Re-growth of *Mycobacterium tuberculosis* populations exposed to antibiotic combinations is due to the presence of isoniazid and not bacterial growth rate.

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Abstract
Modulation of growth rate in *Mycobacterium tuberculosis* is key to its survival in the host; particularly with regard to its adaptation during chronic infection when the growth rate is very slow. The resulting physiological changes will influence the way this pathogen interacts with the host and responds to antibiotics. Therefore, it is important that we understand how growth rate impacts antibiotic efficacy, particularly with respect to recovery/relapse. This is the first study that has asked how growth rates influence the mycobacterial responses to combinations of frontline antimycobacterials, isoniazid (INH), rifampicin (RIF), and pyrazinamide (PZA), using continuous cultures. Time-course profiles of log-transformed total viable counts for cultures, controlled at either a fast growth rate (23.1. mean generation time (MGT)) or slow growth rate (69.3h MGT), were analysed with the fitting of a mathematical model by nonlinear regression that accounted for the dilution rate in the chemostat, and profiled kill rates and recovery in culture. Using this approach, we show that populations growing more slowly were generally less susceptible to all treatments. We observed a higher kill rate associated with INH (compared to RIF or PZA) and the appearance of re-growth. In line with this observation, re-growth was not observed with RIF-exposure, which provided a slower bactericidal response. The sequential additions of RIF and PZA did not eliminate re-growth. We consider here that faster, early bactericidal activity is not what is required for successful sterilisation of *M. tuberculosis*, but instead slower elimination of bacilli followed by reduced recovery of the bacterial population.

Introduction
Improvement in TB treatment is urgently needed with the aim of shortening the
period of antibiotic therapy without increasing relapse rates or encouraging the
development of antibiotic-resistant strains (1). Although new combinations of
antibiotics with novel modes of action are being evaluated, optimal dosing and
treatment duration can be investigated further for existing antibiotics. Understanding
how growth rate impacts the activity of frontline antibiotics, so that they can be
delivered using an alternative approach or in a different timely fashion can
contribute to the development of regimens that contain a combination of both
conventional and new antibiotics (2)(3)(4)(5).

One of the requirements of a new antibiotic combination is its ability to target
heterogeneous populations of bacteria, particularly those that develop antibiotic
tolerance during the course of treatment and are thought to contribute to relapse,
post-treatment (6). Fluctuations in the growth rate in *Mycobacterium tuberculosis*
enable the organism to adapt to different environmental niches in the host;
particularly with regard to its adaptation during chronic infection when the growth rate
becomes very slow. The resulting genotypic and phenotypic changes will influence
the way this pathogen interacts with the host and responds to antibiotic treatment.
Therefore, it is important that we understand how growth rate impacts antibiotic
efficacy. The current paradigm concerning the effect of growth rate or growth phase
on the response of *M. tuberculosis* to antibiotic therapy relies heavily upon the notion
that fast-growing bacteria are more susceptible to the action of antibiotics than slow-
growers (7)(8)(9)(10). The slow-growing proportion of the *M. tuberculosis* population
is thought to be refractory to the bactericidal action of anti-tuberculosis antibiotics
due to phenotypic tolerance, and persistence through treatment (10). Informative *in
vitro* evaluations using batch models have determined the activity of antibiotics
against relevant phenotypes, including non-replicating persistent bacteria (11)(12)(13)(14). However, it is challenging to dissect out the direct cause and effect of a single stimulus in batch cultures. The only models that can be used to determine the effects of growth rate on drug responses are controlled and defined continuous cultures in chemostats (14)(15)(16)(17). These growth systems enable us to control the growth rate with minimal changes to the physiochemical environment, thereby allowing the effect of different growth rates to be compared. In this study, we investigate the contribution of growth rate to early bactericidal kill and the re-growth/recovery of the bacterial population when exposed to INH, PZA, and RIF, delivered singly or in combination. To enable us to perform these analyses we have derived a mathematically discriminative approach for the analyses of drug responses in chemostat culture, which account for dilution effects, and provide predictive and quantitative insights from bacterial responses.

Results

The antibiotics, INH, RIF, and PZA, were added during culture steady-states at minimum inhibitory concentrations (MIC) to replicate-cultures for each single antibiotic or antibiotic combination at each growth rate except for the triple combination, where a single culture was performed at each growth rate. A static concentration of antibiotic was maintained in culture throughout each time-course. Viable counts were performed throughout the culture time-courses and for a minimum of 14 MGT, which is equivalent to 970.2h and 322.2h for slow-growth and fast-growth, respectively. Time-course profiles of log-transformed viable counts for each culture were analysed with the fitting of a mathematical model by non-linear regression, where parameters representing gradients on the logarithmic scale were
determined for each culture to describe the logarithmic transformation of the viable count profile over time. These overall gradients comprise an estimated $k_{\text{net}}$ rate constant parameter that accounts for the net bacterial death/re-growth and a fixed $k_{\text{chemo}}$ rate constant imposed by the chemostat culture according to fast-growth or slow-growth conditions (Equation 1 and 2). A minimum of at least one gradient is needed to describe a log-viable count profile: the initial $\alpha$ gradient typically indicating a bactericidal or bacteriostatic response, where $\alpha = (k_{\text{net,} \alpha} - k_{\text{chemo}})$. If more than the $\alpha$ gradient is needed to adequately describe the log viable count profile (i.e. if more than one exponential phase is present) then additional gradient parameters are estimated accordingly, where $\beta = (k_{\text{net,} \beta} - k_{\text{chemo}})$. Additional gradients indicate responses other than the initial net kill e.g. regrowth/recovery at a higher net growth rate, net kill at a different rate to that initially observed, or a re-established steady-state growth. The $k_{\text{net,} \alpha}$ and $k_{\text{net,} \beta}$ elimination rate constants were compared between pairs of culture conditions to determine the impact of growth rate in each treatment case, whilst accounting for the chemostat dilution rate, $k_{\text{chemo}}$, for each growth rate. P-values from pairwise $Z$-tests comparing relevant pairs of experiments were calculated (Table 2) to determine which growth rate-specific responses were significantly different.

The rate of kill was faster in fast-growing *M. tuberculosis*

Early bactericidal activity was observed in all cultures (as shown by negative $k_{\text{net,} \alpha}$ kill rate constants; Table 1) except for bacteriostatic responses to single RIF exposures in slow-growers ($k_{\text{net,} \alpha}$ close to zero; Figure 2i). Slow-growing cultures exposed to INH at an MIC level, either singly or in combination (Figure 2 g, j, k), all showed similar initial killing rates to each other (ranging from $k_{\text{net,} \alpha}$ values of -0.03 to
Apart from the RIF/INH/PZA triple combination ($k_{net,α} = -0.036$ and $-0.01$, for slow-growth and fast-growth respectively) fast-growers showed an initial net bacterial killing rate of greater magnitude than the slow-growers for any given antibiotic therapy. Fast-growers or slow-growers exposed to RIF alone (Figure 2 c, i) and fast-growers exposed to the triple combination (Figure 2e) demonstrated mono-phasic elimination over time with slower kill rates compared to all INH and INH/RIF-exposed cultures (Figure 2 a, g) (Table 1).

The further addition of PZA to the RIF/INH combination in a slow-growing culture (Figure 2 k) showed a similar kill rate to singly used INH (Figure 2 g) and the RIF/INH dual combination (Figure 2 j) ($P = 1.11$ and 1.28, respectively), indicating that there were no additional killing benefits by the addition of PZA. However, when making a similar comparison in a fast-growing culture, the kill rate for RIF/INH/PZA (Figure 2 e) was slower ($k_{net,α} = -0.01$ for the triple combination vs. $k_{net,α} \sim -0.1$ ($P = 0.07$ INH, $P < 0.01$ RIF/INH) than for the double combination (Figure 2 d) and INH single treatment (Figure 2 a), indicating that further addition of PZA to the fast-growing population reduced the beneficial effects of INH-killing. This was indicative of a potential antagonistic effect between INH/RIF and PZA.

**Increasing the INH concentration does not eliminate re-growth**

Increasing the exposure of INH from MIC to 16-fold higher (8mgL$^{-1}$, a concentration more reflective of patient serum levels in clinic) gave rise to a faster initial killing rate in slow-growing cultures ($k_{net,α} -0.041$ vs. $-0.110$ $P = 0.12$; for MIC vs. 16x MIC respectively) (Figure 2 b, h). However, the concentration increase made little difference to the initial killing rate in fast-growing culture ($k_{net,α} -0.118$ vs. $-0.104$ $P = 0.12$).
0.81 for MIC vs. 16x MIC respectively), suggesting perhaps the kill rate for INH at MIC is maximal for fast-growing bacteria and indicates that fast-growing bacteria are more susceptible to INH. The responses to 16x MIC INH exposure were also the only trends that followed a tri-phasic profile, with two distinguishable initial bactericidal phases indicating that the higher INH concentration provoked a different response in the bacterial population than at the MIC. As discussed above, in all four of these situations (fast or slow culture with MIC or 16x MIC INH), a re-growth/recovery phase was observed with the re-growth rate approximately equivalent in size to the dilution rate for each culture, leading to a relatively flat second and final phases to the viable count profiles, implying a re-establishment of steady-state. However, at a higher INH concentration, for both growth rates, the apparent time of onset of the net re-growth/recovery phase was later and occurred from a starting point of a lower viable count.

Re-growth in response to INH is not eliminated by PZA and/or RIF

The addition of RIF to INH did not alter the kill rate or the re-growth rate compared to INH alone for the fast growers or the slow growers. Re-growth in the RIF/INH combination was apparent after 100 hours post-exposure in fast-growing cultures (Figure 2 d) and after approximately 350 hours in slow-growers (Figure 2 j), with \( k_{\text{net}_\beta} \) values of 0.029 and 0.013, respectively. The addition of PZA in the triple combination of PZA/RIF/INH eliminated re-growth in the fast-growth rate culture (Figure 2 e) by a reduction in the re-growth rate (from \( k_{\text{net}_\beta} = 0.013 \) to \( k_{\text{net}_\beta} = 0.007 \)). However, re-growth was not eliminated entirely. What was surprising was a lack of a reduction in kill rate in the slow-growth culture with the addition of PZA. These findings show that PZA appears to have more of an effect in reducing re-growth of
fast-growing cultures than slow-growers. The fast-growing culture treated with RIF/INH/PZA was the only situation in which a combination containing INH did not result in re-growth. It is possible that the slower kill rate in the fast-growers exposed to the triple combination contributed to a lack of re-growth as this was also observed for RIF-exposure, where the killing rate was also more gradual.

Discussion

The rate of kill was faster in fast-growing *M. tuberculosis*

Apart from the triple combination, for any given antibiotic treatment, there was a more rapid initial bacterial killing in fast-growers than in slow-growing cultures. It is widely accepted that bacteria in stationary phase/non-replicating phases are less susceptible *in vitro* to the frontline antibiotics, RIF and INH (12)(18)(19)(20).

However, the effect of growth rate on the effectiveness of drug combinations, has never been looked at in *M. tuberculosis* and is particularly important for organisms that can survive in the host for long periods of time in slow growing or non-growing conditions, which are characteristic of TB disease progression. Some of these studies have included the use of mathematical models, which described three bacterial states representing fast-multiplying, slow-multiplying, and non-multiplying bacteria in order to identify the responses to antibiotics (12). These mathematical models have predicted exposure–response relationships by inferring bacterial cell states within batch cultures where interpretation of drug responses was confounded by many factors other than growth rate. Continuous culture provides an invaluable method for determining growth rate effects, whilst controlling other parameters in the system (14)(15)(17). A limitation of these systems is that the interpretation of
bacterial responses is complicated by the continuous flow of medium into the chemostat and loss of cells in the effluent, particularly as this dilution rate is different for the two growth rates. We have accounted for this limitation in the mathematical model described here. Antibiotic activity in other bacteria is also impacted by growth rate, as shown in previous experiments using the chemostat, but very few of these studies have used mathematical models to quantify and determine the bacterial responses (17)(16)(21)(22). Levin and Udekwu (23) developed a quantitative model framework, using parameter values from literature (or best a priori estimates), for generating simulations, hypotheses and interpretations of bacterial responses in batch or continuous cultures. This model accounts for culture dilution rates and drug dilution rate (drug clearance in pharmacokinetic terms) and described various considerations that could be incorporated in future experimental design, such as the effect of secondary resources from dead cells and metabolites that have been released, wall subpopulations (biofilms), the impact of reduced cell density on the MIC. Other plausible mathematical models could be used to describe these phenotypic responses and bacterial population dynamics. However, given that we observed viable count data, rather than any quantification of specific sub-populations and antibiotic-exposures were performed at one or two drug concentrations, more complex mathematical models, were not applied at this stage.

**Increasing the concentration of INH does not remove re-growth**

Exposure to INH in the chemostat led to rapid bactericidal responses followed by re-growth/recovery and this was irrespective of growth rate. Recovery in the chemostat was attributable to a population of bacteria that either increased their growth rate or maintained a growth-rate that was dictated by the flow rate of the growth medium.
These experiments indicate that bi-exponential killing and persistence through INH exposure in clinical or in vivo studies is not explained by a reduced growth rate. We previously showed under both these growth rates that recovery was coincident with a substantial increase in mutant frequency and that the bactericidal activity of INH was being arrested, in-part, by the emergence of katG resistance mutations and not a reduction in the log-phase populations; irrespective of growth rate (14)(24). This finding was also reflected in another study by Gumbo et al., in 2007, using the hollow fibre pharmacodynamic model (25). However, we also showed the response to INH alone is not entirely explained by an increase in mutant frequency but also substantial genotypic changes that were growth rate specific (14). Biphasic kill observed in TB-infected guinea pigs treated with INH was found to be associated with the emergence of antibiotic tolerant persisting populations that were not resistance mutants (26). Similarly, relapse has previously been observed in mice after the cessation of treatment. Of all treatment groups that included INH at least 30% of the mice relapsed (as defined by isolation of M. tuberculosis from the spleen) whereas in treatment groups without INH, tubercle bacilli could only be isolated from 8% of mice (27). The lack of reduced recovery at higher concentrations has also been observed previously at a range of INH concentrations both in vitro and in vivo (18) (28).

**Addition of RIF and PZA does not eliminate the re-growth associated with INH**

Despite the response to RIF being slower and monophasic, it was unable to remove the recovery elicited by INH when used in combined exposure, in either growth rate. Similarly, a slower rate of kill by RIF has been shown previously in vitro and in mice, with no relapse observed (28). These findings were also reflected in a study by Hu et
al., in 2016, where RIF-containing regimens reduced persistence in mice (20). These findings combined with the fact that RIF has recently been shown to be effective at higher doses indicates that the removal of INH in treatment, and an increased dose of RIF is worthy of investigation as an attractive option for new drug combinations (29)(30)(31)(32). It would be interesting to see if increasing the concentration of RIF in our chemostat system leads to changes in the bacteriostatic vs bactericidal response, and whether this is growth rate-specific or not. A murine study by Andries et al., in 2010 ranked the bactericidal and sterilising potencies of several regimens (and individual antibiotics) and the comparison of the two ranks highlighted that bactericidal activity is not predictive of sterilisation (33). Their study also confirmed that RIF possessed more sterilising activity than expected from the bactericidal efficacy, further emphasising the need to move away from early bactericidal activity as a priority measure of drug candidate potential. A new drug combination needs to have good sterilising potential for genuine clinical cure/efficacy but prediction of this is challenging. Improved bacterial markers of drug-tolerant persistent sub-populations are required that can be measured early in the development of a drug combination and progress has been made in this area (34). Our results also suggest that the addition of PZA in the triple combination reduced the beneficial effects of INH killing of fast-growth bacteria, indicating that there could be antagonism between PZA and INH in chemostat culture. PZA could be bacteriostatic against the fast-growers, resulting in a halt in replication, which in turn reduced the fast killing effects of INH (35). It has been shown that growth inhibition by bacteriostatic antibiotics is associated with supressed cellular respiration, whereas cell death from exposure to bactericidal antibiotics causes accelerated respiration (36). A reduced cellular respiration induced by PZA exposure could be an explanation for the antagonism
observed between INH and PZA. It may be that slower INH-killing of fast-growers lead to a lack of re-growth. INH has been also shown to have an antagonistic effect on the efficacy of PZA in mice (20)(37)(28)(38), providing further support for the removal of INH either entirely or after an appropriate duration of treatment, and it would therefore be interesting to see, using our continuous culture models, whether removal of INH after one to two days (with continued exposure to PZA and RIF) reduces the presence of re-growth.

Concluding remarks

Re-growth is associated with the presence of INH and not bacterial growth rate. The association between INH and recovery/relapse combined with the knowledge that *M. tuberculosis* will overcome the effects of INH after an initial killing phase, by the development of resistance and/or drug tolerance in any *in vitro* or *in vivo* model, supports the suggested replacement of INH with drugs that kill more slowly and do not lead to relapse. The chemostat model, and the method of data analysis described here can contribute to the direct comparison of drug combinations, and provide information about the relationship between bactericidal activity and recovery.

Materials and Methods

Reagents

Primary stock solutions of RIF, INH, and PZA were prepared at 10gL\(^{-1}\) in 100% DMSO. These were frozen in aliquots (100µL) at -20°C. A working stock of RIF was prepared at 1gL\(^{-1}\) (diluted from the 10gL\(^{-1}\) stock using water) was also frozen at -20°C. When required, the stocks were diluted to the desired concentration in water and filter sterilised (0.2µm pore size).
Strains and their growth

*M. tuberculosis* (strain H37Rv) was used in all experiments. Bacilli were enumerated on 7H10 agar plus OADC supplement.

Continuous culture of *M. tuberculosis*

*M. tuberculosis* (strain H37Rv) was grown in chemostats under controlled conditions as described previously (14). We cultured *M. tuberculosis* using CAMR Mycobacterium medium MOD2, which contained glycerol as the limiting nutrient (39). Continuous cultures were performed at two different growth rates to steady-state under defined and controlled conditions at pH6.9, at a temperature of 37°C and at a dissolved oxygen tension of 10% (14). The cultures achieved a MGT of 23.1h (fast-growth) or a MGT of 69.3h (slow-growth), where the fractional washout/replacement rate of medium in the continuous culture was 0.03 hr⁻¹ and 0.01 hr⁻¹, respectively. Antibiotics were added during steady-state at minimum inhibitory concentrations (MIC; 0.5mgL⁻¹ of INH, 0.032mgL⁻¹ of RIF, and 250mgL⁻¹ of PZA) to two replicate cultures for each single antibiotic or antibiotic combination at each growth rate except for the triple combination, where a single culture was performed at each growth rate. A static concentration of antibiotic was maintained in culture throughout each time-course. In the case of INH, exposures to 16 x MIC (8 mgL⁻¹) were also assessed. Viable counts were performed throughout the culture time-courses as described previously, and for a minimum of 14 MGT which is equivalent to 970.2h and 322.2h for slow-growth and fast-growth, respectively (14). Further triplicate fast-growth or slow-growth cultures were established without antibiotic exposure to
provide baseline information about the differences in viability between the two growth rates.

### Viability measurements

The viability of the cultures was measured at each MGT using the Miles and Misra viable count method (40) with the following modification: the plate was divided into quadrants for the dilutions. In each quadrant, three 20μL aliquots of the appropriate dilution were spotted and then left to dry at room temperature. Colonies were counted after 3 weeks incubation at 37°C.

### Mathematical modelling

The growth rate, death rate, and chemostat washout rate (by continuous culture), of a given population of *M. tuberculosis* in the continuous culture incubation were described mathematically as first-order processes: i.e. processes where the rates of bacterial growth, death or chemostat elimination in the continuous culture at a given instant of time were all proportional to the size of that population of *M. tuberculosis* at that given instant of time and governed by first order rate constants: $k_g$, $k_d$ and $k_{chemo}$ for the growth, death and chemostat washout respectively. Combined, these component rates give an expression for the overall rate of change of the size of the *M. tuberculosis* population, (MTb; Equation 1):

$$\frac{d(MTb)}{dt} = (k_g \cdot MTb) - (k_d \cdot MTb) - (k_{chemo} \cdot MTb)$$

Equation 1 can be simplified as outlined in Equation 2:

$$\frac{d(MTb)}{dt} = (k_{net\_a} - k_{chemo}) \cdot MTb$$
Equation 2 summarises that the rate of change of the *M. tuberculosis* population was governed by an overall net 1\textsuperscript{st} order rate constant "α", itself comprised of a net bacterial growth/death rate constant (\(k_{\text{net,α}}\)) and the chemostat continuous fractional washout rate constant (\(k_{\text{chemo}}\), fixed to 0.01h\(^{-1}\) and 0.03h\(^{-1}\) for slow-growing or fast-growing cultures respectively. Equation 2 (an ordinary differential equation) was integrated into an exponential, closed form solution, describing the *M. tuberculosis* population as a direct function of time and the initial size of the population (Equation 3):

\[
\text{Equation 3: } \text{MTb}(\text{time}) = A * \exp(\alpha \cdot \text{time})
\]

(where \(A = \) the initial size of the *M. tuberculosis* population)

In this form, \(\alpha\) is the gradient of a plot of the natural log transform of the count of the *M. tuberculosis* population vs. time, or the apparent gradient of a plot of the *M. tuberculosis* population vs. time on a semi log scale, and has units of time\(^{-1}\). \(A\) is the y-axis intercept of the same transformed profile/plot. This mathematical approach allows for a more precise partitioning of the overall behaviour of the bacterial population over time ("seen" at the level of the raw data via the "\(\alpha\)" rate constant) into the processes of bacterial growth/death (governed \(k_{\text{net,α}}\)) and the fixed, known, and experimentally controlled process of the chemostat continuous culture washout (governed by \(k_{\text{chemo}}\)). If it is apparent from a plot of the logarithmic transformation of the observed viable count data that more than one exponential phase (i.e. more than one gradient on the log-scale) is required to describe the observations then Equation...
3 (and its underlying assumptions) can be doubled up into a bi-exponential equation (Equation 4).

\[
\text{Equation 4: } MTb(\text{time}) = A.\exp(\alpha . \text{time}) + B.\exp(\beta . \text{time})
\]

Here B and \( \beta \) represent the initial value and overall net 1st order rate constant describing the behaviour of a 2nd sub-population of \( M. \) \( \text{tuberculosis} \) contributing to the total observed viable count. This second sub-population may have had a slower or faster overall net growth or death rate than the other, and/or began from a different initial starting point. This 2nd sub-population may have contributed to potential re-growth that was not obvious until later in the time-course, or perhaps a 2nd slower elimination phase under drug therapy. Further exponential terms (e.g. using G, \( \gamma \) parameters) may be added if data has extra phases able to support their estimation.

A schematic of the processes considered by the overall mathematical model in Equation 4 applied in this work is given in Figure 1. The mathematical models of Equation 3 or Equation 4 were applied to time-course profiles of viable counts from continuous culture to provide an estimate of the \( \alpha \) elimination rate, and if present the \( \beta \) re-growth rate, and/or their corresponding \( k_{\text{net,}\alpha} \) or \( k_{\text{net,}\beta} \) values given the known, fixed \( k_{\text{chemo}} \) washout rate constant. These parameter estimates compare net growth or elimination rates between different experimental conditions and are used to simulate a line of best fit to provide a visual check of how effectively the mathematical model is describing the data. Observations of the minimum viable count in culture were also compared to the initial value at the start of treatment to estimate an observed “maximum reduction” in viable count, to be used in conjunction with the mathematical model parameter values estimated from the data to provide
any extra insight into drug activity. Parameter estimation from applying the model to the data used the nonlinear least-squares optimisation function “lsqnonlin” as part of the “pracma” package in the R statistical software language (version 3.4.2), with an unweighted objective function. Standard errors of parameter estimates were calculated using the method outlined by Landaw and DiStefano with the Jacobian of model parameter sensitivities estimated using a numerical central difference method (41). Standard errors (SE) were expressed as % relative to the parameter estimate in question (%RSE), with a %RSE less than ~50% being considered an acceptable degree of precision of an estimate given the data in question. The replicate experiments were treated as a naive pool for data analyses rather than using the average of replicate data at each time point (42). The significance of differences between model parameter estimates under different experimental conditions was examined with pairwise Z-tests.

**Interpretation of $k_{\text{net}_\alpha}$ and $k_{\text{net}_\beta}$ values**

If gradients $k_{\text{net}_\alpha}$, $k_{\text{net}_\beta}$, $k_{\text{net}_\gamma} < 0$, then the net rate of change of the *M. tuberculosis* population is negative and a plot of the *M. tuberculosis* population viable count in the continuous culture vs. time would show an exponential decrease over time. This is the case for all negative $k_{\text{net}}$ values, $k_{\text{net}} = 0$ (i.e. bacteriostasis), or any positive $k_{\text{net}}$ values that are not large enough in magnitude to cancel out the imposed, fixed $k_{\text{chemo}}$ (i.e. net growth, but not fast enough to completely overcome the chemostat washout rate). A negative gradient value is therefore indicative of either bacteriostatic or bactericidal activity. If a gradient $= 0$, then the net rate of change of the *M. tuberculosis* population is zero and a plot of the population log-viable count vs. time will be flat. For this situation, $k_{\text{net}}$ must equal $(-1) \times k_{\text{chemo}}$. A gradient of zero (or a
value that a very close to zero) indicates growth equal to the imposed rate of chemostat washout, as seen in the steady-state control, no-drug cultures. Estimates of \( k_{\text{n}} \) that are very close to zero can show poor relative standard error precision for the parameter estimate. If \( \text{gradient} > 0 \), then the net rate of change of the \( M. \) \( \text{tuberculosis} \) population is positive and a plot of the population viable count vs. time shows an exponential increase over time. For this \( k_{\text{n}} \) must be both positive and larger in absolute value than \( k_{\text{chemo}} \). A positive gradient is therefore an indication of re-growth/recovery or of an \( M. \text{tuberculosis} \) population, in the overall culture.

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599 **Figures and legends**
Figure 1: A bi-exponential, two-state mathematical model applied to viable count data obtained from continuous cultures of *Mycobacterium tuberculosis* that have been treated with static concentrations of INH, RIF, and PZA, either singly or in combination. (Total Mtb = Mtb1 + Mtb2, α = k_{g1} - k_{d1} - k_{chemo} - k_{net, α} - k_{chemo}, β = k_{g2} - k_{d2} - k_{chemo} - k_{chemo} - k_{net, β} - k_{chemo}). Kg: Bacterial replication (growth), Kd: Bacterial death, Kchemo: Bacterial washout due to dilution rate of chemostat. The sign and magnitude of knet depending, typically: A or α govern the Kill phase of antibiotic treatment, B or β: the re-growth phase of antibiotic treatment. A 2-state model was adequate to describe the dataset profiles for most cultures. However, some cultures demonstrated single or triple exponential phases and required either one-state or three-states for the data to be adequately described.
Figure 2: The viability of *M. tuberculosis* growing under a fast-growth rate (23.1h MGT) (a-f), or a slow-growth rate (69.3h MGT) (g-l) in continuous culture and exposed to either: INH 0.5 mg mL\(^{-1}\)(a, g), INH 8 mgL\(^{-1}\)(b, h), RIF 0.032 mgL\(^{-1}\)(c, i), INH 0.5 mgL\(^{-1}\) & RIF 0.032 mgL\(^{-1}\)(d, j), INH 0.5 mgL\(^{-1}\) & RIF 0.032 mgL\(^{-1}\) & PZA 250 mgL\(^{-1}\)(e, k), or control / no-antibiotic (f, l). Total viable counts were determined by plating (Log\(_{10}\)CFU ml\(^{-1}\); black circles), the mathematical model was fitted to the data, governed by the estimated \(k_{\text{net}, \alpha/\beta}\) and intercept parameters (solid black line), and the underlying imposed chemostat washout rate (grey dashed line) was used as a comparison.
Table 1: Values for \( k_{\text{net},\alpha} \), \( k_{\beta} \), and \( k_{\gamma} \) (if present), with the % error for slow-growing or fast-growing continuous cultures of *Mycobacterium tuberculosis* exposed to INH, RIF, and PZA, singly and in combination. * \( k_{\text{net},\alpha} \) estimate is very close to zero; i.e. the overall observed \( \alpha \) -elimination rate of the viable count is approximately equal to the \( k_{\text{chemo}} \) wash-out rate), leading to an inflated relative standard error of this estimate.

| Treatment     | Slow-growing \( (k_{\text{chemo}} = -0.01) \) | Fast-growing \( (k_{\text{chemo}} = -0.03) \) |
|--------------|---------------------------------------------|---------------------------------------------|
|              | \( k_{\text{net},\alpha} \) (h\(^{-1}\)) | \( k_{\text{net},\beta} \) (h\(^{-1}\)) | \( k_{\text{net},\gamma} \) (h\(^{-1}\)) | \( k_{\text{net},\alpha} \) (h\(^{-1}\)) | \( k_{\text{net},\beta} \) (h\(^{-1}\)) | \( k_{\text{net},\gamma} \) (h\(^{-1}\)) |
|              | est. | %RSE | est. | %RSE | est. | %RSE | est. | %RSE | est. | %RSE | est. | %RSE |
| RIF          | 0.001* | 288* | -  | -  | -  | -  | -0.020 | 26  | -  | -  | -  |
| INH          | -0.041 | 52   | 0.013 | 10  | -  | -  | -0.118 | 47  | 0.033 | 7  | -  |
| RIF&INH      | -0.030 | 19   | 0.013 | 22  | -  | -  | -0.102 | 18  | 0.029 | 37 | -  |
| RIF&INH&PZA  | 0.036 | 55   | 0.007 | 44  | -  | -  | -0.010 | 38  | -  | -  | -  |
| INH (16x MIC)| -0.110 | 35   | -0.003 | 13  | 0.014 | 4  | -0.104 | 19  | -0.009 | 89 | 0.037 | 6  |
| CONTROL      | 0.013 | 9.12 | -  | -  | -  | -  | 0.031 | 4.3 | -  | -  | -  | -  |
Table 2: P-values for pairwise comparisons of $k_{\text{net}_\alpha}$ in fast-growing (23.1 MGT; FAST) and slow-growing (69.3 MGT; SLOW) continuous cultures of *M. tuberculosis* exposed to MIC levels of INH, RIF, and PZA singly and in combination. $k_{\text{net}_\alpha}$
comparisons were also made for 16 x MIC INH and $k_{\text{net}_\beta}$ comparisons were made for responses that were triphasic.