**Title:** Gene transmission and antibiotic usage are the key factors responsible for the positive correlation between antibiotic resistance gene diversity and virulence gene diversity in human metagenomes

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## Abstract

Antibiotics have been used on a large scale in veterinary and medical contexts. The massive and continuous exposure to antimicrobial drugs has led to the emergence and spread of antibiotic resistance, which is nowadays one of the greatest threats to public health. Although antibiotics are used to treat bacterial infections, it is not only pathogenic bacteria that are affected. Upon exposure to antimicrobial drugs, bacteria that are resistant to the antibiotic administered are selected. Therefore, resistance and pathogenicity may be associated. It was recently shown that there is a positive correlation between the diversity of antibiotic resistance genes and the diversity of pathogenicity genes in human metagenomes from different geographical origins. Our study aimed to understand the factors that lead to the establishment of this positive correlation in human populations using computer simulations. Our results suggest that this positive correlation emerges if the contagion probability between individuals is higher or, at least, not much lower than the loss rate of resistance genes. We argue that the loss rate may be very low, or even null, leaving contagion probability between people as the main factor. Moreover, antibiotic usage throughout tens of years selected resistance genes, which was key to establishing resistance genes diversity across human metagenomes, hence contributing to explain the positive correlation between antibiotic resistance gene diversity and virulence gene diversity.

## Introduction

Since the 1940's antibiotics have been used, not only in health contexts in veterinary and medicine but also in livestock and agriculture as growth promoters (Castanon, 2007). As an incredible example of Darwinian selection, bacterial strains around the world have gradually become resistant to several antibiotics. As a consequence, there were about 875 500 disability-adjusted life-years and more than 33 000 deaths in European Economic Area due to antibiotic resistance in 2015 (Cassini et al., 2019).

Bacterial communities are often very complex, eventually comprising both pathogenic and non-pathogenic bacteria. The human microbiome, defined as the set of microorganisms that colonize humans (body's surfaces and biofluids, including tissues such as skin, mucosa, and, most importantly, the gastrointestinal tract) is composed of about 3.8□×□10^13 bacterial cells (Sender, Fuchs, & Milo, 2016) spanning thousands of taxa. Therefore, even when the objective of uptaking antibiotics is to inhibit growth or kill virulent bacteria, many non-pathogenic (mutualistic or commensal) strains and species are certainly affected. Most likely, the same happens when antibiotics are used as growth promoters.

Unlike mutualistic and commensal bacteria, pathogenic bacteria share the ability to colonize and cause damage to the host. They can antagonize the host's immune response or alter cell physiology and tissue function. To achieve this goal, they rely on the existence of pathogenic mechanisms or strategies for coordinating the action of virulence factors. Bacterial virulence factors are usually proteins that allow pathogenic bacteria to bind and adhere to host cell targets, invade cells and tissues, exert a toxic effect on tissues and cells, acquire iron from host tissues (Chen, Xiong, Sun, Yang, & Jin, 2012) or trigger an immune response such as inflammation (Wilson et al., 2002).

As such, we can argue that to colonize a new biome, the bacterial pathogen needs to encode and express a set of virulent factors to have an active pathogenic mechanism. For example, *Staphylococcus aureus* is responsible for skin and soft tissue infections in humans and encodes many different virulence factors. Some of them are unique to the pathogenesis of skin abscesses and should take action and contribute to the development of this specific infection: IsdAB for protection against reactive oxygen species; Coa and vWbp as factors of immune evasion; ClfAB, SdrCDE, FnBPAB, Eap and Emp as ECM binding molecules; IsdA for resistance to antimicrobial peptides; Spa and IsdBH for antibody binding and altering phagocytosis; Hla for Cytolysis (Kobayashi, Malachowa, & DeLeo, 2015). This means that the

presence of most of these 13 virulence molecules is an indication of the presence of an abscess and symptoms of skin infection by *S. aureus*.

The onset of infection with symptoms drives humans to seek medical help and, in the case of bacterial infections, to antibiotic prescriptions. Thus, it is the onset of symptoms of infectious diseases that triggers the ingestion of antibiotics following a pattern of cause and consequence (David, Sá-Pinto, & Nogueira, 2019). Although exposure to antibiotics in therapeutic doses aims to target pathogens, all resistant bacteria are selected. However, as the selective pressure for antibiotics is actively exercised only when there is pathogenicity, it is expected that both resistance and pathogenic phenotypes are associated. The same will happen at the genotypic level, and it is to be expected that the diversity of genes encoding bacterial resistance and virulence will be correlated. This point has been demonstrated in human gut microbiomes, where the correlation between the diversity of antibiotic resistance and virulence factors are stronger in the human gut of individuals from industrialized areas and having western world lifestyles, with increased exposure to antibiotics (Escudeiro, Pothier, Dionisio, & Nogueira, 2019).

The transmission of microbes from mother to child is already well documented in the bibliography (Blaser & Falkow, 2009; Ferretti et al., 2018; Nayfach, Rodriguez-Mueller, Garud, & Pollard, 2016; Nogueira, David, & Pothier, 2019; Yassour et al., 2018). A recent study highlighted the fact that the oral and gut microbiomes of individuals belonging to the same household share similarities in bacterial strains, regardless of the individuals' genetic relationship (Brito et al., 2019). This suggests that bacteria in human microbiomes can either have a shared exposure or that they can be transferred from person to person on the social network. This second hypothesis is supported by a study that showed that social interactions shape the chimpanzee's microbiomes (Moeller et al., 2016).

In this work, we have simulated the co-transfer of bacterial antibiotic resistance genes and virulence factor genes in a human to human transmission on a network. We show evidence that the positive correlation between the diversity of antibiotic resistance coding genes and those coding for virulence appears mostly when metagenomes lose resistant genes at a slower rate than the transmission between individuals.

## Methods

Our model simulates a group of contacting individuals forming a network to understand the correlation between the diversity of virulence genes and antibiotic resistance genes during the transmission of bacteria from person to person from individual microbiomes. Human interactions are complex and very difficult to simulate. Simplifications in the model allow us to comprehend the effect of specific factors in our simulations, which would otherwise be extremely difficult to detect. As these simplifications do not allow us to make quantitative inferences, we make qualitative analyses. In other words, we study whether there is a correlation and, if so, whether the slope is positive or negative. We intend to explore the effect of different parameters on this correlation.

### *Human network*

Our networks of human contacts were built on a regular network basis, following the methodology of Watts and Strogatz (ref. (Watts & Strogatz, 1998)). Consider a regular network, each node represents a human being, and where each node is linked to the *n* nearest nodes. Each link on each node has a certain probability *p* of being reconnected to another randomly chosen node. The parameter *p* represents the probability of each connection to be modified. The type of network is defined by the value assigned to the parameter *p* (for example, a regular network is obtained for p = 0, whereas p = 1 results in a random network). Unless noted, we performed all simulations presented here with p = 0.5.

### *Human metagenome*

Our model considers three types of genes belonging to the human metagenome, namely, antibiotic resistance genes, virulence genes, and the third category of genes that we call “other” genes. This last category represents genes that do not confer resistance to antibiotics and that are also not coding for virulence. It works as a control, to help us understanding to what extent a certain correlation occurs only between virulence genes and antibiotic resistance genes. None of these types of groups are house-keeping genes.

The genes that code for proteins with similar functions are grouped into families. We consider that each family of the three types of genes can be either present (1) or absent (0) in the metagenome of each individual. It is worth mentioning that our

focus is on the presence of a family, and not on the number of genes in a family, present in the metagenome.

We divide virulence genes into groups of related families, each group having the same number of families. When a person has one of these groups of virulence genes complete (because she/he is receiving genes from her/his contacts), that person feels sick and takes an antibiotic.

Resistance genes are also divided into groups, each group having the same number of families. Each group represents resistance genes associated with an antibiotic (in our simulations, we do not consider resistance to multiple drugs). There will be as many groups as there are antibiotics considered in the simulations.
The “other” genes are not divided into groups.

*Human-human contacts*

Individuals are connected in a network, leading to a flow of genes (due to person to person transmission of part of the microbiome) from one individual to another. Upon contacts, there is a certain probability that each gene will be transmitted to another individual. An expected result of the contact is an increase in the diversity of genes present in the metagenome of each individual.

*Immune system and antibiotic consumption*

From time to time, individuals experience symptoms of illness caused by an increased load of virulence genes. In our model, we assume that an individual is considered sick whenever a group of families of virulence genes becomes complete. This represents the presence of a complete set of genes that encode a pathogenic mechanism of infection. In the simulations, the immune system has two different effects: (i) causing virulence genes of the completed group to disappear from the individual metagenome with an associated probability; (ii) according to the number of virulence genes selected in (i) to be eliminated, the same number of both resistance and “other” genes are selected to be eliminated with an associated probability.
In addition to the action of the immune system, antibiotics are administered to target pathogenic bacteria. The metagenome of individuals undergoing antibiotic treatment changes. Bacteria carrying resistance genes to the antibiotic administered will be selected, whereas the remaining bacteria will be eliminated. Even bacteria that harbor resistance genes in their genome will be eliminated unless they are resistant to that specific antibiotic. Since we are only focusing on the effect of antibiotics and not on

their mechanisms of action, all antibiotics considered here can be used to treat sick individuals. Thus, the administered antibiotics will be chosen randomly from those considered in the simulations. In our simulations, upon antibiotic consumption, the following occurs: (i) resistance genes associated to that antibiotic become “activated” (see below for an explanation) in the individual metagenome, (ii) resistance genes associated with other antibiotics are lost (becoming “inactivated”) with a given probability; (iii) virulence genes and “other” genes disappear from the metagenome with a given probability.

The concepts “activation” and “inactivation” of resistance genes refer to a central assumption in this work. We assume that all families of resistance genes are present in all metagenomes, sometimes activated, sometimes inactivated. When they are inactivated, these gene families are present but not transmissible to other individuals in the network. A gene family becomes “activated” in a given individual in two ways: (i) when the associated antibiotic is taken; or (ii) if received from another person (node) of the network. It may become inactivated again in three ways: (i) if an antibiotic of another type is taken, or (ii) by the action of the immune system, or (iii) according to the loss rate of resistance genes as explained next.

As previously described, we assume that in an environment containing antibiotics only resistant bacteria survive, constituting a clear advantage of carrying these genes (by assuming this, we are discarding the appearance of antibiotic-resistant mutants or mechanisms of indirect resistance (Wright, 2005)). However, carrying resistance genes come at a fitness cost (Andersson & Levin, 1999; Gama, Zilhao, & Dionisio, 2018). Consequently, in an antibiotic-free environment, the fitness cost of resistance usually leads to a decrease in the number of resistant bacteria to low levels (Levin et al., 1997). To simulate this cost, we consider that a percentage of resistance genes is lost according to a “loss rate” when the individual does not take antibiotics.

*Co-transfer of virulence and resistance genes*

As explained above, when two individuals come into contact, there is the transmission of bacteria of the microbiome that harbor virulence genes, resistance genes, and “other” genes. In some simulations, however, we consider the possibility of co-transfer of virulence and resistance genes. This means that, if a given individual transmits at least one virulence gene, the probability of transmitting resistance genes is slightly higher than if there was no co-transfer. Similarly, if an individual transmits resistance

genes, the probability of transmitting virulence genes increases. There is no co-transfer of “other” genes in any situation.

*Flow*

Each simulation is composed of several cycles. In each cycle, all procedures described previously are considered (pseudocode in Table 1 and a flowchart in Figure 1).
The program code described here was implemented in the Python programming language. We performed exploratory simulations to parameterize our model. A set of parameters was set as default (Table 2).

*Statistical analysis*

To know if the diversity of antibiotic resistance genes is correlated with the diversity of virulence genes, we consider that $Y$ (diversity of resistance genes) has an affine relationship with $X$ (diversity of virulence genes). This is mathematically expressed as:

$$Y = \beta_0 X + \beta_1$$

In this equation, $\beta_0$ represents the slope of the linear regression, while $\beta_1$ represents the point at which the line crosses the y-axis.
To analyze the linear model, we consider the hypothesis to be that there is a correlation between the diversity of antibiotic resistance genes and the diversity of virulence genes. Consequently, the null hypothesis is that there is no correlation. We define a = $1x10^{-6}$, rejecting the null hypothesis for the P-value < a.

## Results

In simulations, on each person's metagenome, we consider three types of non-housekeeping genes: antibiotic resistance genes, virulence genes, and the so-called "other" genes. The "other" genes are used for control simulations (see Methods).

It is assumed that people are organized in a network of contacts. Between two connected people, there is a likelihood of contagion during which there is the transmission of some bacteria from an individual's microbiome to that of another individual. That is, in each cycle (during which each person has a single opportunity to transmit genes or to be infected by each neighbor in the network, see Methods) any gene present in one of the person's metagenomes has a certain probability of being transmitted to each person to which she/he is connected.

In each cycle, a person receives virulence genes from her/his contacts. Whenever a group of virulence genes is completed in a person's metagenome (representing a pathogenic mechanism necessary to trigger an infection), the following happens: (i) the immune system acts by eliminating part of that group of genes, as well as some genes of the other two types of genes; (ii) in the meantime, that person feels sick and takes an antibiotic; the antibiotic eliminates part of the virulence genes (by eliminating the bacteria encoding them, and for which the antibiotic is targeted to) as well as "other" genes and some resistance genes not associated to the used antibiotic. The "other" genes do not influence the decision to take antibiotics.

If antibiotics are not being used, there is no selective pressure within each person's metagenome to maintain antibiotic resistance genes, and these genes are lost according to a certain "loss rate". That is, we assume that these genes are costly to bacterial cells.

## Number of pathogenic mechanisms and the likelihood of contagion

If a group of virulence genes is complete, it means that there is a pathogenic mechanism for a certain bacterial infection in an individual. Except for the specific case of the proliferation of opportunistic bacteria (not simulated in this work), we assumed that individuals are not affected by more than two infectious diseases at the same time. Accordingly, in our simulations, we have explored parameters that lead individuals to have no more than two full groups.

We performed simulations with different contagions probabilities and counted the number of complete groups (infections) that each individual has per cycle. As we can see in Table 3, when the contagion probability is 0.25%, there is an individual in a certain cycle (out of eight million possibilities) with three groups of virulence genes completed. For this reason, in our simulations, we have settled the contagion value to be less than 0.20% or less.

## Effect of the relative values of the contagion probability and the resistance gene loss rate

We tested the effect of different combinations of contagion probability and resistance gene loss rate. For that, we fixed all the other parameters (see Table 2). The results obtained after 8000 cycles (see Methods) are shown in Supp. Table1. Fig.2 shows that, if the contagion probability is higher, the same, or only slightly lower than the transmission rate, the slope between the diversity of virulence genes and the diversity of resistance genes is positive, as well as the slope between the diversity of “other” genes and the diversity of resistance genes (Supp. Table1, Fig. 2). This can be understood in the following way. In each cycle, some individuals do not need to take an antibiotic in that cycle (because there is no complete group of virulence genes in their microbiota). In these individuals, the diversity of virulence genes and the "other" genes are only affected by the contagion probability. For some time, virulence and “other” genes accumulate because we assume that they do not confer a fitness cost to bacterial cells. However, the diversity of resistance genes only increases if the effect of contagion is "stronger" than that of gene loss, leading to a positive correlation; otherwise, the diversity of resistance genes decreases, leading to a negative correlation.

The above explanation can be illustrated as follows. If we fix the contagion probability and increase the rate of loss of resistance genes, the slope decreases. After a certain point, the slope becomes negative.

At first, one might expect to observe a negative slope whenever the contagion probability is lower than the loss rate. This is not the case; for example, if the contagion probability is 0.05% and the loss rate is 0.10%, the slope is still positive (Fig. 2), despite presenting a slope close to zero (but significantly different from zero, see Supp. Table 1). The reason for this is that, in each transmission cycle, any individual contacts four other individuals, and, during each of these contacts, it shares some bacteria from

their microbiomes. Each individual, in turn, can only be medicated with antibiotics once, at the end of a cycle, which implies that the rate of loss of resistance genes applies only once in a cycle. Therefore, the impact of the contagion rate is higher than the rate of loss of resistance genes.

It was recently shown that the correlation between the diversity of resistance genes and virulence genes in human populations from different parts of the world is positive (Escudeiro et al., 2019). Therefore, in the next sections, we focus on positive correlations. We chose a probability of contagion of 0.10% and a rate of loss of 0.05% of resistance genes.

## The effect of antibiotic uptake

Then, we verified the role of antibiotic consumption in establishing the correlation between the diversity of resistance genes and virulence genes. As explained in the methods section, antibiotics are taken by an individual when one of the groups of virulence genes is complete. The reasoning is that a complete group of virulence genes in a given person implies that that person becomes ill. Now, to assess the role of antibiotic consumption, we performed further simulations, this time assuming that no one consumes antibiotics, even if groups of virulence genes are complete in the metagenome.

In the absence of antibiotics, the diversity of virulence and “other” genes in metagenomes accumulate overtime in each metagenome. However, such accumulation is not perpetual because, as soon as one of the groups of virulence genes is completed, the immune system is activated to eliminate this entire group of virulence genes, as well as some genes from all three gene types (see methods for details). Without antibiotic use, the diversity of resistance genes solely depends on the contagion probability, on the rate of loss of resistance genes, and the immune system. The results of these simulations are shown in Figure 3.

These simulations show that, even without the use of antibiotics, there is a correlation between the diversity of resistance genes and the diversity of virulence genes, and also with the diversity of “other” genes (virulence genes: R = 0.360, slope = 0.769 , P-value = $5.05 \times 10^{-32}$; "other" genes: R = 0.428, slope = 0.631, P-value = $7.22 \times 10^{-46}$).

However, Fig. 3 shows that both virulence genes and "other" genes tend to accumulate in each person (all individuals have approximately 60 or more of those genes). This concentration of genes is not seen when antibiotics are taken (Figure 2A) nor in real human populations (Escudeiro et al., 2019).

## Co-transfer (co-selection) is not determinant to obtain a positive correlation

The positive correlation between the diversity of virulence and antibiotic resistance genes observed in human gut metagenomes (Escudeiro et al., 2019) may suggest that they are co-selected, that is, co-transferred between people. In this section, we address this possibility. In this context, co-transfer means that the transfer of at least one virulence gene is followed by an increase in the probability of transmitting resistance genes. Likewise, the transfer of at least one resistance gene implies an increase in the probability of transmitting virulence genes. This co-transfer could be favored if there was some kind of genetic link between the two types of genes. To understand the relevance of such co-transmissibility, we compared the simulations with those of the "other" genes, taking them as a control group (the “other” genes are not co-transferred).

Figure 4 shows that the diversity of resistance genes correlates with the diversity of both the virulence and "other" genes when co-transfer is 0.1% or 1%. However, the impact on the diversity of virulence and “other” genes differs.

The slope of the linear regression between virulence and resistance genes is similar for the two simulations (slope = 0.604, with 0.1% co-transfer; slope = 0.686, with 1% co-transfer). However, in the case of the linear regression between the diversity of resistance and “other” genes, the slope increases when the probability of co-transfer increases to 1% (slope = 0.566, without co-transfer; slope = 0.583 with 0.1% co-transfer; slope = 1.532 with 1% co-transfer).

Therefore, the results are very different, in particular, if one compares Fig. 2 (probability of co-transfer is 0%) with Fig. 4B (probability of co-transfer is 1%). The reason for this effect is the following. When someone takes an antibiotic, there is a selection of resistance genes associated with that antibiotic and elimination of some virulence and “other” genes (see Methods). Because there is some degree of co-transfer of resistance and virulence genes, the latter is indirectly positively selected. This means that, in these metagenomes, one of the groups of virulence genes is going to be completed faster than if there was no co-transfer. As a consequence of having a

complete set of virulence genes from a pathogenic mechanism, these people get sick, take an antibiotic, again selecting for resistance genes, indirectly selecting for virulence genes (due to co-transfer), and counter-selecting the “other” genes. With this cyclical process, the diversity of resistance genes remains correlated with the diversity of virulence genes (as seen also for the case where there was no co-transfer), but the “other” genes almost disappear from the population (Fig. 4B). This striking behavior of the “other” genes suggests a future analysis of metagenomes.

## Discussion

The human microbiota comprises hundreds of bacterial species. Therefore, even if antibiotics target a specific bacterial pathogen, hundreds of other bacterial strains and species are affected. Antibiotics are also frequently given to healthy animals and plants as growth-promoters. With these two processes, the frequency of antibiotic-resistance raises in metagenomes. Meanwhile, most metagenomes from sick or healthy people harbor virulence genes. All these facts, together with the contagion of microbiomes between people, contribute to the positive correlation between the diversity of virulence and antibiotic-resistance genes among human populations (Escudeiro et al., 2019). The objective of this work was to understand what causes this positive relation.

Our results suggest that the positive correlation is established if the contagion probability is not much lower or is higher than the loss rate of antibiotic-resistance genes. This loss rate is a direct consequence of the fitness cost imposed by antibiotic-resistance genes.

It should be noted that this is a qualitative result. Neither the size of the simulated population nor the contacts' network considered is real. However, further simulations (Supp. Table 2) suggest that our main conclusion is the same with bigger populations. Other types of networks also do not change the final relationship. The results presented here were generated with a rewiring probability of $p = 0.5$ (see Methods section). With other networks (regular, $p = 0$) or random ($p = 1$), the only difference is the time needed to achieve a stable distribution as seen in Fig. 2, being much longer in the regular network and slightly faster in the random network (see Supp. Fig. 3).

Likewise, the total number of virulence, resistance, and "other" gene families did not change the main conclusion (Supp. Table 4). Neither the effect of antibiotics on virulence, resistance, and "other" genes (we tested the same and different effects between the various types of genes), nor the number of antibiotics considered, change the slope sign (see Supp. Tables 5, 6 and 7).

According to Fig. 3, when there is no antibiotic intake, the diversity of virulence genes and "other" genes accumulate in metagenomes. This is because, without antibiotics, only the immune system decreases their diversity. Meanwhile, the diversity of resistance genes decreases. Thus, there is a wider range of resistance genes

diversity than of the other two groups of genes. When we compare our results with those obtained by Escudeiro and colleagues, we realize that the simulations that best support their results are the ones where sick individuals take antibiotics (Fig. 2A) because the diversity of virulence genes in human metagenomes is not concentrated at high values (Escudeiro et al., 2019).

The contagion probability is an important parameter. In recent years, the interest in human microbiomes strongly increased, yet we still do not know how much is the contagion probability of non-housekeeping genes such as the ones studied here. We know, for example, that human microbiomes are more similar among humans living together, irrespective of the genetic relatedness. This suggests that transmission is a key factor of the microbiome constitution (Rothschild et al., 2018).

Sarowska et al recently reviewed the fate of the so-called extraintestinal pathogenic E. coli (ExPEC), which are facultative pathogens of the normal human intestinal flora. Many virulence genes are involved in the pathogenicity of ExPEC and some of them are mostly encoded on pathogenicity islands or mobile genetic elements such as plasmids. One of the author's conclusions is precisely the difficulty in assigning ExPEC transmission to people due to the delay between ExPEC colonization and infection: ExPEC can live in human intestines for months or even years before starting an infection (Sarowska et al., 2019). The same problem applies to the transmission rate of antibiotic-resistance genes: very few data on transmission rates between people (Andersson & Hughes, 2017).

We have seen that what is important is the relationship between the contagion rate and loss rate. How much is the loss rate in a human metagenome? Several longitudinal studies have shown that antibiotic-resistance genes often take tens of days, sometimes months, to become undetected in human guts (Horcajada et al., 2002; Lautenbach et al., 2006; O'Fallon, Gautam, & D'Agata, 2009; Rogers et al., 2012; Weinreich, Delaney, Depristo, & Hartl, 2006). While still harboring resistance-genes, people most probably contact with several other people during this time. Yet, it is still unclear what is the relationship between contagion and loss rates.

One way around this problem is by acknowledging that the loss of antibiotic-resistance genes is a consequence of the fitness cost of resistance determinants on bacterial cells (compared to otherwise isogenic susceptible cells). Several studies have shown that resistance determinants, here broadly comprising resistance mutations and resistance genes often encoded in plasmids, impose a fitness cost on their hosts (giving the sensitive strains a growth advantage) (Andersson & Levin, 1999). However,

several mechanisms have been shown to decrease or even eliminate fitness costs. For example, compensatory mutations, masking the deleterious effects of resistance mutations, have been observed in several studies (Bjorkman, Nagaev, Berg, Hughes, & Andersson, 2000; Maisnier-Patin & Andersson, 2004; Nilsson et al., 2006; Schrag, Perrot, & Levin, 1997). Moreover, an important fraction of resistance mutations can be beneficial in certain resistance genetic backgrounds and sometimes double resistances entail no measurable cost (Trindade et al., 2009). Furthermore, if plasmids are often costly, they also have been shown to adapt to their hosts in just a few hundreds of generations (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio, Conceicao, Marques, Fernandes, & Gordo, 2005; Modi & Adams, 1991). In some of these evolutionary experiments, plasmids became costless (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio et al., 2005; Modi & Adams, 1991). Moreover, some plasmids increase the fitness of bacteria already resistant to an antibiotic; likewise, some resistance mutations increase the fitness of plasmid bearing cells (Silva et al., 2011). The same may happen with two plasmids: one of them compensating for the cost of the other (San Millan, Heilbron, & MacLean, 2014; Silva et al., 2011). Furthermore, plasmids may interact with other plasmids in such a way as facilitating their transfer (Gama, Zilhao, & Dionisio, 2017a, 2017b). Finally, it has been suggested that most plasmids are perhaps costly if their fitness effect is measured within a short time of its isolation from nature (Gama et al., 2018; Lau, Malkus, & Paulsson, 2013). All these works suggest that the fitness cost of resistance determinants are often very low or null, allowing their permanence in microbiomes for months. This implies that the loss rate is indeed much lower than the contagion probability. Therefore it is mostly the contagion between people that leads to the positive correlation between virulence and resistance genes. However, for such correlation to occur, some human metagenomes have to harbor resistance genes.

Before the antibiotic era, most bacteria were sensitive to antibiotics (Datta & Hughes, 1983; Hughes & Datta, 1983). Antibiotic-resistance genes have been gradually selected through the massive usage of antibiotics since the 1940s. Due to the spread of such genes all over the world, we assumed in our simulations that the uptake of antibiotics selects resistance genes already present in tiny quantities or concentrations at every human metagenome. That is, we assumed that they are present, but that their quantity in each metagenome is so low, that they are not transmitted to their contacts (these genes can be transmitted only if they were previously selected by an antibiotic).

As shown in Supp. Table 8, if we, instead, assume that initially, only 10% of the metagenomes contain antibiotic-resistance genes, the final correlations with virulence or “other” genes are the same (the only difference is it takes more cycles to stabilize). Therefore, our assumption that antibiotic-resistance genes are present in tiny quantities in all metagenomes does not change the final relationship between the diversity of virulence genes and antibiotic-resistance genes.

Therefore, contagion between people and the “activation” of resistance genes through the antibiotic consumption are chief to explain the positive correlation between antibiotic resistance gene diversity and virulence gene diversity in human metagenomes.

## Concluding remarks

This work suggests that the positive correlation between the diversity of virulence and resistance genes observed in human gut metagenomes is established if the contagion probability is not much lower or is higher than the loss rate of antibiotic-resistance genes. Massive antibiotic usage for tens of years was also paramount because that is the mechanism that selected resistance genes in human metagenomes.

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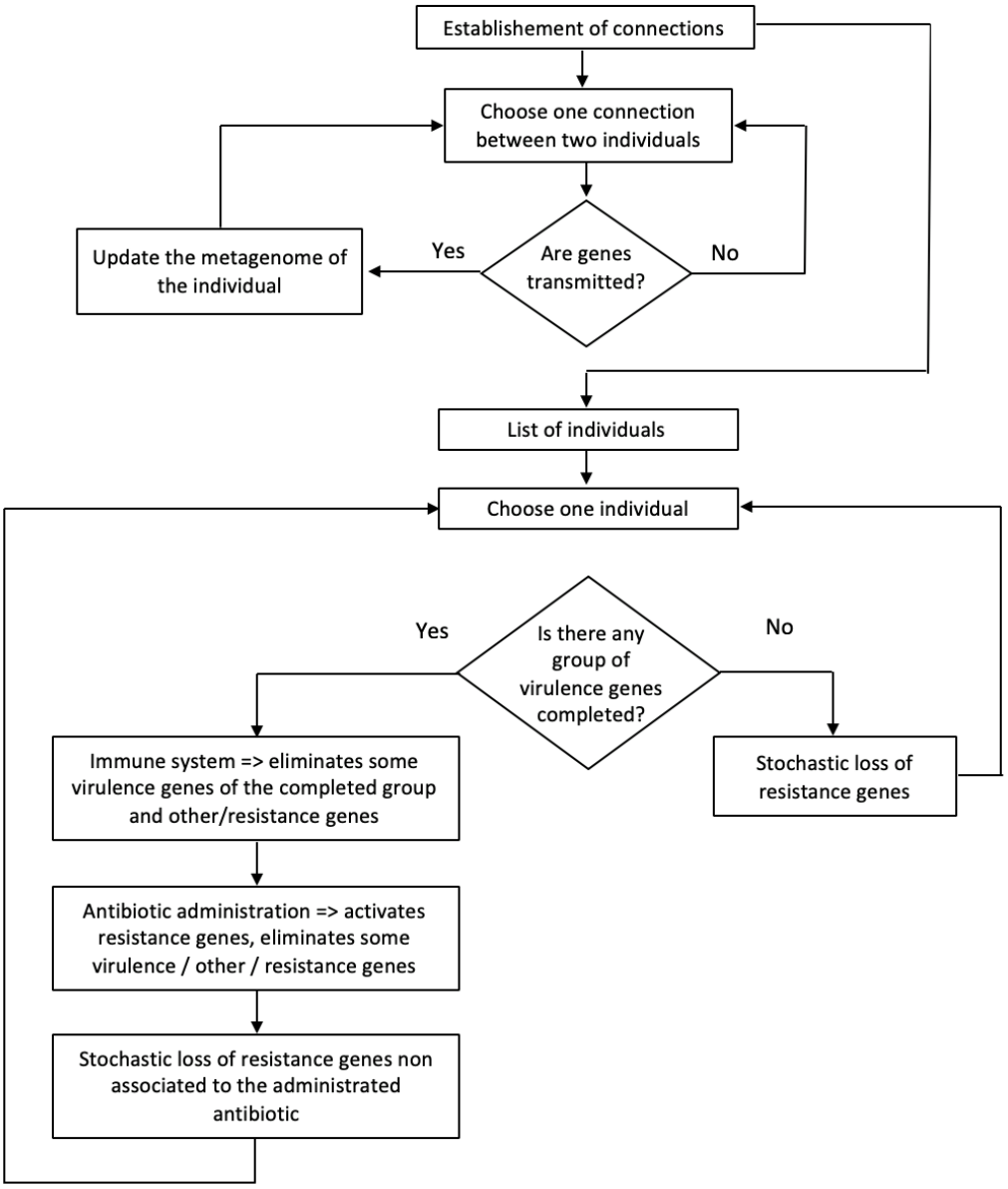
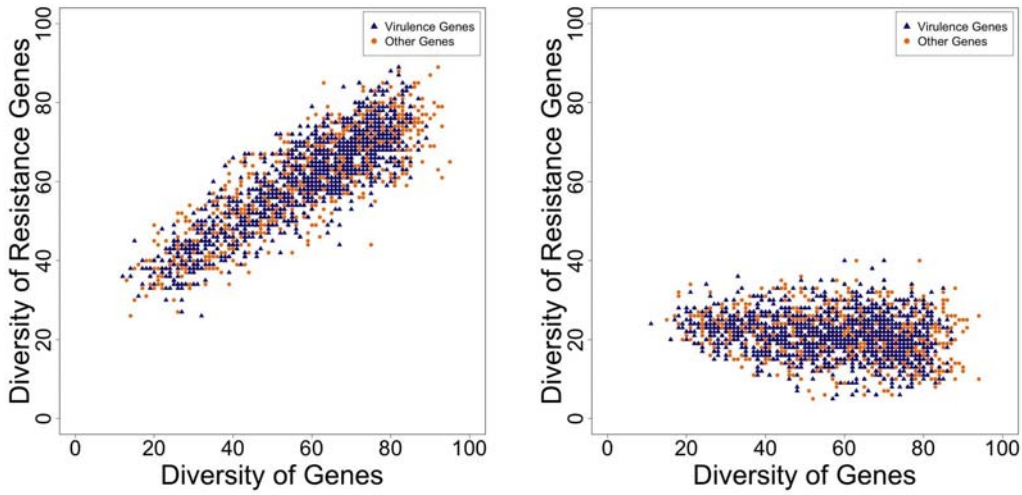
**Figure 1:** Flowchart of the program.



A contagion probability = 0.1% and resistance genes loss rate = 0.05%

B contagion probability = 0.1% and resistance genes loss rate = 0.3%




C

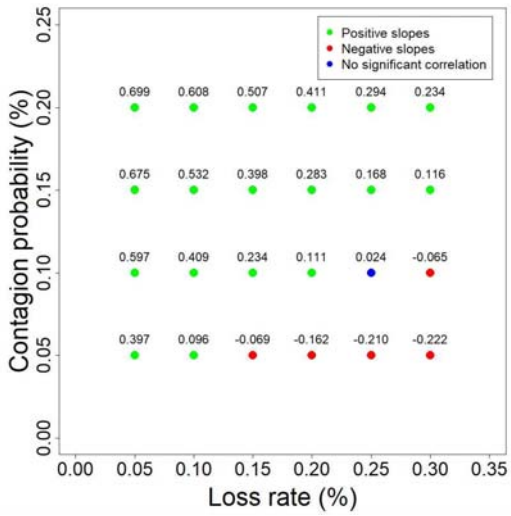



**Figure 2. Effect of the relative values of the contagion probability and the resistance genes loss rate.** A and B: the relationship between the diversity of resistance genes and the diversity of the other two gene types. The vertical axes represent the diversity of resistance genes. The horizontal axes represent the diversity of virulence or the diversity of "other" genes. Each blue and orange dot represents the diversity of virulence genes and the "other" genes present in an individual metagenome, respectively. A: contagion probability = 0.1% > resistance genes loss rate = 0.05%, resulting in a positive slope; (virulence genes: R = 0.861, slope = 0.597, p-value = $2.22 \times 10^{-295}$; "other" genes: R = 0.858, slope = 0.566, p-value = $3.32 \times 10^{-291}$) B: contagion probability = 0.1% < resistance genes loss rate = 0.3%, resulting in a negative slope; (virulence genes: R = -0.195, slope = -0.065, p-value = $4.62 \times 10^{-10}$; "other" genes: R = -0.198, slope = -0.063, p-value = $2.84 \times 10^{-10}$) C: Relationship of the sign of the slope with the relative values of contagion probability and loss rate. The horizontal axis represents the values of the resistance genes loss rate (%). The vertical axis represents the

values of the contagion probability (%). The green dots represent the simulations where the correlations have positive slopes; the red dots represent the simulations where the correlations have negative slopes; the blue dots represent the simulations where the correlations are not significant (p-value < $1x10^{-6}$). For the same contagion probability, the increment of the resistance genes' loss rate leads to the decrease of the slopes.

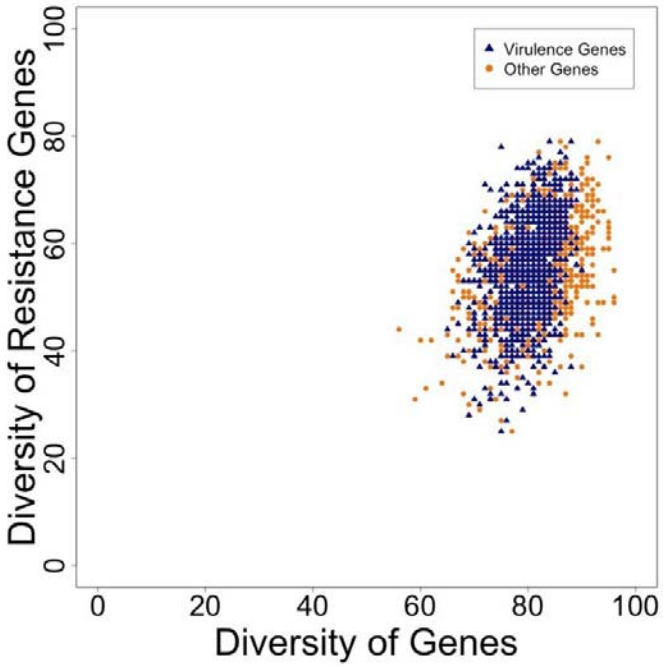



**Figure 3. Relationship between resistance genes and virulence or "other" genes when there is no antibiotic use.** As in Fig.2, the vertical axes represent the diversity of resistance genes and the horizontal axes represent the diversity of virulence or the diversity of "other" genes. Each blue and orange dot represents the diversity of virulence genes and of the "other" genes present in an individual metagenome, respectively. When there is no antibiotic exposure, virulence and "other" genes accumulate once the only factor that leads to their decrease is the effect of the immune system. On the other hand, besides the effect of the immune system, there is also a decrease in the diversity of resistance genes due to the loss rate. Thus, there is a wider range of resistance genes diversity than the other two groups of genes (virulence genes: R = 0.360, slope = 0.769, p-value = $5.05 \times 10^{-32}$ ; "other" genes: R = 0.428, slope = 0.631, p-value = $7.22 \times 10^{-46}$). Simulations were performed using the parameters described in Methods (Table 2), with contagion probability of 0.1% and loss rate probability of 0.05%.

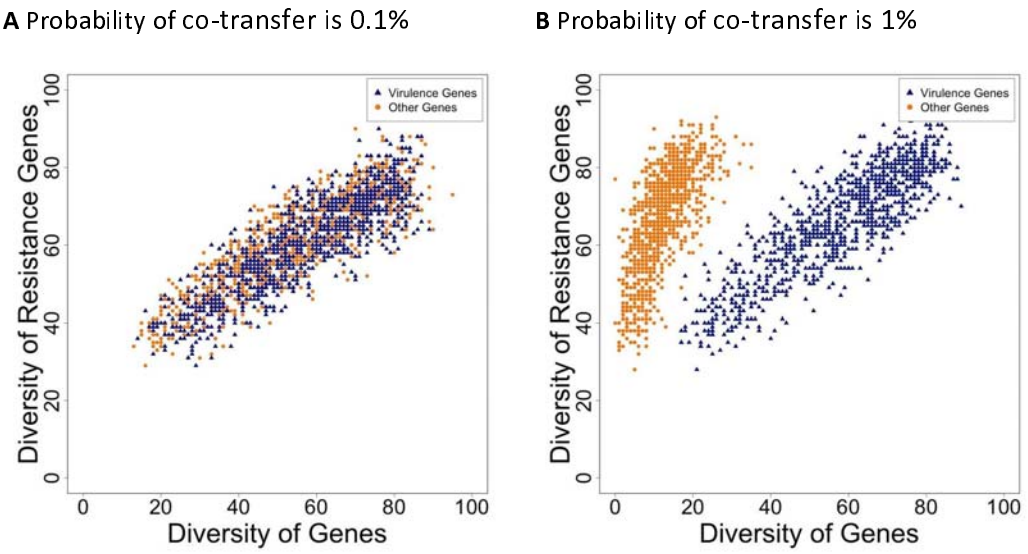



**Figure 4. Influence of the co-transfer of virulence and antibiotic resistance genes in the correlation between virulence and antibiotic resistance genes, and "other" genes and antibiotic resistance genes.** The vertical axes represent the diversity of resistance genes. The horizontal axes represent the diversity of virulence or the diversity of "other" genes. Each blue and orange dot represents the diversity of virulence genes and "other" genes present in an individual metagenome, respectively. A. With a co-transfer probability of 0.1%, there is a correlation between the diversity of resistance genes and the diversity of both virulence and "other" genes (virulence genes: R = 0.862, slope = 0.604, P-value = $1.09 \times 10^{-296}$; "other" genes: R = 0.865, slope = 0.583, P-value = $4.35 \times 10^{-301}$). B. With co-transfer probability of 1%, there is also a correlation between the diversity of resistance genes and the diversity of both virulence and "other" genes, but the diversity of "other" genes strongly decreases (virulence genes: R = 0.865, slope = 0.686, P-value = $8.94 \times 10^{-302}$; "other" genes: R = 0.701, slope = 1.532, P-value = $9.32 \times 10^{-149}$). The results were obtained using the parameters described in Methods (Table 2).

| **Table 1.** Pseudocode of the program. | |
|---|---|
| **Process** | **Pseudo Code** |
| Gene transfer | For each connection between two individuals do (for each individual of the connection do (get the genes present in each individual metagenome; transmit genes to the other individual of the connection according to the contagion probability)) |
| Screening of individuals | For each individual do (for each group of virulence genes do (check if the group is completed)) |
| Immune system effect | For each group of virulence genes completed do (eliminate virulence genes according to the immune system curing probability).<br>Select as many resistance genes as virulence genes present in the completed groups; eliminate resistance genes according to the immune system curing probability.<br>Select as many “other” genes as virulence genes present in the completed groups; eliminate “other” genes according to the immune system curing probability. |
| Antibiotic effect | Select an antibiotic randomly.<br>“Activate” all resistance genes associated with the chosen antibiotic.<br>Eliminate resistance genes non-associated with the chosen antibiotic according to the antibiotic curing probability.<br>Eliminate virulence genes according to the antibiotic curing probability.<br>Eliminate “other” genes according to the antibiotic curing probability. |
| Loss rate of resistance genes under antibiotic consumption | Eliminate resistance genes not associated with the chosen antibiotic according to the loss rate probability. |
| Loss rate of resistance genes without antibiotic consumption | Eliminate resistance genes according to the loss rate probability. |

**Table 2.** Parameters and default values used in simulations.

| **Parameters** | **Default value** | **Changing values** |
|---|---|---|
| Rewiring connectivity probability p | 0.5 | 0 or 1 |
| Number of individuals | 1000 | NA |
| Number of virulence genes | 100 | NA |
| Number of resistance genes | 100 | NA |
| Number of "other" genes | 100 | NA |
| Number of antibiotics | 5 | NA |
| Contagion probability (%) | NA | 0.05, 0.10, 0.15, 0.20 |
| Co-transfer probability (%) | NA | 0, 10 |
| Probability of eliminating genes under the immune system (%) | 50 | NA |
| Probability of eliminating genes under antibiotic intake (%) | NA | 0, 70 |
| The loss rate of resistance genes (%) | NA | 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 |

**Table 3.** Full groups of virulence genes according to the contagion probability.

| | | Number of full groups of virulence genes (in 8 000 000 possibilities) | | | | |
|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** |
| **Contagion probability (%)** | **0.05** | 3415 | 2 | 0 | 0 | 0 |
| | **0.1** | 9545 | 14 | 0 | 0 | 0 |
| | **0.15** | 15746 | 34 | 0 | 0 | 0 |
| | **0.2** | 21886 | 85 | 0 | 0 | 0 |
| | **0.25** | 28389 | 146 | 1 | 0 | 0 |