antibiotic resistance or susceptibility are based on measuring the growth of bacterial cultures under varying antibiotic concentrations. This allows certain and accurate demonstration of the relative degree of resistance. However, the method is costly, time-consuming, and can only test a single culturable bacterial species at a time versus a particular antibiotic or combination treatment. It also might miss gene systems that do not provide resistance in their native host, under native regulation, but that *do* when transferred to other bacteria. Conversely, purely genotypic tests can only demonstrate the "potential" for resistance – the presence of a gene does not guarantee its expression or its ability in each context to cause clinically relevant resistance. However, metagenomic analysis either through sequencing or through PCR analysis of individual resistance gene families can screen even unknown or unculturable bacterial species present in a sample for the presence of such genes, and can be done quickly and cheaply for a large number of biological samples in parallel. Functional metagenomics is a hybrid method where DNA fragments from a bacterial community are used to transform susceptible host cells which are then screened for inserts conferring resistance. Not only does this demonstrate active, functional resistance, but also selects for such resistance determinants that can be effectively transferred between different bacterial lineages. It also allows discovery of entirely novel resistance gene systems, and makes no assumption about any previously available knowledge [74]. However, it cannot quantify antibiotic resistance potential in a community, meaning communities cannot be compared fairly. A combined approach of functional metagenomics for resistance gene discovery, shotgun sequencing for quickly quantifying known resistance genes in each community, and susceptibility tests for validation in critical applications, appears to be the most fruitful route at present.

B. Defining the antibiotic resistance potential

Full details on the method and dataset employed for the resistome analysis results presented here are given in Forslund et al. [75], which these results extend. Briefly, metagenomic sequencing reads, following quality control and trimming, were mapped to a collection of reference genes created by assembly and clustering of a large set of reads. For the collection of reference genes, these have been annotated according to whether they belong to any gene family annotated in the ARDB database [4] as contributing to antibiotic resistance. Gene abundances were counted from a threshold of 10% base coverage in order to eliminate spurious matches. Taxonomic composition of the samples, relative to a collection of 3,496 reference genomes, and based on mapping reads to a set of 10 marker genes, were likewise estimated. Sequencing datasets were the same as in Forslund et al. [75] plus the additional studies referenced here. For each antibiotic considered, the filtered abundances of reference genes active against each antibiotic were binned to provide the raw gene-level base count of resistance capacity (X_{drug}) for that antibiotic. Species where any of these gene families were found in at least one reference genome are considered potential carrier species for resistance to the antibiotic. From the taxonomic composition of the samples the amount of potential carrier species sequence (T_{drug}) was computed for each antibiotic in each sample. The simplest measure of the antibiotic resistance potential then is defined as $potential_{drug} = X_{drug}/T_{drug}$, corresponding to the average fraction of the potential carrier species genomes in the sample that code for each resistance. For the more detailed analyses of the Illumina-sequenced datasets, potential stochastic effects of low species abundances were accounted for by finding $T_{threshold}$, the smallest value of T_{drug} across the samples that fulfills $T_{threshold} > 0.01 \times max\ (T_{drug})$ and $T_{threshold} > 0.001 \times T_{sample}$, where T_{sample} is size of each sample. Samples with $T_{drug} < T_{threshold}$ were discarded with respect to that antibiotic; otherwise X_{drug} and T_{drug} were both proportionally downsampled by randomly discarding average read-length sized blocks of mapped nucleotides, such that T_{drug_down} is the same for all samples, yielding a downsampled resistance potential as the ratio of X_{drug_down} to T_{drug_down}. An overall, "global" antibiotic resistance potential using all resistance genes at once is correspondingly defined as $potential_{global} = X_{global}/T_{global}$ or the ratio of X_{global_down} to T_{global_down}.

associated with antibiotic exposure. Screens within the DANMAP antibiotic resistance surveillance project revealed a drastic decrease in resistance in isolates from food production animals over the years 1995–1999. This is attributable to the Danish ban on agricultural growth promoters [36, 54]. Varga et al. [89] were able to model quantitatively the resistance of bacteria isolated from pig farms based on the antibiotic use practices of each farm. Higher carriage as a result of antibiotic treatment has further been demonstrated through intervention-type metagenomic studies, which have also demonstrated the relative mobilization across co-occurring bacteria upon exposure to antibiotics, mediated by vectors such as bacteriophages.

Box 3

Metagenomic analysis of resistance: a brief overview over relevant literature

A. Evidence of anthropomorphic impact on environmental resistomes

In 2011, Kristiansson et al. [76] sequenced bacteria from river sediments near antibiotic production plants, revealing many different resistance genes and an increased abundance of mobile genetic elements. This suggests that antibiotic exposure also selects for the capacity for gene transfer. Qualitatively similar results have been shown throughout Rocky Mountains river systems [77, 78] and from Cuba [79], with qualitatively different impact of human waste water plants and cattle feedlot operations, respectively. Analysis of a human waste water plant also revealed a vast repertoire of resistance genes both midway through and in the final effluents [80], supporting a role of sewage processing in enabling resistance gene dissemination. Bacteria from sediments from Chinese fish farms, a habitat linking the wider marine environment to the antibiotic-intensive practice of aquaculture [52], have been sequenced [81]. In these analyses, members of several resistance gene families were detected, and many of these were found to be identical or nearly identical to sequences from human pathogens, especially those associated mobile element regions. Using archived soil samples from Dutch sites ranging from the 1940s to the present, Knapp et al. [28] showed a steady increase in four out of five sampled soil sites in resistance gene carriage: furthermore, there was some indication that irrigation, rather than manure fertilizer use, may more strongly contribute to this trend. Several studies [82–86] suggest that manure as fertilizer enriches for resistance-carrying mobile elements in the soil, hence increasing the likelihood for transfer to human or animal food via crops.

B. Evidence of antibiotic exposure increasing chance of lateral (resistance) gene transfer

Looft et al. [90] reared piglets with or without exposure to antibiotics, and found more antibiotic resistance genes in bacteria colonizing the exposed animals. Moreover, prophages are induced to a higher degree under antibiotic exposure of the pig gut [91], implying that gene transfer may become more likely in such situations. Fundamentally the same results are seen in mice [92], where bacteriophages from treated mice are able to confer resistance on susceptible bacteria. Antibiotic resistance genes are also widely found in phage DNA identified from animal fecal waste, including from cattle, pig and poultry production [93]. This would allow gut ecosystems to serve well as hotspots for antibiotic resistance transfer. Durso et al. [94] analyzed a collection of environmental, agricultural, and human-associated metagenomes, finding resistance genes in all environments, as expected, but more often in human or animal fecal samples, and least often in marine environmental samples. This might in part reflect biases in which bacteria have been studied, but it also matches expectations of higher resistance being found where human impact is strong. Shoemaker et al. [27] assessed phenotypic resistance and resistance gene presence in human gut *Bacteroides* samples taken over a time span of three decades. This revealed an increase in carriage of the tetracycline resistance gene *tetQ* from about 30% of strains to about 80% of strains during this period, as well as an increase of the macrolide resistance genes *ermF* and *ermG* from $<2\%$ to 23% carriage during the same period. These genes are, if anything, even more prominent a decade later, as revealed by metagenomic analysis [75]. This mirrors the results of Houndt and Ochman [26], who measured antibiotic susceptibility of strains of enteric bacteria sampled from humans as well as wild and domesticated animals between 1885 and 1987. The researchers found that while 20% of recent strains displayed high-level resistance, no strains from the pre-antibiotic era did. Moreover, Shoemaker et al. [27] noted that the similarity of resistance genes across *Bacteroides* strains was very high, consistent with a mostly horizontal spread between lineages, which would be expected to respond well to anthropogenically induced selection pressure. Subsequent studies support pervasive resistance transfer in the gut [88, 95].

Taken together, available studies (see Box 3B for further details) strongly imply that agricultural use of antibiotics selects for resistant microflora, which spreads across food production ecosystems by a variety of means, likely aided by migrating scavengers such as gulls, cockroaches, rodents and others [96]. In this way, it is possible for bacteria that have become resistant in one food production context (e.g. growth promotion or prophylaxis of farm animals) to enter the food chain through some other, parallel but associated channel (e.g. manure or waste water added to soil, then contaminating vegetables). This underscores the interconnected nature of the resistomes of the various food production ecosystems.

Should gene transfer between resistomes be expected?

A potential caveat applying to "farm-to-fork" resistance concerns is whether livestock-colonizing strains and human colonizers are distinct, and if they are, whether this difference is sufficient to prevent lateral gene transfer [97]. Studies comparing expected and observed strain variability (see Box 3B for further details) support a model where such transfer of resistance genes is in fact common in the gut ecosystem, as well as an increase in such carriage during the course of the antibiotic era.

The relative dissimilarity of many resistance genes found through functional metagenomic analysis of human gut microbes [3] to those of pathogens might call into question the resistome model wherein environmental resistance determinants migrate into the gut and from there on to human pathogens in diverse body sites. However, later work by Forsberg et al. [98] applied functional metagenomic analysis of antibiotic resistance genes to soil bacteria screened for multidrug resistance. This approach, instead, found a large number of environmental resistance genes from different classes that were wholly or almost identical to genes found in clinical isolates, hence providing strong evidence for influx of resistance capacity from the environment into human pathogens. By identifying highly similar regions within bacterial genomes taken from different environments, including human colonizers and pathogens, environmental samples, and samples from different stages of food production, Smillie et al. [99] found signs of recent resistance gene exchange between human isolates and bacteria in many other niches, including farm isolates. Intriguingly, they also demonstrated higher degrees of bacterial lateral gene transfer involving human isolates than between environmental isolates. This perhaps reflects how the human lifestyle – which bridges the many distant niches of our habitat – provides arenas for transfer of resistance genes between bacterial genomes and between different microbial communities vastly more often than would otherwise occur.

Metagenomic insights into human resistomes

Functional metagenomics verifies and expands human-associated resistomes

The human oral resistome was first investigated by Diaz-Torres et al. [100] through functional metagenomic screens for tetracycline, amoxicillin, or gentamicin resistance, thereby revealing a wide diversity of resistance genes active against these antibiotics in saliva samples from healthy human donors. Sommer et al. [3] performed further functional metagenomics screening of fecal and oral samples from two human donors, screening inserts for genes causing resistance in *E. coli* to 13 different antibiotics. These genes were compared with previously known resistance genes, and on the whole, were often very different from their closest homologs in pathogens, thus implying a non-negligible diversity of antibiotic resistance genes in the microbial "dark matter". Enriching for strains culturable under aerobic conditions instead revealed a subset of resistance genes more similar to known gene diversity, hence implying that bacterial genome sequencing efforts as well as gene annotation favors certain groups of easily studied bacteria at the expense of groups that may be more abundant in actual human microbiomes. In these data, resistance genes with high similarity to those previously found in pathogens were also more likely to be flanked by mobile gene elements such as transposases. This finding is consistent with easily disseminated genes being more likely to have been previously encountered and annotated. Further smaller-scale (four donors) functional metagenomic screens of the human gut resistome have also been undertaken by Cheng et al. [101], who found a variety of previously unknown resistance determinants.

Geographic origin strongly impacts composition of human-associated resistomes

The first attempt at a population-level analysis of resistance gene prevalence in the human gut was carried out by Seville et al. [102]. Their study used macroarray probes to test for 14 tetracycline and macrolide resistance genes in fecal and saliva samples from twenty healthy volunteers from each of six European countries (England, Finland, France, Italy, Norway, and Scotland). Several of the tested genes emerged as common in the human microbiomes, and were also associated with transposable elements, which may help explain their spread. The resolution of the study makes inference of significant country differences difficult, but notably the fecal samples from France and Italy show strongly elevated levels of some tetracycline and erythromycin genes compared with the Scandinavian or UK samples. This is consistent with later findings on much larger datasets (see [75] and the present work).

Large-scale metagenomic studies provide a window into the gut resistome

In the most comprehensive metagenomic resistome analysis to date, we recently [75] screened all the high-resolution human gut metagenome datasets then available for antibiotic resistance determinants represented in the ARDB database [4]. We analyzed the antibiotic resistance potential, which we define as the amount of genetic material that is active against each antibiotic relative to the sample fraction estimated to come from potential carrier species (see Box 2B for further details). This yielded two major findings; first, that resistance gene abundance overall is significantly higher for antibiotics approved for animal (e.g. food production) use – an observation that holds even when controlling for the fact that more resistance genes are known for these antibiotics. Second, amongst different donor properties that could conceivably affect resistance gene carriage, donor country of origin plays a central role, such that for many antibiotics, their resistance potential is significantly higher in some countries than others.

In this review, we have further revisited these data and augmented that analysis with three additional large-scale datasets that have since been released, including 25 metagenomes from elderly Irish donors [103], 368 metagenomes from Chinese type 2 diabetes patients and controls [104], and from 145 elderly Swedish female type 2 diabetes patients and controls [105]. Figures 1 and 2 provide an overview of these datasets, including their size and origin, as well as donor demographic properties. Within these results, some degree of population-level resistance potential characterization can be carried out for a total of 10 different countries from three continents (though for some of these, the small number of samples together with relatively shallow sequencing make them primarily useful as qualitative controls rather

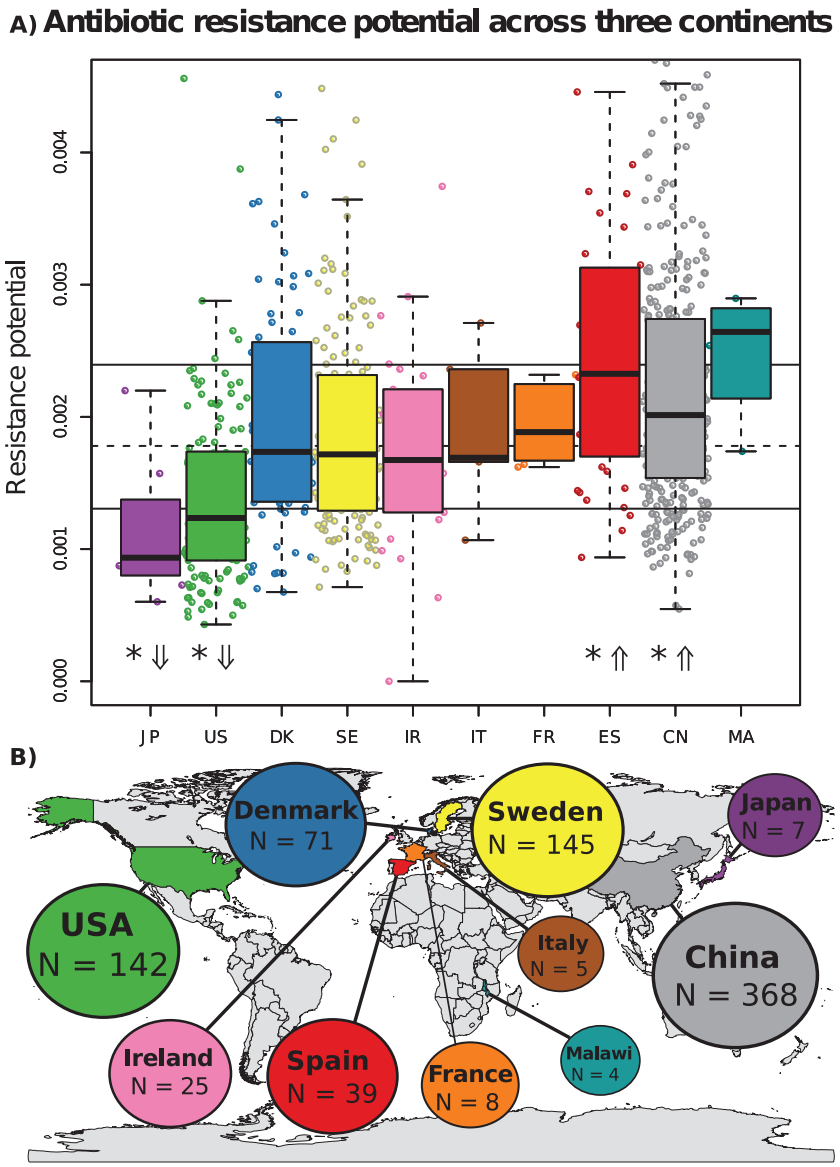

Figure 1. Overview of available gut resistomes. Population-level human gut metagenomes are available at high-resolution from 10 different countries, and there are significant differences between these in the prevalence of antibiotic resistance genes in the sequenced microbiomes. **A:** The boxplots (with individual samples shown as scatterplots, and with a few outliers not shown due to scale) show overall antibiotic resistance potential across the presently available human gut metagenome datasets (excluding small-scale or 16S studies). Resistance potential is defined as amount of resistance gene-encoding sequence material, relative to the amount of sequence that is mapped to bacterial species known to sometimes carry resistance genes. Horizontal dashed lines correspond to the overall population, showing 25%, 50%, and 75% quantiles of resistance potentials. Each country subset is compared to this global population under a MWU test for significantly higher and lower dataset median resistance potential, respectively. Two countries show significantly (Benjamini–Hochberg–Yekutieli-corrected false discovery rate <5% [119]) higher and lower potentials, respectively. **B:** The map legend shows the origin of the samples, along with the color key also used in other figures and the amount of samples available from each country.

than for detailed novel discovery). Results of such a characterization, showing the total resistance capacity integrated over all antibiotics, are shown in Figure 1A. In particular, there are significant differences between countries in this regard. The previous demonstration of elevated resistance capacity in samples from Spain is seen again, as are elevated resistance potentials in the Chinese samples. For the source studies, which include disease cohorts (inflammatory bowel disease or diabetes, respectively), the country-level trends are borne out for both patients and controls, with little evidence of any effect of health status.

Donor properties other than country of origin have only minor influence on the resistome

Given the demographic diversity of presently available datasets, which span different geographic regions and differ in size, age, and sex distribution, as well parameters such as body mass index (Figs. 1 and 2), it is relevant for technical reasons to ask whether any biases between datasets in these regards may contribute to the observed country differences. For purposes of understanding resistome evolution, it is also interesting to see what role individual donor histories play. We might ask whether or not the gut resistome of an individual becomes gradually enriched over a lifetime. Similarly, statistical differences in diet between men and women, or between lean and obese donors, might conceivably mediate a "farm-to-fork" connection to different extents. We can look at these metadata properties (age, gender, and body mass index (BMI)) and investigate the resistance capacity of the 790 gut metagenomes for which we have metadata available as a function of these variables. The results are shown in Fig. 2A. In no case, either for individual antibiotics or for the overall resistance potential, do these donor properties provide significantly better explanatory power beyond considering only country of origin, after multiple testing has been controlled for. Plotting the overall resistance potential

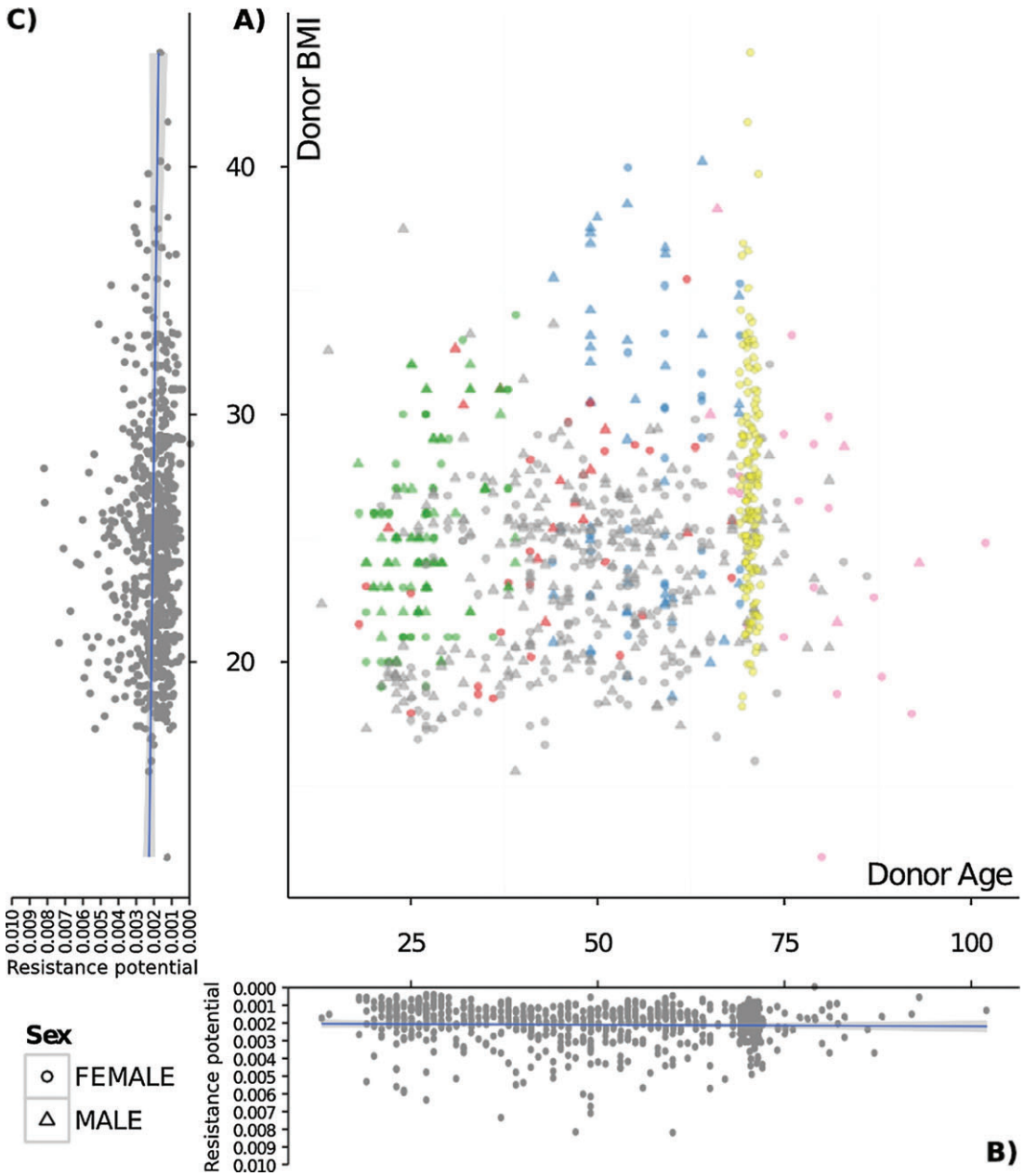

Figure 2. Age and BMI are poor predictors of resistance potential. Available deep-sequencing gut metagenome datasets show notable donor demographic biases, span demographic diversity well when taken together, and as a whole reveal little influence of donor metadata properties other than country of origin. Although each study individually covers only a limited demographic, aggregation allows us to cover a wide range of age and BMI. For 790 Illumina-sequenced gut microbiomes where donor metadata is available, age is plotted on the *X*-axis, and BMI on the *Y*-axis. Point shape denotes donor gender, point color denotes country of origin, and point size correspond to the overall resistance potential of each sample (as shown in Fig. 1). Both for the overall resistance capacity and for each antibiotic separately, no significant effect of age, gender, or BMI is found (likelihood ratio comparison of nested mixed-effects models comparing models with country and each other demographic as independent variables, compared to simpler models dependent on country only, Benjamini–Hochberg–Yekutieli false discovery rate >5% in all cases). This is also shown in the graphs to the left and below the main plot (**A**), which shows overall resistance potential of each sample regressed against donor age (**B**) or BMI (**C**), respectively. Taken together, while support for country-level factors remain, it cannot be reduced to an effect of donor demographic properties, nor is there much support for any strong effect of those properties on antibiotic resistance potential.

against the continuous measures of age (Fig. 2B) or BMI (Fig. 2C) also show no clear or obvious trends. While it cannot be ruled out that such factors play a role at least in some sub-demographics, it is not strongly seen in the available datasets, and also does not suffice to explain the observed country differences.

The donor attributes that should most directly influence the individual resistomes are medical treatment with antibiotics and the influx of bacterial strains that have evolved resistance in food production settings. Unfortunately, of the presently available datasets, none consistently has antibiotic exposure metadata beyond

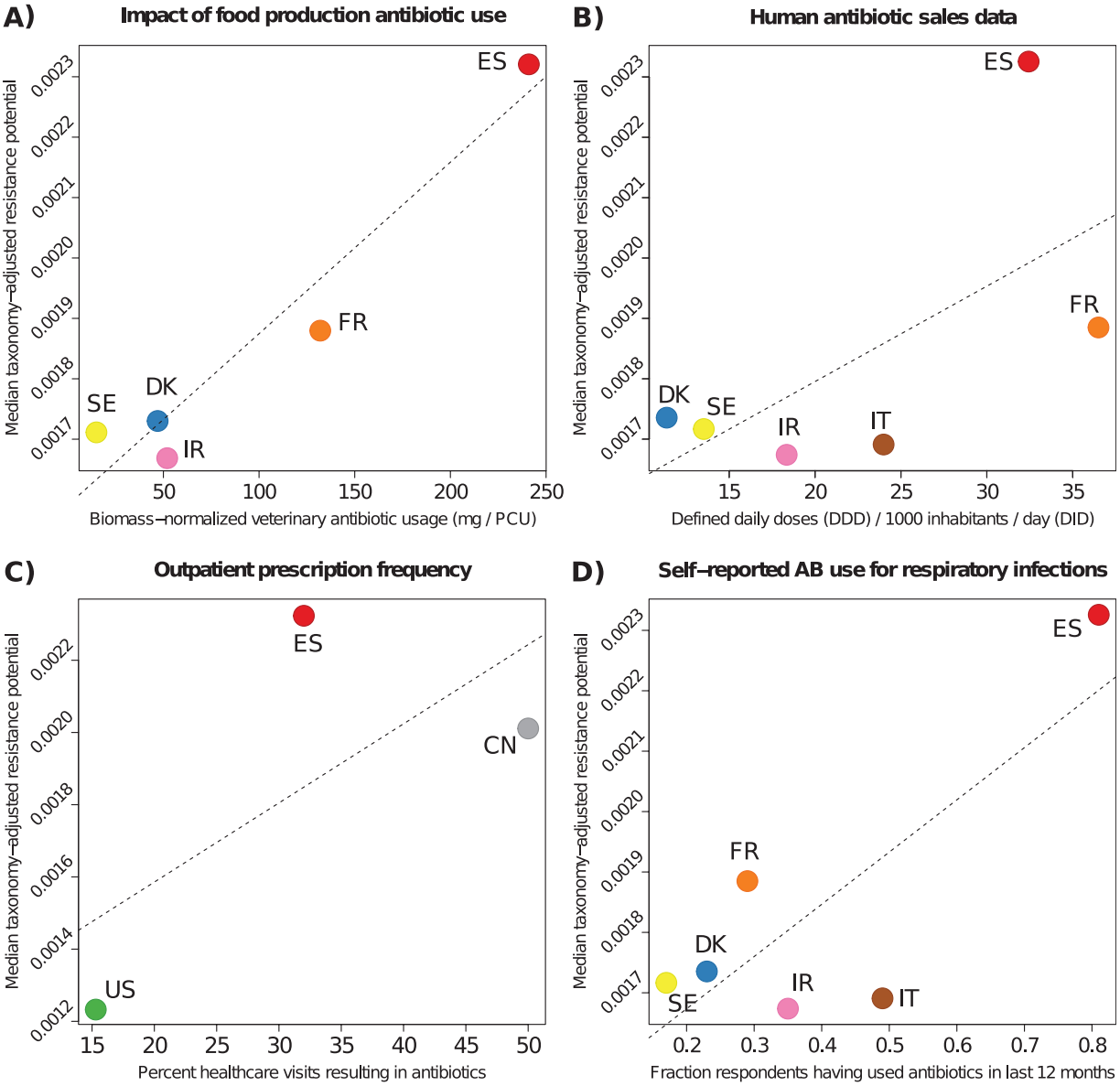

Figure 3. Overall resistance levels linked to antibiotic exposure. Pairing country-level statistics of indirect exposure to antibiotics paired with resistance potential in the gut metagenomes indicate a relationship between these factors. While no individual-level exposure data exists at a scale allowing statistical analysis, population-corrected veterinary (food production) antibiotic sales data (**A**), human medical antibiotics sales data (**B**), fraction of healthcare visits resulting in antibiotic prescriptions (**C**), and frequency of antibiotic use in the last year as measured in surveys (**D**), are available for some of the countries for which we can characterize the gut resistome at a population level. These plots show the median resistance potential (as in Fig. 1) on the *Y*-axis, and the respective country-level antibiotic exposure measures on the *X*-axis. Trendlines for fitting the medians to these dependent variables are shown. Only the veterinary sales data fit is significant ($p < 0.01$) given the presently available data.

noting that participants were required to have had no recent antibiotic usage; furthermore, only the Claesson et al. [103] study has any systematic dietary information. However, it is notable that median overall resistance potential is significantly higher for samples from southern Europe, given on the one hand the implications described above on fermented food products potentially being a favored vector for "farm-to-fork" resistance transmission, and on the other, the prevalence of such foods in the "Mediterranean Diet" [55].

Country-level resistance potentials correlate with local antibiotic use

In the absence of comprehensive individual-level antibiotic exposure metadata, we can attempt to link country-level estimates of resistance potential to country-level estimates of antibiotic exposure via different routes, indirectly testing the influence of different factors on the gut resistome. Figure 3 pairs metagenomic resistance potential with mostly European measures of antibiotic use or exposure –

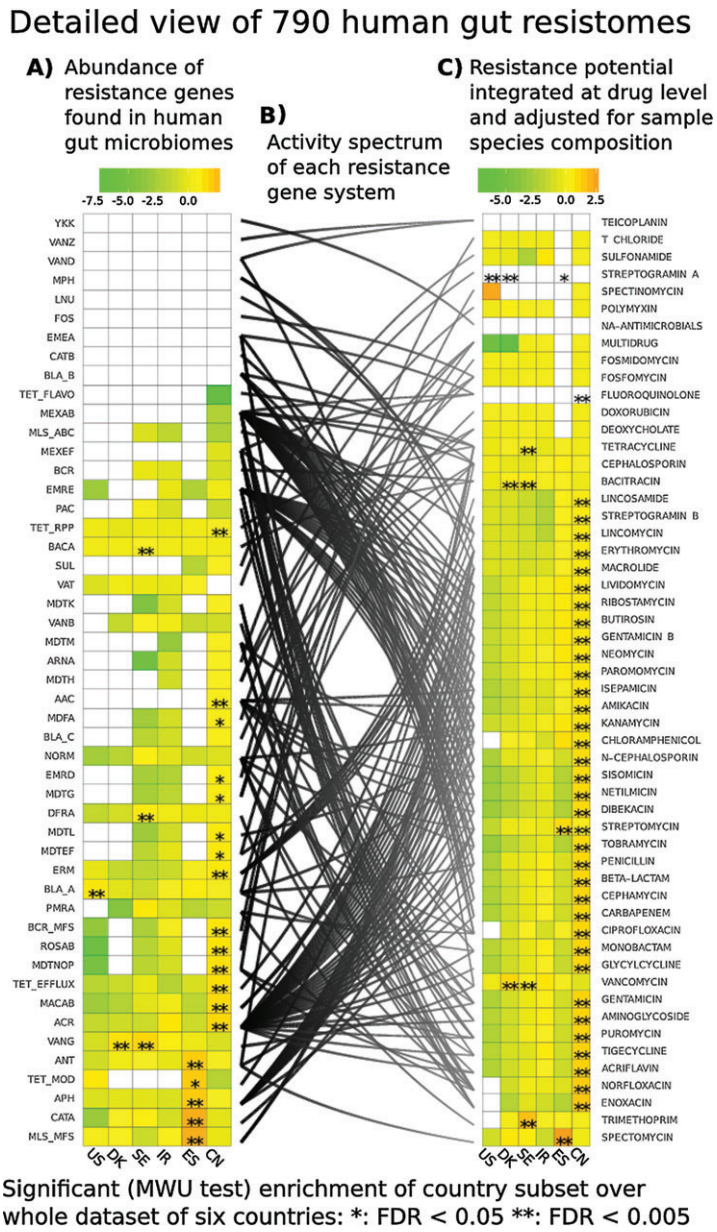

Figure 4. Significant differences in resistance potential are seen between six countries from three continents, broken down by antibiotic resistance type or by antibiotic. There are strong and significant differences between countries in the prevalence of particular resistance gene families, many of which remain when compensating for the effects of the community composition of the samples. This figure shows a detailed breakdown of the 790 resistomes within the six Illumina-sequenced gut metagenome datasets presently publically available: [72] (39 Spanish and 71 Danish samples), [73] (139 US samples + 3 US samples from [120]), [103] (25 Irish samples), [104] (368 Chinese samples), and [105] (145 Swedish samples). **A:** The heatmap to the left shows median sample fractions encoding each resistance type (as defined by the ARDB [4] database; resistance types together related gene families such as those that interact within the same operon, or which are close homologs) across the six countries. The color of each cell corresponds to country median fraction of sequence material mapping to each resistance type, divided by the median across all samples, shown in logarithmic scale. White cells represent the resistance type being absent in a majority of samples (i.e. median across the full dataset is zero). For each resistance type, its relative abundance in the samples from each country was tested against the full set of samples for whether it was significantly (MWU test, Benjamini–Hochberg–Yekutieli-corrected [119], * → FDR < 0.05; ** → FDR < 0.005) enriched in each country subset. Resistance types are sorted by highest abundance over any country subset. **B:** Network of the many-to-many relationship between antibiotic resistance genes and the antibiotics to which they provide resistance; incorporating broad specificity, cross-resistance, and antibiotics sharing the same chemical structure. **C:** The heatmap to the right shows median sample antibiotic resistance potential (as defined in [75]; proportion of sequence material from potentially ARG-carrying species that map to ARGs) across the same datasets as in (A), across the antibiotics for which resistance capacity was found. Similarly as in (A), country subsets were compared for significantly higher levels from the dataset as a whole, using the same testing methodology (MWU test, Benjamini–Hochberg–Yekutieli-corrected, * → FDR < 0.05; ** → FDR < 0.005). The color of each cell corresponds to country median resistance potential divided by median resistance potential across all samples, shown in logarithmic scale, with white cells representing antibiotics where a majority of samples have zero resistance potential. Antibiotics are sorted by highest resistance potential over any country subset.

the difficulty of finding statistics measured uniformly across countries limits this type of analysis to these states in most cases. The highest agreement is observed for total biomass-normalized veterinary antibiotics sales data from the ESVAC project [106] (Fig. 3A). A trend is also visible between median total resistance potential and over-the-counter antibiotic sales [107] (Fig. 3B) as well as the fraction of respondents in a survey reporting having taken antibiotics in the last 12 months [108] (Fig. 3D) and statistics on what fraction of healthcare visits results in antibiotic prescriptions [109–111] (Fig. 3C); however, only the veterinary sales figures yield a significant fit to the resistance potential. Available evidence hence suggests a multifactorial model, wherein both medical and food production antibiotic exposure contributes to the enrichment of resistance capacity in the human gut. At present, research lends most support to the "farm-to-fork" hypothesis.

The deep-sequencing Illumina studies available additionally provide enough resolution to reliably determine the distribution of multiple individual resistance genes at once, relative to the antibiotic resistance profiles they contribute to. We present such a higher-resolution view in Fig. 4, displaying inter-country differences in both the relative abundance of resistance gene types (corresponding to resistance gene families or to resistance operons as defined in the ARDB database [4]) and the resistance potentials to the antibiotics against which they protect. There are several options for testing the significance of such differences. Figure 4 shows a comparison of data from each country to the global population

of samples, whereas Supplementary Tables S1A–K show separate comparisons of each country subset to the others.

Features of resistome composition can be traced to local factors

Broadly speaking, for the countries included in Forslund et al. [75], the same observations can be made here. The Irish dataset is mostly similar to the Danish MetaHIT and US HMP samples, with the exception of elevated (though not significantly so) levels of some efflux pump systems (*AcraB-TolC* and *MFS* systems) – possibly a sign of enriched prevalence of these genes in the environment of homes for the elderly as well as in medical/hospital environments. The Swedish dataset likewise resembles the Danish, Irish, and US datasets, though with slightly higher resistance potential levels overall. Swedish and Danish samples show significantly higher abundance of the *VanG* vancomycin resistance operon. This observation matches that of Sørum et al. [112] who noted that vancomycin resistance in enterococci remained elevated in samples from Norwegian farmers even after a vancomycin analog growth promoter had been banned, perhaps persisting because of genetic linkage with post-segregational killing systems. The Swedish samples further exhibit relatively higher abundance of *DFRA* trimethoprim resistance genes as well as *BACA* bacitracin resistance genes, neither of which follow from higher abundance of carrier species within this dataset – in fact, the Swedish samples are less abundant in such bacteria, implying either increased carriage of *DFRA* in applicable species or introduction of these genes into species where it previously was not described.

The Chinese samples stand out as carrying significantly higher resistance potentials for many antibiotics than any of the Western datasets, and also significantly higher than those found in a smaller-scale Japanese dataset also included. This difference derives from many different resistance gene families that include examples of all three major mechanism classes for resistance. By analyzing solely metagenomic data, we can only speculate on the reasons behind this. A recent study [113] using a similar methodology to ours – though not controlling for taxonomic composition – independently replicate these findings: both the higher resistance capacity found in Spanish than Danish samples, and higher resistance capacity than in both of these in a subset of 38 Chinese samples from the Qin et al. [104] study. It thus seems unlikely that the finding of these country differences results from methodological artifacts. Statistics of food production usage of antibiotics are volatile, and dependent on methodology; as such, no direct comparison between the West and China is possible in this regard. It is known, however, that Chinese food production does make heavy use of antibiotics; very strong enrichment of resistance genes was found on Chinese pig farms [21, 114], and there are similar reports [81] from the large and antibiotics-intensive aquaculture sector [52]. Furthermore, there is strong enrichment of resistance-carrying bacteria found in feral versus food production animals in Hong Kong [115]. Antibiotics for human medical use are generally available over the counter without prescription in Chinese pharmacies, and studies of Chinese antibiotic prescribing practices (see review and meta-analysis in Yin et al. [111]) show that ~50% of visits to a health professional result in prescriptions, compared with ~15% in the United States [109] or 32% in Spain [110]. Given these observation, the relatively high resistance potentials in the Chinese samples, also suggested previously by Hu et al. [113] emerges as a likely consequence of medical and food production antibiotic use.

Conclusions and prospects

Evidence points to anthropogenic, rather than spontaneously occurring, causes behind increased antibiotic resistance in both environmental and human microbiomes. Specific transfer events [116–118] and closely related sequences in environmental and clinical isolates [94, 98–99] reveal potential impact on medical outcomes. Despite the complexity of the evolution of the resistome, we find the highest gut resistance potentials in geographic contexts where both medical and food production antibiotic use are very high ([75, 113], and the present work, see also [65, 66]). This strongly suggests that those practices enrich human gut resistomes, likely resulting in more frequent treatment failures for bacterial diseases. Disentangling these components will soon become possible as more metagenomic resistome analyses become available, including fine-grained, donor-level metadata on lifetime antibiotic exposure and diet history. Future epidemiological studies could be linked to these results and provide a final link in the chain.

Putting the human gut resistome into a wider context, recent work points to the high prevalence of lateral gene transfer in host-associated microbiome environments [27, 91–92, 99], leading to transfer into the human microbiome of antibiotic resistance genes from natural environments [14–15, 94]. Furthermore, such studies particularly emphasize the enhanced transfer of resistance genes in environments subject to anthropogenic antibiotic exposure [21, 28, 76, 81–83, 89]. Pathogen resistance elements highly similar to those of environmental or agricultural isolates have previously been found, and as we have shown, there are strong, prevalent differences in antibiotic resistance potential between countries correlating with antibiotic exposure. While many details are still unknown, it therefore still seems clear despite some claims to the contrary that we should hope the trend towards more restrictive use of antibiotics within food production and medicine continues.

Acknowledgments

K.F. was supported by a fellowship from the Alexander von Humboldt foundation. The authors are thankful to Matt Rogon for help with figure formatting.

References

1. **Waksman SA**. 1973. History of the word "antibiotic." *J Hist Med Allied Sci* **28**: 284–6.
2. **Wright GD**. 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* **5**: 175–86.
3. **Sommer MOA, Dantas G, Church GM**. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **325**: 1128–31.

4. **Liu B, Pop M**. 2009. ARDB – antibiotic resistance genes database. *Nucleic Acids Res* **37**: D443–7.
5. **D'Costa VM, McGrann KM, Hughes DW, Wright GD**. 2006. Sampling the antibiotic resistome. *Science* **311**: 374–7.
6. **Benveniste R, Davies J**. 1973. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci USA* **70**: 2276–80.
7. **Cundliffe E**. 1989. How antibiotic-producing organisms avoid suicide. *Annu Rev Microbiol* **43**: 207–33.
8. **Davies J, Davies D**. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**: 417–33.
9. **D'Costa VM, Griffiths E, Wright GD**. 2007. Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr Opin Microbiol* **10**: 481–9.
10. **Riesenfeld CS, Goodman RM, Handelsman J**. 2004. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* **6**: 981–9.
11. **McGarvey KM, Queitsch K, Fields S**. 2012. Wide variation in antibiotic resistance proteins identified by functional metagenomic screening of a soil DNA library. *Appl Environ Microbiol* **78**: 1708–14.
12. **D'Costa VM, King CE, Kalan L, Morar M**, et al. 2011. Antibiotic resistance is ancient. *Nature* **477**: 457–61.
13. **Allen HK, Donato J, Wang HH, Cloud-Hansen KA**, et al. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* **8**: 251–9.
14. **Allen HK, Moe LA, Rodbumrer J, Gaarder A**. 2009. Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil 243–251. *ISME J* **3**: 243–51.
15. **Bhullar K, Waglechner N, Pawlowski A, Koteva K**, et al. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One* **7**: e34953.
16. **Baquero F, Tedim AP, Coque TM**. 2013. Antibiotic resistance shaping multi-level population biology of bacteria. *Front Microbiol* **4**: 1–15.
17. **Davies J**. 2006. Are antibiotics naturally antibiotics? *J Ind Microbiol Biotechnol* **33**: 496–9.
18. **Aminov RI**. 2011. Horizontal gene exchange in environmental microbiota. *Front Microbiol* **2**: 158.
19. **Dantas G, Sommer MO**. 2012. Context matters – the complex interplay between resistome genotypes and resistance phenotypes. *Curr Opin Microbiol* **15**: 577–82.
20. **Cordero OX, Wildschutte H, Kirkup B, Proehl S**, et al. 2012. Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science* **337**: 1228–31.
21. **Zhu YG, Johnson TA, Su JQ, Qiao M**, et al. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci USA* **110**: 3435–40.
22. **Martineau F, Picard FJ, Lansac N, Ménard C**, et al. 2000. Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* **44**: 231–8.
23. **Zhu LX, Zhang ZW, Wang C, Yang HW**, et al. 2007. Use of a DNA microarray for simultaneous detection of antibiotic resistance genes among staphylococcal clinical isolates. *J Clin Microbiol* **45**: 3514–21.
24. **Eitel Z, Soki J, Urban E, Nagy E**. 2012. Investigation of the main antibiotic resistances and their correlation with the presence of antibiotic resistance genes in clinical Bacteroides strains [abstract]. p. 163, Anaerobe Society of the Americas, San Francisco, CA.
25. **Hughes VM, Datta N**. 1983. Conjugative plasmids in the bacteria of the 'pre antibiotic' era. *Nature* **302**: 725–6.
26. **Houndt T, Ochman H**. 2000. Long-term shifts in patterns of antibiotic resistance in enteric bacteria. *Appl Environ Microbiol* **66**: 5406–9.
27. **Shoemaker NB, Vlamakis H, Hayes K, Salyers AA**. 2001. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. *Appl Environ Microbiol* **67**: 561–8.
28. **Knapp CW, Dolfing J, Ehlert PAI, Graham DW**. 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol* **44**: 580–7.
29. **Barlow G, Nathwani D**. 2005. Is antibiotic resistance a problem? A practical guide for hospital clinicians. *Postgrad Med J* **81**: 680–92.
30. **Stone PW**. 2009. Economic burden of healthcare-associated infections: an American perspective. *Exp Rev Pharmacoecon Outcomes Res* **9**: 417–22.
31. **Carlet J**. 2012. The gut is the epicentre of antibiotic resistance. *Antimicrob Resist Infect Control* **1**: 39.
32. **Bush K, Courvalin P, Dantas G, Davies J**, et al. 2011. Tackling antibiotic resistance. *Nat Rev Microbiol* **9**: 894–86.
33. **Cunha CB, Varughese CA, Mylonakis E**. 2013. Antimicrobial stewardship programs (ASPs): the devil is in the details. *Virulence* **4**: 147–9.
34. **Barton MD**. 2000. Antibiotic use in animal feed and its impact on human health. *Nutr Res Rev* **13**: 279–99.
35. **Dibner JJ, Richards JD**. 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84**: 634–43.
36. **Marshall BM, Levy SB**. 2011. Food animals and antimicrobials: impacts on human health. *Clin Microbiol Rev* **24**: 718–33.
37. **Collignon P, Wegener HC, Braam P, Butler CD**. 2005. The routine use of antibiotics to promote animal growth does little to benefit protein undernutrition in the developing world. *Clin Infect Dis* **41**: 1007–13.
38. **Swann MM, Baxter KL, Field HI**. 1969. *Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine*. HMSO.
39. **Phillips I**. 1999. The use of bacitracin as a growth promoter in animals produces no risk to human health. *J Antimicrob Chemother* **44**: 725–8.
40. **Pugh DM**. 2002. The EU precautionary bans of animal feed additive antibiotics. *Toxicol Lett* **128**: 35–44.
41. **Casewell M, Friis C, Marco E, McMullin P**, et al. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother* **52**: 159–61.
42. **Teuber M, Meile L, Schwarz F**. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek* **76**: 115–37.
43. **Teuber M**. 1999. Spread of antibiotic resistance with food-borne pathogens. *Cell Mol Life Sci* **56**: 755–63.
44. **van den Bogaard AE, Stobberingh EE**. 2000. Epidemiology of resistance to antibiotics: links between animals and humans. *Int J Antimicrob Agents* **14**: 327–35.
45. **Witte W**. 2000. Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents* **16**: S19–24.
46. **Collignon P, Powers JH, Chiller TM, Aidara-Kane A**, et al. 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies for the use of antimicrobials in food production animals. *Clin Infect Dis* **49**: 132–41.
47. **Wegener HC**. 2003. Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**: 439–45.
48. **Phillips I, Casewell M, Cox T, De Groot B**, et al. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53**: 28–52.
49. **Oliver SP, Murinda SE, Jayarao BM**. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne Pathog Dis* **8**: 337–55.
50. **Russell SM**. 2003. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors, and populations of *Campylobacter* spp. and *Escherichia coli*. *Poult Sci* **82**: 1326–31.
51. Scientific Advisory Group on Antimicrobials of the Committee for Medicinal Products for Veterinary Use. 2009. Reflection paper on the use of third and fourth generation cephalosporins in food producing animals in the European Union: development of resistance and impact on human and animal health. *J Vet Pharmacol Ther* **32**: 515–33.
52. **Heuer OE, Kruse H, Grave K, Collignon P**, et al. 2009. Human health consequences of use of antimicrobial agents in aquaculture. *Clin Infect Dis* **49**: 1248–53.
53. **Carlet J, Collignon P, Goldmann D, Goossens H**, et al. 2011. Society's failure to protect a precious resource: antibiotics. *Lancet* **378**: 369–71.
54. **Aarestrup F**. 2012. Sustainable farming: get pigs off antibiotics. *Nature* **486**: 465–6.
55. **Devirgiliis C, Barile S, Perozzi G**. 2011. Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes Nutr* **6**: 275–84.
56. **Ruimy R, Brisabois A, Bernede C, Skurnik D**, et al. 2010. Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents. *Environ Microbiol* **12**: 608–15.
57. **Verraes C, Van Boxstael S, Van Meervenne E, Van Coillie E**, et al. 2013. Antimicrobial resistance in the food chain: a review. *Int J Environ Res Public Health* **10**: 2643–69.
58. **Sánchez Valenzuela A, Lavilla Lerma L, Benomar N, Gálvez A**, et al. 2013. Phenotypic and molecular antibiotic resistance profile of

Enterococcus faecalis and *Enterococcus faecium* isolated from different traditional fermented foods. *Foodborne Pathog Dis* **10**: 143–9.
59. **Jernberg C, Löfmark S, Edlund C, Jansson JK**. 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* **156**: 3216–23.
60. **Raum E, Lietzau S, von Baum H, Marre R**, et al. 2008. Changes in *Escherichia coli* resistance patterns during and after antibiotic therapy: a longitudinal study among outpatients in Germany. *Clin Microbiol Infect* **14**: 41–8.
61. **Jernberg C, Löfmark S, Edlund C, Jansson JK**. 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J* **1**: 56–66.
62. **Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M**, et al. 2010. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* **5**: e9836.
63. **Austin DJ, Kristinsson KG, Anderson RM**. 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci USA* **96**: 1152–6.
64. **Lindbaek M, Berild D, Straand J, Hjortdahl P**. 1999. Influence of prescription patterns in general practice on anti-microbial resistance in Norway. *Br J Gen Pract* **49**: 436–40.
65. **Bronzwaer SL, Cars O, Buchholz U, Mölstad S**, et al. 2002. A European study on the relationship between antimicrobial use and antimicrobial resistance. *Emerg Infect Dis* **8**: 278–82.
66. **Goossens H, Ferech M, Vander Stichele R, Elseviers M**, et al. 2005. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* **365**: 579–87.
67. **Walson JL, Marshall B, Pokhrel BM, Kafle KK**, et al. 2001. Carriage of antibiotic-resistant fecal bacteria in Nepal reflects proximity to Kathmandu. *J Infect Dis* **184**: 1163–9.
68. **Bartoloni A, Pallecchi L, Rodríguez H, Fernandez C**, et al. 2009. Antibiotic resistance in a very remote Amazonas community. *Int J Antimicrob Agents* **33**: 125–9.
69. **Costelloe C, Metcalfe C, Lovering A, Mant D**, et al. 2010. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. *BMJ* **340**: c2096.
70. **Meyer E, Gastmeier P, Deja M, Schwab F**. 2013. Antibiotic consumption and resistance: data from Europe and Germany. *Int J Med Microbiol* **303**: 388–95.
71. **Tyson GW, Chapman J, Hugenholtz P, Allen EE**, et al. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**: 37–43.
72. **Qin J, Li R, Raes J, Arumugam M**, et al. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**: 59–65.
73. HMP Consortium. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* **486**: 207–14.
74. **Pehrsson EC, Forsberg KJ, Gibson MK, Ahmadi S**, et al. 2013. Novel resistance functions uncovered using functional metagenomic investigations of resistance reservoirs. *Front Microbiol* **4**: 145.
75. **Forslund K, Sunagawa S, Kultima JR, Mende DR**, et al. 2013. Country-specific antibiotic use practices impact the human gut resistome. *Genome Res* **23**: 1163–9.
76. **Kristiansson E, Fick J, Janzon A, Grabic R**, et al. 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS One* **6**: e17038.
77. **Yang S, Carlson K**. 2003. Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Res* **37**: 4645–56.
78. **Pruden A, Arabi M, Storteboom HN**. 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ Sci Technol* **46**: 11541–9.
79. **Graham DW, Olivares-Rieumont S, Knapp CW, Lima L**, et al. 2011. Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ Sci Technol* **45**: 418–24.
80. **Szczepanowski R, Linke B, Krahn I, Gartemann KH**, et al. 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155**: 2306–19.
81. **Yang J, Wang C, Shu C, Liu L**, et al. 2013. Marine sediment bacteria harbor antibiotic resistance genes highly similar to those found in human pathogens. *Microb Ecol* **65**: 975–81.
82. **Ghosh S, LaPara TM**. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* **1**: 191–203.
83. **Binh CTT, Heuer H, Kaupenjohann M, Smalla K**. 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol Ecol* **66**: 25–37.
84. **Yang H, Byelashov OA, Geornaras I, Goodridge LD**, et al. 2010. Characterization and transferability of class 1 integrons in commensal bacteria isolated from farm and nonfarm environments. *Foodborne Pathog Dis* **7**: 1441–51.
85. **Heuer H, Schmitt H, Smalla K**. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microbiol* **14**: 236–43.
86. **Stokes HW, Gillings MR**. 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol Revi* **35**: 790–819.
87. **Stanton TB, Humphrey SB, Stoffregen WC**. 2011. Chlortetracycline-resistant intestinal bacteria in organically raised and feral Swine. *Appl Environ Microbiol* **77**: 7167–70.
88. **Kazimierczak KA, Scott KP, Kelly D, Aminov RI**. 2009. Tetracycline resistome of the organic pig gut. *Appl Environ Microbiol* **75**: 1717–22.
89. **Varga C, Rajić A, McFall ME, Reid-Smith RJ**, et al. 2009. Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic fecal *Escherichia coli* isolated from Alberta finishing swine farms. *Prev Vet Med* **88**: 185–92.
90. **Looft T, Johnson TA, Allen HK, Bayles DO**, et al. 2012. In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* **109**: 1691–6.
91. **Allen HK, Looft T, Bayles DO, Humphrey S**, et al. 2011. Antibiotics in feed induce prophages in swine fecal microbiomes. *MBio* **2**: e00260–11.
92. **Modi SR, Lee HH, Spina CS, Collins JJ**. 2013. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* **499**: 219–22.
93. **Colomer-Lluch M, Imamovic L, Jofre J, Muniesa M**. 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. *J Antimicrob Chemother* **55**: 4908–11.
94. **Durso LM, Miller DN, Wienhold BJ**. 2012. Distribution and quantification of antibiotic resistant genes and bacteria across agricultural and non-agricultural metagenomes. *PLoS One* **7**: e48325.
95. **de Vries LE, Vallès Y, Agersø Y, Vaishampayan PA**, et al. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLoS One* **6**: e21644.
96. **Martiny AC, Martiny JB, Weihe C, Field A**, et al. 2011. Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. *Front Microbiol* **2**: 238.
97. **Martínez JL**. 2012. Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens. *Front Microbiol* **2**: 265.
98. **Forsberg KJ, Reyes A, Wang B, Selleck EM**, et al. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**: 1107–11.
99. **Smillie CS, Smith MB, Friedman J, Cordero OX**, et al. 2011. Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* **480**: 241–4.
100. **Diaz-Torres ML, Villedieu A, Hunt N, McNab R**, et al. 2006. Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. *FEMS Microbiol Lett* **258**: 257–62.
101. **Cheng G, Hu Y, Yin Y, Yang X**, et al. 2012. Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS Microbiol Lett* **336**: 11–6.
102. **Seville LA, Patterson AJ, Scott KP, Mullany P**, et al. 2009. Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microb Drug Resist* **15**: 159–66.
103. **Claesson MJ, Jeffery IB, Conde S, Power SE**, et al. 2012. Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488**: 178–84.
104. **Qin J, Li Y, Cai Z, Li S**, et al. 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490**: 55–60.
105. **Karlsson FH, Tremaroli V, Nookaew I, Bergström G**, et al. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* **498**: 99–103.
106. **Grave K, Torren-Edo J, Mackay D**. 2010. Comparison of the sales of veterinary antibacterial agents between 10 European countries. *J Antimicrob Chemother* **65**: 2037–40.
107. **Cars O, Mölstad S, Melander A**. 2001. Large variation in antibiotic utilisation between the countries in the European Union. *Lancet* **357**: 1851–2.

108. **Borg MA**. 2012. National cultural dimensions as drivers of inappropriate ambulatory care consumption of antibiotics in Europe and their relevance to awareness campaigns. *J Antimicrob Chemother* **67**: 763–7.
109. **Roumie CL, Halasa NB, Grijalva CG, Edwards KM**, et al. 2005. Trends in antibiotic prescribing for adults in the United States—1995 to 2002. *J Gen Intern Med* **20**: 697–702.
110. **Bjerrum L, Cots JM, Llor C, Molist N**, et al. 2006. Effect of intervention promoting a reduction in antibiotic prescribing by improvement of diagnostic procedures: a prospective, before and after study in general practice. *J Gen Intern Med* **62**: 913–8.
111. **Yin X, Song F, Gong Y, Tu X**, et al. 2013. A systematic review of antibiotic utilization in China. *J Antimicrob Chemother* **68**: 2445–52.
112. **Sørum M, Johnsen PJ, Aasnes B, Rosvoll T**, et al. 2006. Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. *Appl Environ Microbiol* **72**: 516–21.
113. **Hu Y, Yang X, Qin J, Lu N**, et al. 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat Commun* **4**: 2151.
114. **Wu NAN, Qiao MIN, Zhang B**. 2010. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ Sci Technol* **44**: 6933–9.
115. **Ho PL, Chow KH, Lai EL, Lo WU**, et al. 2011. Extensive dissemination of CTX-M-producing *Escherichia coli* with multidrug resistance to 'critically important' antibiotics among food animals in Hong Kong, 2008–10. *J Antimicrob Chemother* **66**: 765–8.
116. **Levy S, FitzGerald G, Macone A**. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *N Engl J Med* **295**: 583–8.
117. **Holmberg SD, Wells JG, Cohen ML**. 1984. Animal-to-man transmission of antimicrobial-resistant Salmonella: investigations of U.S. outbreaks, 1971–1983. *Science* **225**: 833–5.
118. **Hummel R, Tschäpe H, Witte W**. 1986. Spread of plasmid-mediated nourseothricin resistance due to antibiotic use in animal husbandry. *J Basic Microbiol* **26**: 461–6.
119. **Benjamini Y, Yekutieli D**. 2001. The control of the false discovery rate in multiple testing under dependency. *Ann Stat* **29**: 1165–88.
120. **Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL**, et al. 2008. A core gut microbiome in obese and lean twins. *Nature* **457**: 480–4.