Title: Correlation among antibiotic resistance and virulence traits in human gut microbiomes

Pedro Escudeiro[1], Joël Pothier[2], Francisco Dionisio[1, 3] and Teresa Nogueira[1]

[1] cE3c – Centro de Ecologia, Evolução e Alterações Ambientais, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

[2] Atelier de Bioinformatique, ISYEB, UMR 7205 CNRS MNHN UPMC EPHE, Muséum National d'Histoire Naturelle, CP 50, 45 rue Buffon F-75005 Paris, France

[3] Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal.

Corresponding author:

Teresa Nogueira
cE3c – Centro de Ecologia, Evolução e Alterações Ambientais, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal
Phone. +351 217500577
Fax. +351 21750002
teresainogueira@gmail.com

Keywords: antibiotic resistance – virulence – microbiome - metagenomics – human gut – co-occurrence

Abstract:

Human beings have used large amounts of antibiotic, not only in medical contexts but also, for example, as growth factors in agriculture and livestock, resulting in the contamination of the environment. Even when pathogenic bacteria are the targets of antibiotics, hundreds of non-pathogenic bacterial species are affected as well. Therefore, both pathogenic and non-pathogenic bacteria have gradually become resistant to antibiotics. We tested whether there is still co-occurrence of resistance and virulence determinants. We performed a comparative study of environmental and human gut metagenomes issuing from different individuals from different human populations across the world. We found a great diversity of antibiotic resistance determinants (ARd) and virulence factors (VFd) in metagenomes. Importantly, even after correcting for metagenome sizes, there is a correlation between ARd and VFd. In the human gut there are less ARd and VFd than in more diversified environments, and yet the ARd/VFd correlations are stronger. They can vary from very high in Malawi, where antibiotic consumption is unattended, to non-existent in the uncontacted Amerindians. We conclude that there is co-occurrence of resistance and virulence determinants, suggesting a possible co-selective phenomenon. Therefore, by selecting for resistant bacteria, we may be selecting for more virulent strains, as a side effect of antimicrobial therapy.

Introduction:

Antibiotics are present in microbial communities, not only as a result of the natural lifestyle of microorganisms but also due to the usage of these drugs in agriculture, food industry, livestock, or in human health (Castanon 2007). Therefore, antibiotics can affect bacterial communities as a whole, comprising both pathogenic and non-pathogenic bacteria. Take, for example, the human microbiome, defined as the set of microorganisms that colonize humans. This microbiome is composed of about 3.8e+13 bacterial cells (Sender *et al* 2016) spanning thousands of taxa and colonizing our body's surfaces and bio fluids, including tissues such as skin, mucosa and most importantly, the gastrointestinal tract. Thus, even when virulent bacteria are the targets of antibiotics, the administration of these drugs may also affect many non-pathogenic mutualistic or commensal bacterial species present in individuals undergoing treatment (Francino 2015).

The resistome (the collection of all antibiotic resistance (AR) genes, which exist in both pathogenic and non-pathogenic bacteria (Wright 2007)) is frequently encoded on mobile genetic elements. Similarly, the virulome, the set of genes encoding virulence can also be encoded on the mobilome (Svara and Rankin 2011; Nogueira *et al* 2012; Nogueira *et al* 2009; Smith 2001). Therefore, many bacterial virulence factors (VF) are easily spread in bacterial populations by horizontal gene transfer, converting mutualistic or commensal bacteria into potential opportunistic pathogens. Several examples of highly virulent and multi-resistant disseminated clones have already been reported throughout the literature (for a review see (Beceiro *et al* 2013)).

A given bacterial population may constitute a life insurance to other bacterial populations present in the same microbiome through at least two different mechanisms. First, if antibiotic resistance genes are coded by plasmids or other mobile genetic elements, they may transfer to pathogenic cells and save them from the negative effects of antibiotics. Indeed, these genetic elements can spread into the bacterial community by horizontal gene transfer, even crossing species (Sommer *et al* 2010; Sommer *et al* 2009; Andersson and Hughes 2014; Vega and Gore

2014). Curiously, some bacterial strains from different species have been shown to be extremely good donors of certain plasmids. As such, they are able to amplify the number of plasmids in a bacterial community and spread those plasmids to other bacterial cells (Dionisio *et al* 2002), a phenomenon probably explained by interactions between different plasmids (Gama *et al* 2017a, 2017b).

Secondly, even bacterial cells not coding for antibiotic resistance determinants nor receiving mobile elements may be protected by cells coding for certain drug resistance genes. Indeed, gene products that inactivate antibiotics by degrading or modifying antibiotic molecules are also decreasing their concentration in the local environment (for a review about mechanisms of possible indirect resistance see (Wright 2005). This mechanism of indirect resistance has been shown to occur in different systems and to be pervasive (Nicoloff and Andersson 2016; Sorg *et al* 2016; Domingues *et al* 2017).

In this paper we ask whether there is co-occurrence of antibiotic and virulence among bacterial populations. For this, we have chosen to study metagenomes. There are three main reasons to use metagenomes to address this question. First, it is known that, for millions of years, bacteria had to cope with the presence of many other species, mostly competing with them (Foster and Bell 2012), but also cooperating, both phenomena relying on the full set of genes of the metagenome (Ponomarova and Patil 2015; Rosenthal 2011; Foster and Bell 2012). Secondly, horizontal gene transfer promotes the genetic relationship between species thus enforcing cooperation (Nogueira *et al* 2009) avoiding the emergence of cheaters within the microbiome. Third, the study of metagenomes gives us access to the repertoire of genes involved in adaptation to the environment, given that many of these traits are often encoded in the mobilome and thus can be shared by different, eventually unrelated bacteria. In this context, mining for genes coding for antibiotic resistance and virulence traits in metagenomes is a reliable way to access the selective pressures the population is subject to, as well as the co-occurrence of genetic traits of the whole microbiome.

The present study aims at understanding the relationship between antibiotic resistance genes and those coding for virulence. Here we show that there is indeed a linkage between the dissemination of virulence factors and genes coding for antibiotic resistance, within natural microbiomes, and that this relationship is influenced by the behaviours of human populations, spanning from very different geographical locations across the world.

Materials and methods:

Metagenomic datasets

Our human gut query cohort included 110 previously studied, and publicly available metagenomes pertaining to individuals from different regions of Venezuela, Malawi, and the USA, as well as a broad age span (0.05 to 53 years) (Yatsunenko *et al* 2012). All the metagenomes files were generated by the same team and project, and by using the same bioinformatics pipeline. Our environmental cohort comprised 64 previously selected, and publicly available environmental metagenomes, belonging to 12 different biomes (Delmont *et al* 2011). Although Delmont's team report using a dataset comprised of 77 metagenomes, there are only 70 MG-RAST accession numbers present in the article's appendix, of which only 64 are publicly available. Both project's metagenomes were downloaded from MG-RAST (Meyer *et al* 2008) under FASTA format, making use of successive calls to its Application Programming Interface (API) (Wilke et al 2015) using the respective MG-RAST's accession numbers available in the aforementioned bibliography. Each FASTA file comprised clustered protein-coding sequences, retrieved from MG-RAST's file-formatting pipeline (550.cluster.aa90.faa files). The protein sequences enclosed in these files are clustered at 90% identity, containing non-redundant translated sequences (Yatsunenko *et al* 2012). These protein-coding formatted FASTA files contain the translations of one representative from each cluster. Thus, the numbers of protein sequences for a given metagenome used here represent its richness.

BLAST, VFDB and Resfams

For every metagenome present in our query, a BLASTP (Altschul *et al* 1997) search was performed against the VFDB database for bacterial virulence factors families (Chen *et al* 2012) and the Resfams AR Proteins database of bacterial antibiotic resistance protein families (Gibson *et al* 2014). An alternative approach made use of the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop 2009), but several hindrances concerning its sub-classification by functional antibiotic resistance protein families made us discard the possibility of using such database to address of AR and VF protein family co-occurrence in metagenomes.

The BLAST+ executables package was downloaded on the 17th of November 2015 (ncbi-blast-2.2.31+ version). The VFDB was downloaded on the 11th of November 2013 (31 classified FASTA files of bacterial virulence factor sub-families), and the Resfams database was downloaded on the 29th of January 2016, (123 classified FASTA files of bacterial antibiotic resistance protein subfamilies. Every protein enclosed in each of the addressed metagenomes, was used as a query in order to search for similarities to either AR or VF protein-coding traits. Hence, we aimed at retrieving the best hit (best scored alignment) that enabled us to assign an AR or VF function to each of the aforementioned metagenomic proteins. Every BLASTP search was performed with a very stringent e-value cut-off of 1e-15 (10 orders of magnitude lower than the conservative commonly used E-value of 1e-5). Next, we filtered the resulting output files, as to only retrieve the alignment hits with >60% coverage of the query size, and whose query and subject length ratio is between 0,75 and 1,5. When using a combination of this very stringent e-value of 1e-15 together with coverage, and length ratio filters, we expect to only retrieve true homologues, avoiding false positives. Furthermore, from all the generated alignments between our metagenomic query cohort and the preceding databases, we computed the representative counts for the different gene families that coded for either AR or VF traits. Thus, the amounts of different classes (gene families) that are present in a given metagenome represent its diversity in terms of AR or VF traits.

We have also removed hits for proteins that aligned with both antibiotic resistance and virulence factor proteins (25.9% of hits against the VFDB, and 29,9% of the hits against the Resfams database). The abovementioned filters and algorithms were implemented making use of Unix scripting languages (GNU Awk version 4.0.1, and Z-Shell version 5.0.2), under a Linux environment.

Statistical Analysis

To test for relationships in metagenomes between the presence of both antibiotic resistance and virulence factors traits we proceeded as follows. Our expectation is that the diversity number for homologues of antibiotic resistance protein families (henceforth denoted as ARd) and the diversity number for homologues of virulence factors protein families (denoted as VFd) in each metagenome (clustered at 90% identity) increases with the protein family richness of the latter (being that the protein family richness here means the total number of cluster representative proteins. Given the potential diversity of these protein families, one does not expect ARd and VFd to level off with a metagenome's protein family richness. Therefore, we assume a linear relationship between ARd and metagenome's protein family richness, with a fixed 0 intercept. The same for VFd. Our results will show that these assumptions are reasonable. Thus, to avoid spurious correlation, we corrected the diversity of ARd and VFd in a metagenome by taking into account its protein family richness: we have standardized the different representative counts that issued from these samples according to the protein family richness of their respective metagenomes, therefore escaping "statistical inevitability".

Hence we define α as the slope of the regression line of ARd on the protein family richness (henceforth denoted as *Size* solely in the equations) of the metagenomes (with a fixed 0 intercept) and β as the slope of the regression line of VFd on the protein family richness of the metagenomes (also with a fixed 0 intercept):

$$ARd = \alpha.Size \qquad \text{Equation 1A}$$

$$VFd = \beta.Size \qquad \text{Equation 1B}$$

Thus a metagenome i of a given protein family richness (Size(i)) is expected to have $ARd\ (i) = \alpha.Size\ (i)$ antibiotic resistance protein families' homologues and $VFd(i) = \beta.Size(i)$ virulence factors protein families' homologues. Or:

$$\frac{ARd(i)}{\alpha.Size(i)} = 1 \qquad \text{Equation 2A}$$

$$\frac{VFd(i)}{\beta.Size(i)} = 1 \qquad \text{Equation 2B}$$

Naturally, it would be the case if ARd and VFd were only correlated with the protein family richness of the metagenomes. This is our null hypothesis, H0: data points are evenly present in the four quadrants around point $(\frac{ARd(i)}{\alpha.Size(i)}, \frac{VFd(i)}{\beta.Size(i)})$=(1,1). However, some of the metagenomes do not match these predictions. Therefore, one may ask whether a given metagenome with more ARd than expected for its protein family richness has less or more VFd than expected for its protein family richness. There are two alternative hypothesis: 1) data points distribute over upper right and lower left quadrants around $(\frac{ARd(i)}{\alpha.Size(i)}, \frac{VFd(i)}{\beta.Size(i)})$=(1,1) (i.e. the more diverse are AR genes, the more diverse are VF genes); or 2) they distribute over upper left and lower right quadrants around $(\frac{ARd(i)}{\alpha.Size(i)}, \frac{VFd(i)}{\beta.Size(i)})$=(1,1) (i.e. the more diverse are AR genes, the less diverse are VF genes, and vice versa) This is the alternative hypothesis H1: data points distribute over two quadrants around.

The Spearman rank correlation coefficient (r_s) was used to test the association between VFd and ARd. All possible associations between VFd and ARd hits for sub-families were also generated (n = 123 * 31 = 3813). As to control the expected proportion of rejected null hypotheses, the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) was applied to the Spearman's r_s p-values generated this way. The correlation coefficient (r) and the slope of the linear fits were also calculated as to access the strength and direction of the relationship between the standardized ARd and VFd on the generated scatterplots, as well as to address the ratio between these variables. Associations with $r_s > 0.5$, that also had $r > 0.5$ and a r_s P-value < 0.001 were considered valid. All statistical analysis was conducted with the R programming language (version 3.2.2). Scatterplots and marginal boxplots were generated using R's ggplot2, grid, gtable and scales packages.

Results:

Antibiotic resistance (AR) gene families in the metagenomes

We started by evaluating the quantity of different AR genes present in the metagenomes under study. We wanted to know how does the diversity of antibiotic resistance gene families' homologues (that is, ARd, the number of different families) vary with the metagenome protein family richness (equation 1A). To answer this question, we used a data set of environmental metagenomes issuing from diverse ecosystems and biomes, such as oceans, coral atolls, deep oceans, Antarctic aquatic environments and snow, soils, hyper saline sediments, sludge's, microbial fuel cell biofilms, and animal microbial populations (Delmont *et al* 2011). In this dataset, there is a broad variation in AR gene diversity (ARd) (Fig. 1.A), even for a given fixed metagenome protein family richness For example, when the number of sequences of a given metagenome is about 1.75e+5, the number of ARd can range from almost zero (chicken cecum or cow rumen) to about 3000 (acid mine biofilms). However, there is a close to linear relationship between ARd and the protein family richness of the metagenomes. Indeed, we draw a regression line, with $r = 0.7543$, $r_s = 0.6426$, $P = 1.037e{-}08$ (r_s is the Spearman rank correlation coefficient, slope = $\alpha_e \approx 0.0048$).

We also draw a fit line for the subset of metagenomes belonging to the human faeces biome. Another dataset composed by 110 human gut metagenomes, sampled from healthy individuals with ages ranging from 0.05 to 53 years of age, spanning different regions of the world such as: USA, Malawi, and the Venezuelan Amazon was also studied (Yatsunenko *et al* 2012). In this dataset (see Supplemental Fig. A) the frequency of antibiotic resistance protein families is strongly dependent on the protein family richness of the metagenome ($r = 0.9142$, $r_s = 0.9424$, $P = 2.2e{-}16$, slope = $\alpha_g \approx 0.0052$).

Virulence factor (VF) gene families in the metagenomes

Bacterial virulence factors are proteins that enable pathogenic bacteria to parasitize a host, including gene products involved in adhesion and invasion, secretion, toxins, and iron acquisition systems (Chen *et al* 2012).

The environmental microbiomes of our dataset reveal a great diversity of virulence factors protein families' (VFd) densities (Fig. 1.B). For example, when the number of sequences of a metagenome is about 3.7e+5, VFd varies from about 1000 (for human faeces) to more than 7000 (ocean). However, there is a close to linear relationship between VFd and the protein family richness of the metagenomes. Indeed, we draw a regression line, with $r = 0.6694$, $r_s = 0.5219$, $P= 9.745e{-}06$, where r_s is the Spearman rank correlation coefficient, slope = $\beta_e \approx 0.0083$). Since virulence may also be associated with the colonization of different types of biomes (some virulent traits are involved in adaptation to new environments, by allowing bacteria to adhere and colonize substrates, to access to resources such as iron, among other functions) besides the context of infection, one can expect different types of these genes in environmental microbiomes. Moreover, in human gut microbiomes (see Supplemental Fig. B), we observed a good correlation between VFd and the protein family richness of the metagenomes ($r = 0.6331$, $r_s = 0.7626$, $P = 2.2e\text{-}16$, where r_s is the Spearman rank correlation coefficient, slope = $\beta_e \approx 0.0058$).

The AR / VF correlations

The main purpose of the present work is to evaluate the relationship, if any, between ARd and VFd in microbiomes. Therefore, we excluded from our analysis all the gene products that are both homologues to AR and VF determinants. Thus we avoid the introduction of a bias in the correlation analysis. We computed $\frac{ARd(i)}{\alpha.Size(i)}$ and $\frac{VFd(i)}{\beta.Size(i)}$ for all metagenomes. In Fig. 2 one can see that the two variables are positively correlated: a metagenome with more (less) than expected VFd also has more (less) than expected ARd. Although there is a large variation of ARd (Fig. 1.A), and VFd (Fig. 1.B), in environmental metagenomes, there is a strong correlation

between ARd and VFd (r = 0.7547, r_s = 0.8256, P = 2.2e-16, Spearman rank correlation coefficient) (Fig. 2). When we draw the regression line for the human gut microbiomes from this dataset (human gut), we can see that the ratio ARd/VFd is even higher than for the case of environmental metagenomes. Despite the fact that the values for ARd and VFd are lower in gut metagenomes (see Fig. 1), one can witness a higher ratio ARd/VFd (larger slope) of metagenomes pertaining to the human faeces biome relatively to the slope calculated for all environmental metagenomes (Fig. 2). This suggests that there is a greater accumulation of ARd over VFd for this particular biome than in environmental ones. In Fig. 2, one can see that just a few points fall in the second and fourth quadrants. Points that fall into the second quadrant correspond to metagenomes for which there is more ARd than expected, given the VFd. The points that fall into the fourth quadrant pertain to metagenomes for which there is less ARd than expected, given the VFd.

Fig. 3 shows AR diversity versus VF diversity in the human gut metagenomes for each population (country) and for the three populations together. All the statistics are summarized in Table I and Supplemental Table (for the study using the ARDB database (Liu and Pop 2009)). The correlation between ARd and VFd is lower in the human gut metagenomes dataset (r = 0.5572, r_s = 0.6289, P = 2.2e-16, Spearman rank correlation coefficient) (Fig. 3.A) than in the environmental samples (r = 0.7547, r_s = 0.8256, P = 2.2e-16) (Fig. 2). This correlation is not dependent on the protein family richness of the metagenomes (Fig. 3.A). In order to better understand the results, we can divide the graph into quadrants, using the axes set by coordinates (1,1)(See Equations 2.A and 2.B). We can see that there are many points (metagenomes) located on the first and third quadrant following the regression line. These points represent the metagenomes for which there is a correlation between ARd and VFd; that is, ARd and VFd are both either in excess or in deficit (compared to their expected values given the protein family richness of the metagenome). Nevertheless, some points fall in the second quadrant, (corresponding to metagenomes for which there is more ARd than expected given VFd), and some other points fall into the fourth quadrant (corresponding to metagenomes for which there is less ARd than expected given VFd.

However, we can distinguish different trends upon geographical localization of the human populations under study. The largest contribution to this graph comes from the North American samples, which account for 66/110 (60%) of the individuals (Fig. 3.B).

We have compared these results with those of the gut metagenomes issuing from non-western human populations, having very contrasting lifestyles and exposure to antibiotics: the uncontacted Amerindians, harbouring a resistome similar to that of pre-antibiotic era; and those of the people from Malawi with a high prevalence of infectious diseases and widespread use of unprescribed antibiotics (WHO s.d.; UNICEF s.d.; Forslund *et al* 2013).

While studying metagenomes collected from Amerindians (Venezuela) (21/110, or 19% of the metagenomes of the dataset) and Malawians (23/110, or 21%), we can see two completely different scenarios (Fig. 3.C and 3.D). In the Amerindian gut metagenomes there is no statistically significant correlation between ARd and VFd ($r = 0.326$, $r_s = 0.4519$, $P = 0.04111$, Spearman rank correlation coefficient). This result is relevant, when we compare it to the Malawian gut metagenomes where there is a very strong correlation between ARd and VFd ($r = 0.9088$, $r_s = 0.8706$, $P = 2.533e{-}06$). Interestingly, the slope of the regression line portrayed by the association between ARd and VFd belonging to Malawian human gut metagenomes (Fig. 3.D) is twice as large than those issuing from the Amerindian (Fig. 3.C) and USA populations (Fig. 3.B). That is, the Malawian metagenomes accumulate two times more ARd per VFd than its Amerindian and North-American counterparts.

The co-occurrence of AR and VF belonging to the cell envelope

Our results suggest that co-occurrence of AR and VF might be taking place amidst bacterial communities. We wondered, however, which were the genetic traits that were more prone to this effect. All the AR and VF protein families associations were plotted and analysed. Thus, after all possible associations between subfamilies of AR and VF pairs had been generated (123 * 31 = 3813) a Spearman's rho (r_s) cut-off of 0.5 was applied as to filter the best correlations. The vast majority of associations falls into the functional category of multi-drug efflux pumps (AR's) associated with either secretion systems, as well with iron uptake and adhesion

mechanisms (VF's), respectively (Table II). Amongst the most representative associations between AR and VF traits are those belonging to the cell envelope and the general secretion mechanisms. On the other hand, the AR protein families for beta-lactamases, and general beta-lactam resistance mechanisms, bore no statistically significant correlations whatsoever, attaining very weak Pearson and Spearman correlation coefficients as well.

Discussion:

Antibiotic resistance among environmental and human gut metagenomes

As expected, we found different homologues for antibiotic resistance (AR) gene products belonging to different protein families (AR diversity or ARd) among environmental metagenomes (Fig. 1.A). We have shown that, in the environmental samples, ARd varies a lot from metagenome to metagenome. This may result from the differential microbial community composition of the metagenomes, whose genetic diversity can be grouped according to the adaptation to the environment in question (Delmont *et al* 2011); but also from the fact that the selective pressure for the maintenance of antibiotic resistance genes in environmental microbiome varies widely from environment to environment (Martinez 2008; Martinez and Baquero 2002; Fitzpatrick and Walsh 2016). Human gut metagenomes, on the contrary, have a less diversified repertoire of AR determinants (lower ARd) than that of their environmental counterparts.

We found a very strong correlation between the diversity of AR determinants and the metagenome's protein family richness (Supplemental Fig. A), independently of the geographic origin of the human populations. These similar densities of ARd can indicate that, in human gut microbiomes, the number of different AR genes is not influenced by the human lifestyle, such as diet, medical care, access to antibiotics or other cultural habits (Ghosh *et al* 2013), and/or that the adaptation to the intestinal tract shapes microbial AR diversity as well.

Antibiotic use can vary from country to country (CDDEP s.d.), and, as consequence, individuals from different human populations are under different antibiotic exposition. Forslund and co-workers have demonstrated that there are robust differences in the antibiotic resistance arsenal between countries and that these differences follow the veterinary and human antibiotic use (Forslund *et al* 2013).

Virulence among environmental and human gut metagenomes

In what concerns virulence, we can assert that there is a wide diversity and density differences of VFs in environmental metagenomes, which poses as evidence of the plasticity portrayed by environmental bacteria in order to adapt to different hosts and niches (Fig. 1.B). On the other hand, human gut microbiomes harbour a less diverse VF repertoire, especially in the USA samples (Supplemental Fig. B), which seem to indicate an evolution towards adaptation to the human gut, or lower contact with pathogens, eventually due to vaccinations and sanitation.

Association of AR / VF

According to Yatsunenko and colleagues, once the human gut metagenome is established at the age of 3 years old, it does not diverge much from individual to individual, in what concerns both phylogenetic diversity and functional richness (Yatsunenko *et al* 2012).

The most relevant results shown here are that, when corrected for metagenome's protein family richness, ARd and VFd are strongly correlated both in the environmental samples (Fig. 2 and 3) and in the human gut samples (Fig. 3), with special emphasis on the human gut.

The North American intestinal samples (Fig. 3.B) show a wide variety of associations between ARd and VFd, always presenting a statistically significant correlation between these genetic traits. This result in itself reinforces our hypothesis that antibiotic resistance and virulence are in fact co-associated. In particular there are 5/66 (7.5%) points in the second quadrant of Fig. 3.B representing metagenomes where there is an accumulation of ARd by VFd, thus suggesting that the mobilization of mobile genetic elements as gene cassettes, driving to multirresistance, might be taking place. The USA population, like other industrialized countries, is culturally exposed to antibiotics from health care facilities such as hospitals, antibiotic therapy, and the use of antibiotics in agriculture and livestock.

Amerindians from Venezuela and Malawians share, phylogenetically, more similar human gut microbiomes than the North Americans (Yatsunenko *et al* 2012), which belong mainly to a different enterotype (Lozupone *et al* 2012). Yet, whereas there is no ARd/VFd correlation among the Venezuelan individuals, there is a very strong correlation among the Malawian ones. This result highlights the potential effect of the exposure to antibacterial drugs on promoting the dissemination of antibiotic resistance by horizontal gene transfer, and the potential co-selection of virulence traits within bacterial communities.

The uncontacted Amerindian microbiome represents a “frozen” relic of a pre-antibiotic era of the human resistome, while the Malawian gut microbiome is much more exposed, both to antibiotics, and to colonization by pathogens (WHO s.d.; UNICEF s.d.; Forslund *et al* 2013).

Amerindians have no known access to pharmaceutical drugs, as they usually make use of the traditional indigenous medicine. Malawi is one of the poorest countries in Africa, where most people live with less than one dollar a day, many people cope with AIDS and bacterial infections, and where many children suffer from severe malnutrition (WHO s.d.). Nutrition has been reported to have a big impact on both the human gut microbiome composition and resistome (Ghosh *et al* 2014; 2013; Pehrsson *et al* 2016). In Malawi, UNICEF has been implementing a program of Ready-to-Use-Therapeutic-Food (RUTF) in order to reduce mortality among children. RUTF often contains antibiotics such as co-trimoxazole. It has been questioned, however, whether the success of this therapy is due to re-nutrition or to the combination with antibiotics (Alcoba *et al* 2013; Makoka *et al* 2012) that could have an effect on the microbial gut composition. It has been reported that there is widespread resistance to almost all of antibiotics that are empirically used in Malawi due to the lack of routine microbiological culture and sensitivity testing (Makoka *et al* 2012), but also due to self-medication. Human gut samples from the Malawi people of the dataset used in the present study are described as having a high overall resistance potential with an over representation of cephalosporin and tetracycline resistance genes which may suggest extensive use of old, broad-spectrum antibiotics, a known problem in many developing countries (Forslund *et al* 2013).

The co-representation of AR and VF targeting the cell envelope

Between all the possible statistical AR and VF protein families' correlations, the strongest ones are among proteins belonging to the bacterial cell envelope (Table II). This result is not surprising, as many proteins that belong to the secretory system or to the secretome itself, and thus target the bacterial envelope, are frequently encoded on mobile genetic elements (Nogueira *et al* 2012; Nogueira *et al* 2009). As such, this co-occurrence may also be regarded as a direct consequence of the dynamics of these genetic elements coding for both types of determinants.

In human gut the strongest correlations involve efflux pumps that can extrude antibiotics non-specifically, and adhesion and iron scavenging mechanisms of pathogenicity. One possible explanation for the fact that the most frequent AR and VF associations in human gut involve efflux pumps could be that they allow a fast and efficient response to new man-made antibiotic molecules, while in environmental genomes, specific resistance mechanisms targeted to particular antibiotic molecules may have had more time to evolve to specific strategies.

Another possible explanation is that extrusion can also be implicated in pathogenic mechanisms of human associated pathogens as well as bacteria-host interactions. For example, biofilm formation in a *Staphylococcus aureus* methicillin resistant (MRSA) strain has been known to be essentially reliant on the activity of fibronectin binding proteins (McCourt *et al* 2014), and multi-drug efflux pumps have direct implications with the formation and maintenance of such biofilms (Soto, 2013). Furthermore quinolone resistant *S. aureus* strains up regulate the production of fibronectin binding proteins when subjected to sub-lethal dosages of ciprofloxacin (Bisognano *et al* 2004). It has also been acknowledged that physiological levels of some cations present within the host promote the up regulation of genes encoding putative efflux transporters, oxidoreductases, and mechanisms of iron uptake either in *Acinetobacter baumannii* (Hood *et al* 2010) as in *Burkholderia cenocepacia* (Drevinek *et al* 2008), which could explain the co-association of iron acquisition systems with those of multi-drug efflux pumps.

We show here that there is a correlation between the diversity of antibiotic resistance and virulence traits in human gut microbiomes and in environmental microbiomes. Interestingly, this correlation varies from a human population to another. Human antibiotic exposure due, either to therapy or to the environment and food, can have an effect on selecting for potentially pathogenic bacteria in the human gut microbiomes. It can also drive and shape changes on the gene pool of microbiomes. This means that, by selecting for resistant bacteria, we might be also selecting for more virulent strains, as a side effect of antimicrobial therapy.

Funding

T.N. was a recipient of a grant from Fundação para a Ciência e a Tecnologia (SFRH/BPD/41863/ 2007).

Acknowledgements:

We thank Eduardo P. C. Rocha for critical reading of the manuscript and constant interest and suggestions since the beginning of this work, Guillaume Sapriel for suggestions to improve the methods, Pedro David, Eric Duchaud, Guillaume Achaz, and all the members of our teams for the fruitful discussions, Octávio Paulo, Francisco Pina-Martins and Sophie Brouillet for the technical help, and granting access to the servers.

Conflict of Interest:

The authors declare no conflict of interest.

References

Alcoba G, Kerac M, Breysse S *et al*. Do Children with Uncomplicated Severe Acute Malnutrition Need Antibiotics? A Systematic Review and Meta-Analysis. *PLoS One* 2013, **8** (1): e53184.

Altschul SF, Madden TL, Schäffer AA *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997, **25** (17): 3389-3402.

Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014, **12**: 465-478.

Beceiro A, Tomás M, Germán B. Antimicrobial Resistance and Virulence: a Successful or Deleterious Association in the Bacterial World? *Clin Microbiol Rev* 2013, **26** (2): 185.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc* 1995, ***Series B*** (57): 289–300.

Bisognano C, Kelley WL, Estoppey T *et al*. A recA-LexA- dependent pathway mediates ciprofloxacin-induced fibronectin binding in Staphylococcus aureus. *J Biol Chem* 2004, **279**: 9064–9071.

Casadevall A. Cards of Virulence and the Global Virulome for Humans. *Microbe* 2006, **1** (8): 359-364.

Castanon JIR. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poultry Science* 2007, **86** (11): 2466–2471

CDDEP (n.d.) *Resistance Map.* Retrieved 4 08, 2017 from https://resistancemap.cddep.org/AntibioticUse.php

Chen LH, Xiong ZH, Sun LL *et al*. VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Res 2012,* **40***(Database issue)*: D641–D645.

Dethlefsen L, Huse S, Sogin ML *et al.* The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* 2008, **6**(11): e280.

Delmont TO, Malandain C, Prestat E *et al*. Metagenomic mining for microbiologists. *ISME J* 2011, **5:** 1837-1843.

Dionisio F, Matic I, Radman M *et al.* Plasmids spread very fast in heterogeneous bacterial communities. *Genetics* 2002,**162**: 1525-32.

Domingues IL, Gama JA, Carvalho LM *et al* Social behaviour involving drug resistance: the role of initial density, initial frequency and population structure in shaping the effect of antibiotic resistance as a public good. Heredity 2017 *Heredity* **119**: 295–301

Drevinek P, Holden MT, Ge Z *et al.* Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum. *BMC Infect Dis* 2008, **8:** 121.

Fitzpatrick D, Walsh F. Antibiotic resistance genes across a wide variety of metagenomes. *FEMS Microbiology Ecology* 2016, **92** (2): 1-8.

Francino MP. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Frontiers in Microbiology* 2015, **6**: 1543.

Foster KR, Bell T. Competition, Not Cooperation, Dominates Interactions among Culturable Microbial Species. *Current Biology* 2012, **22**: 1845–1850

Freilich S, Zarecki R, Eilam O *et al.* Competitive and cooperative metabolic interactions in bacterial communities. *Nature Communications* 2011, **2** (1): 589.

Gama JA, Zilhão R; Dionisio F. Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. *Plasmid* 2017a **93**: 6-16.

Gama JA, Zilhão R; Dionisio F. Multiple plasmid interference - Pledging allegiance to my enemy's enemy. *Plasmid* 2017b **93**: 17-23.

Ghosh TS, Gupta SS, Nair GB *et al*. In Silico Analysis of Antibiotic Resistance Genes in the Gut Microflora of Individuals from Diverse Geographies and Age-Groups. *PLoS One* 2013, **8** (12): e83823.

Ghosh TS, Gupta SS, Bhattacharya T *et al.* Gut Microbiomes of Indian Children of Varying Nutritional Status. *PLoS One* 2014, **9** (4): e95547.

Gibson M, Forsberg K, Dantas G. Improved annotation of antibiotic resistance functions reveals microbial resistomes cluster by ecology. *ISME J* 2014, **9** (1): 207-16.

Hood M, Jacobs A, Sayood K *et al*. Acinetobacter baumannii increases tolerance to antibiotics in response to monovalent cations. *Antimicrob Agents Chemothe.* 2010, **54** (3): 1029-41.

Forslund K, Sunagawa S, Kultima JR *et al*. Country-specific antibiotic use practices impact the human gut resistome. *Genome Res* 2013, 23(7): 1163–1169.

Liu B, Pop M. ARDB–Antibiotic Resistance Genes Database. Nucleic Acids Res. 2009 Jan;37(Database issue):D443-7.

Lozupone CA, Stombaugh JI, Gordon J *et al*. Diversity, stability and resilience of the human gut microbiota. *Nature* 2012, **489**: 220-230.

Makoka MH, Miller WC, Hoffman IF *et al*. Bacterial infections in Lilongwe, Malawi: aetiology and antibiotic resistance. *BMC Infect Dis* 2012, **12** (1): 1471-2334.

Martinez JL. Antibiotics and antibiotic resistance genes in natural environments. *Science* 2008, **321:**365-7.

Martínez JL, Baquero F. Interactions among Strategies Associated with Bacterial Infection: Pathogenicity, Epidemicity, and Antibiotic Resistance. *Clin Microbiol Rev* 2002, **15** (4): 647–679.

McCourt J, O'Halloran D, McCarthy H *et al.* . Fibronectin-binding proteins are required for biofilm formation by community-associated methacillin-resistant Staphylococcus aureus strain LAC. *FEMS Microbiol Lett* 2014, **353**: 157–164.

Meyer F, Paarmann D, D'Souza M *et al.* The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 2008, **9**: 386.

Morschhauser J, Kohler G, Ziebuhr W *et al*. Evolution of microbial pathogens. *Philos Trans R Soc Lond B Biol Sci* 2000, **355**: 695-704 .

Nicoloff H, Andersson DI. Indirect resistance to several classes of antibiotics in cocultures with resistant bacteria expressing antibiotic-modifying or -degrading enzymes. *Journal Antimicrob Chemother*, 2016 **71**(1):100-110

Nogueira T, Rankin DJ, Touchon M *et al.* Horizontal Gene Transfer of the Secretome Drives the Evolution of Bacterial Cooperation and Virulence. *Curr Biol* 2009, **19**: 1683-91.

Nogueira T, Touchon M, Rocha EP. Rapid Evolution of the Sequences and Gene Repertoires of Secreted Proteins in Bacteria. *PLoS One* 2012, **7** (11): e49403.

Novick RP. Plasmid Incompatibility. *Microbiology Rev* 1987, **51** (4): 381-395.

Pehrsson EC, Tsukayama P, Patel S *et al*. Interconnected microbiomes and resistomes in low-income human habitats. *Nature* 2016, **533:** 212.

Ponomarova O, Patil KR. Metabolic interactions in microbial communities: untangling the Gordian knot. *Curr Opin Microbiol* 2015, **27**: 37-44.

Rosenthal AZ. RNA-seq reveals cooperative metabolic interactions between two termite-gut spirochete species in co-culture. *ISME J* 2011, **5**: 1133–1142.

Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* 2016, **14**(8): e1002533.

Smillie C, Garcillan-Barcia MP, Francia MV *et al.* Mobility of Plasmids. *Microbiol Mol Biol Rev* 2010, **74** (3): 434–452.

Smith J. The social evolution of bacterial pathogenesis. *Proc. R. Soc. Lond. B* 2001, **268**: 61-69.

Sommer MO, Church GM, Dantas G. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. *Virulence* 2010, **1** (4): 299-303.

Sommer MO, Dantas G, Church GM. Functional Characterization of the Antibiotic Resistance Reservoir in the Human Microflora. *Science* 2009, **325** (5944): 1128-31.

Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm, Virulence, 4:3, 223-229. *Virulence* 2013, **4** (3): 223-229.

Sorg RA, Lin L, van Doorn GS, Sorg M, Olson J *et al*. Collective Resistance in Microbial Communities by Intracellular Antibiotic Deactivation. PLOS Biology 2016 **14**(12): e2000631.

Svara F, Rankin D. The evolution of plasmid-carried antibiotic resistance. *BMC Evol Biol* 2011,**11:** 130.

UNICEF (n.d.) Committing to Child Survival: A Promise Renewed Progress Report 2015

https://www.unicef.pt/docs/pdf_publicacoes/a-promise-progress-report.pdf

Vega NM, Gore. Collective Antibiotic Resistance: Mechanisms and Implications. *Curr Opin Microbiology* 2014, **21**: 28-34

WHO (n.d.) *Global Health Observatory (GHO) data*. Retrieved 7 20, 2016 from http://www.who.int/gho/countries/mwi/en/

Wilke A, Bischof J, Harrison T *et al.* A RESTful API for accessing microbial community data for MG-RAST. *PLoS Comput Biol* 2015, **11** (1): e1004008.

Wright GD. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* 2007, **5**: 175-186.

Wright GD. Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews* 2005, **57**(10): 1451-70

Yatsunenko T, Rey FE, Manary MJ. Human gut microbiome viewed across age and geography. *Nature* 2012, **486** (7402): 222–227.

Legends:

Figure 1. Distribution of the diversity number of antibiotic resistance and virulence factor protein families by environmental metagenomes' protein family richness

The vertical axes represent the total diversity count of AR protein families' homologues (ARd) (1. A) and VF protein families' homologues (VFd) (1.B), present in metagenomes. The horizontal axes represent the protein family richness of the metagenome, that is, the number of cluster representative sequences - see Methods. In 1.A each dot represents one of the 64 metagenomes. The black line represents the simple linear regression of best fit for all of the metagenomes, where the grey shading is the 95% confidence interval. The points are scattered showing that the diversity of AR gene families can vary greatly from metagenome to metagenome. The points' dispersal illustrates different VFd frequencies when taking distinctive environmental metagenomes into comparison. The light line represents the linear regression of best fit for the human faeces metagenomes subset. The Correlation coefficient (r), Spearman's rank correlation coefficient (r_s), P-value obtained from the latter, Coefficient of determination (r^2) and respective P-value are shown on Table I.

Figure 2. Distribution of AR by VF diversity in environmental metagenomes.

Scatter plots with marginal boxplots of $\frac{ARd(i)}{\alpha_e.Size(i)}$ *versus* $\frac{VFd(i)}{\beta_e.Size(i)}$ of each metagenome where α_e and β_e are the slopes for the environmental metagenomes, calculated in Fig. 1.A and 2.A, respectively. The black line represents the simple linear regression line of best fit, where the grey shading is the 95% confidence interval. The black line in the box plots represents the median, and the black diamonds represent the mean. Each dot represents a metagenome, and the grey lines parallel to the axes represent the (1,1) reference coordinates. The correlations seem to depend on the biome. The light line represents the linear regression of the human faeces metagenomes subset. There is a significant correlation involving the 12 different environmental biomes (n = 64, r = 0.7547, r_s = 0.8256, P < 0.001, BH post hoc procedure, lm slope = 0.6283), and that the slope is greater in metagenomes issuing from the human faeces

subset, than in the environmental ones. These statistics along with the Coefficient of determination (r^2) and respective P-value are shown on Table I.

Figure 3. Distribution of AR by VF diversity in Human gut metagenomes.

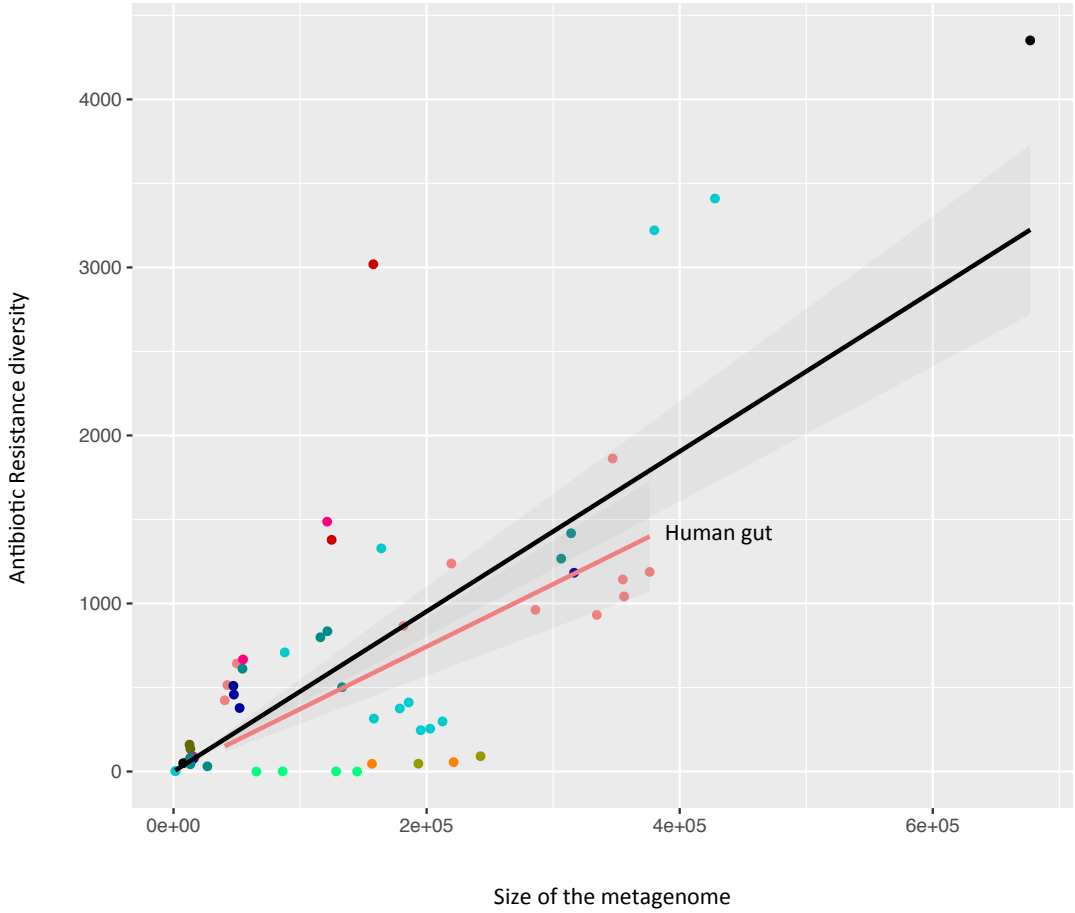
Scatter plots with marginal boxplots of $\frac{ARd(i)}{\alpha_e.Size(i)}$ *versus* $\frac{VFd(i)}{\beta_e.Size(i)}$ of each metagenome where α_g and β_g are the slopes calculated in Supplemental Fig. A and B , respectively. A simple linear regression line of best fit is represented in each graph, where the grey shading is the 95% confidence interval. The black line in the box plots represents the median and the black diamonds represent the mean. Each dot represents a metagenome, and the grey lines parallel to the axes represent the (1,1) reference coordinates. A) Significant correlation in metagenomes of all human populations – Malawi, USA, Venezuela, respectively (n = 110, r = 0.5572, r_s = 0.6289, P < 0.001, BH post hoc procedure, lm slope = 0.2075), coloured according to the metagenome protein family richness. B) Significant correlation in gut microbiomes of USA individuals (n = 66, r = 0.5304, r_s = 0.6367, P < 0.001, BH post hoc procedure, lm slope = 0.1897). C) Non-significant correlation in gut microbiomes of Venezuelan individuals (n = 21, r = 0.326, r_s = 0.4519, P > 0.001, BH post hoc procedure, lm slope = 0.1371). D) Strong significant correlation in gut metagenomes of Malawian individuals (n = 23, r = 0.9088, r_s = 0.8706, P < 0.001, BH post hoc procedure, lm slope = 0.3725). These statistics along with the Coefficient of determination (r^2) and respective P-value are shown on Table I.

Supplemental Figure. Distribution of the diversity number of antibiotic resistance and virulence factor protein families by human gut metagenomes' protein family richness

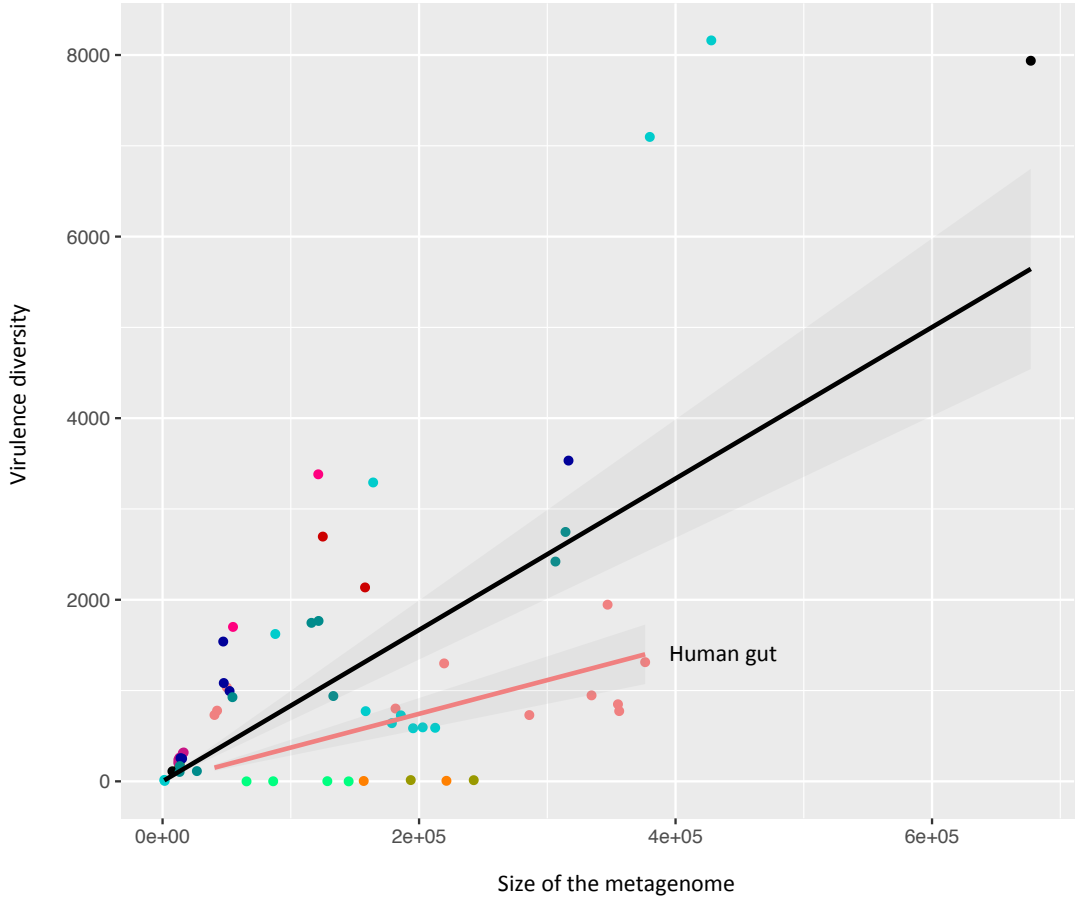
The vertical axes represent the total diversity count of AR protein families' homologues (ARd) (A) and VF protein families' homologues (VFd) (B), present in metagenomes. The horizontal axes represent the protein family richness of the metagenome, that is, the number of cluster representative sequences - see Methods. Each dot represents one of the 110 human gut metagenomes. The lines depict the linear regressions of the metagenomes from Malawi, USA and Venezuela, and the black line depicts the regression line for all metagenomes, where the grey shading is the 95% confidence interval. The Correlation coefficient (r), Spearman's rank

correlation coefficient (r_s), and P-value obtained from the latter, Coefficient of determination (r^2) and respective P-value are shown on Table I.

1. A



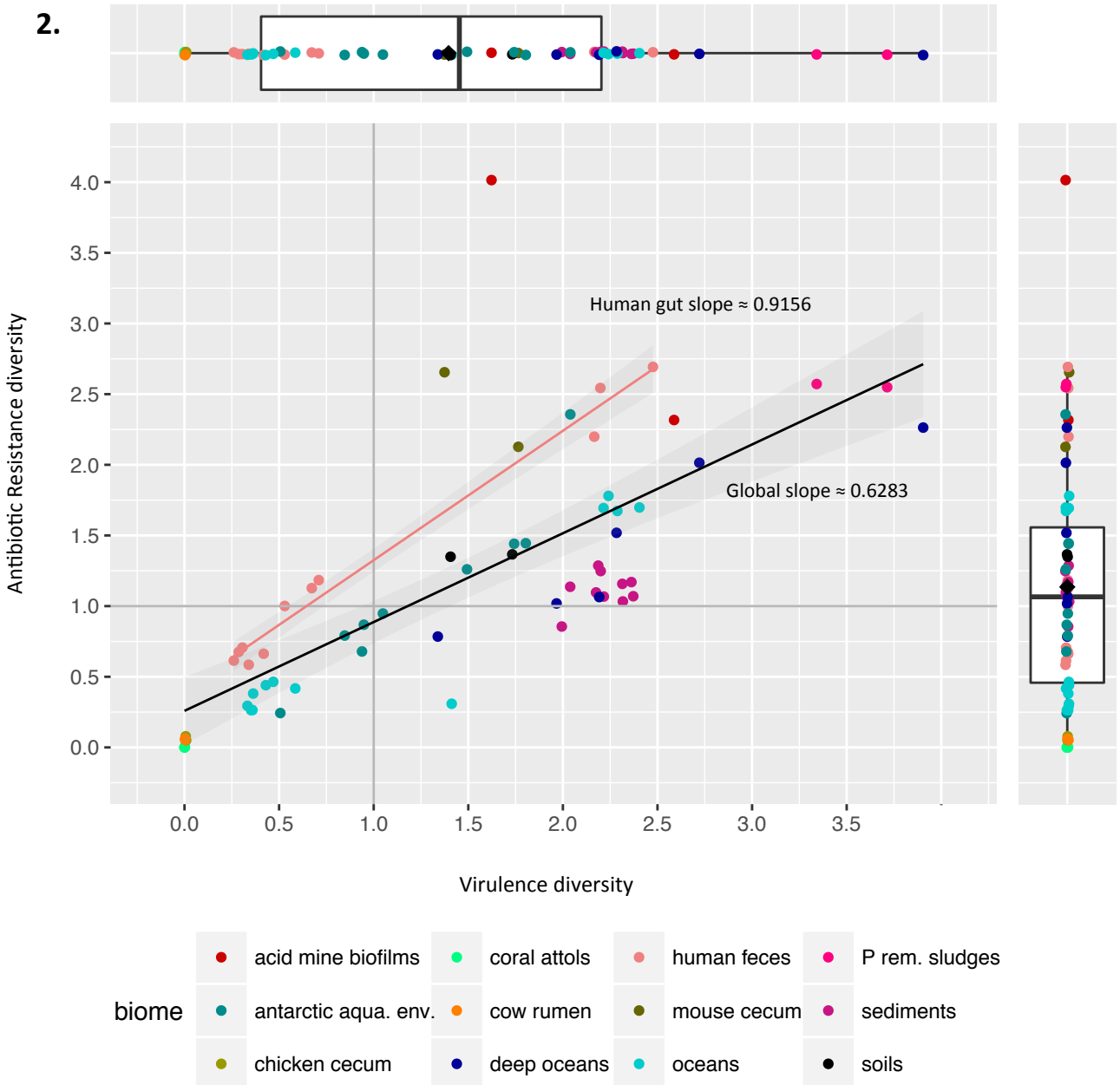
1. B



biome

- acid mine biofilms
- antarctic aqua. env.
- chicken cecum
- coral attols
- cow rumen
- deep oceans
- human feces
- mouse cecum
- oceans
- P rem. sludges
- sediments
- soils

2.


Antibiotic Resistance diversity
Virulence diversity
Human gut slope ≈ 0.9156
Global slope ≈ 0.6283
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
biome
acid mine biofilms
antarctic aqua. env.
chicken cecum
coral attols
cow rumen
deep oceans
human feces
mouse cecum
oceans
P rem. sludges
sediments
soils

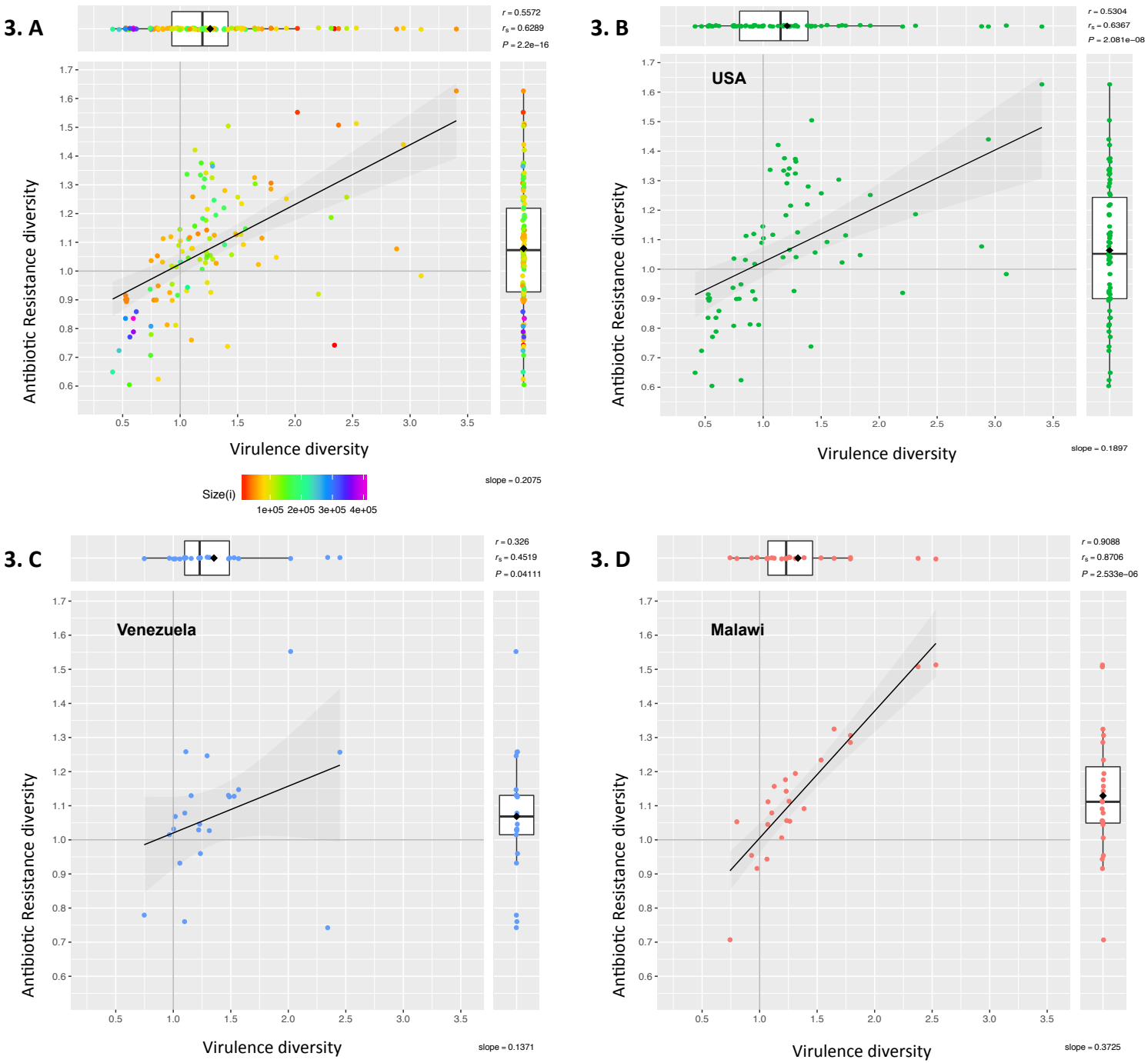

3. A
r = 0.5572
rs = 0.6289
P = 2.2e−16
Antibiotic Resistance diversity
Virulence diversity
Size(i)
1e+05 2e+05 3e+05 4e+05
slope = 0.2075
3. B
r = 0.5304
rs = 0.6367
P = 2.081e−08
USA
Antibiotic Resistance diversity
Virulence diversity
slope = 0.1897
3. C
r = 0.326
rs = 0.4519
P = 0.04111
Venezuela
Antibiotic Resistance diversity
Virulence diversity
slope = 0.1371
3. D
r = 0.9088
rs = 0.8706
P = 2.533e−06
Malawi
Antibiotic Resistance diversity
Virulence diversity
slope = 0.3725

Table I

Resfams vs VFDB

Human

	ARd(i) ~ Size(i)				VFd(i) ~ Size(i)				(ARd(i) / (αg * Size(i))) ~ (VFd(i) / (βg * Size(i)))			
Statistic	All Countries	USA	Malawi	Venezuela	All Countries	USA	Malawi	Venezuela	All Countries	USA	Malawi	Venezuela
r	0,9142	0,9034	0,9413	0,9689	0,6331	0,5768	0,8251	0,8288	0,5572	0,5304	0,9088	0,326
rs	0,9424	0,9394	0,9202	0,9468	0,7626	0,7211	0,8429	0,8987	0,6289	0,6367	0,8706	0,4519
rs p-value	2,20E-016	2,20E-016	5,19E-010	5,16E-006	2,20E-016	8,61E-012	1,73E-006	2,98E-006	2,20E-016	2,08E-008	2,53E-006	0,04111
slope	0,005192	0,005039	0,005494	0,005587	0,005825	0,005259	0,006937	0,007301	0,2075	0,1897	0,3725	0,1371
r-squared	0,9558	0,9443	0,9816	0,9858	0,8301	0,7859	0,9482	0,9247	0,3105	0,2813	0,8259	0,1063
F-statistic p-value	2,20E-016	2,20E-016	2,20E-016	2,20E-016	2,20E-016	2,20E-016	1,25E-015	1,06E-012	2,58E-010	4,64E-006	2,00E-009	0,1493

Environmental

	ARd(i) ~ Size(i	VFd(i) ~ Size(i)
Statistic	All Biomes	All Biomes
r	0,7543	0,6694
rs	0,6426	0,5219
rs p-value	1,04E-008	9,75E-006
slope	0,004762	0,008337
r-squared	0,7213	0,6241
F-statistic p-value	2,20E-016	5,19E-015

	(ARd(i) / (αe * Size(i))) ~ (VFd(i) / (βe * Size(i)))
Statistic	Value
r	0,7547
rs	0,8256
rs p-value	2,20E-016
global slope	0,6283
human gut metager	0,9156
r-squared	0,5695
F-statistic p-value	5,95E-013

Table II

AR mechanism	VF mechanism	Correlation coefficient	Spearman's r_s	Spearman's r_s P-value (BH corrected)
Environmental:				
ABC (ATP-binding cassette superfamily) Antibiotic Efflux Pump	Heme-mediated Iron Uptake	0.8785	0.8598	2.787245e−14
msbA (Lipid A export ATP-binding/permease protein MsbA)	Heme-mediated Iron Uptake	0.8602	0.8738	2.787245e−14
macB (Macrolide export ATP-binding/permease protein MacB)	Heme-mediated Iron Uptake	0.8415	0.8889	2.787245e−14
macB (Macrolide export ATP-binding/permease protein MacB)	Type VI secretion system & effectors	0.8017	0.8367	2.787245e−14
macB (Macrolide export ATP-binding/permease protein MacB)	Fibronectin-binding proteins	0.7463	0.7549	2.665298e−11
TetM TetW TetO TetS (Tetracycline resistance proteins)	Fibronectin-binding proteins	0.7375	0.7620	1.301533e−11
macB (Macrolide export ATP-binding/permease protein MacB)	Type III secretion system & effectors	0.7307	0.8205	2.787245e−14
msbA (Lipid A export ATP-binding/permease protein MsbA)	Fibronectin-binding proteins	0.7304	0.7545	2.726477e−11
msbA (Lipid A export ATP-binding/permease protein MsbA)	Others (Adhesion & Invasion proteins)	0.7005	0.7216	5.711802e−10
TetM TetW TetO TetS (Tetracycline resistance proteins)	Others (Adhesion & Invasion proteins)	0.6594	0.7308	2.603417e−10
Human Gut:				
MFS (major facilitator superfamily) Antibiotic Efflux Pump	Type VI secretion system & effectors	0.8101	0.6806	6.008628e−14
MFS (major facilitator superfamily) Antibiotic Efflux Pump	Siderophore-mediated Iron Uptake	0.7489	0.7353	4.979333e−14
ABC (ATP-binding cassette superfamily) Antibiotic Efflux Pump	Heme-mediated Iron Uptake	0.6477	0.6307	4.979333e−14
ABC (ATP-binding cassette superfamily) Antibiotic Efflux Pump	Fibronectin-binding proteins	0.6390	0.6603	4.979333e−14
mexW mexI (multidrug resistance efflux pump)	Type IV Pili	0.6189	0.5899	4.979333e−14
ABC (ATP-binding cassette superfamily) Antibiotic Efflux Pump	Others (Adhesion & Invasion proteins)	0.6012	0.5639	1.810177e−09
msbA (Lipid A export ATP-binding/permease protein MsbA)	Fibronectin-binding proteins	0.5538	0.5958	4.979333e−14