Quantitative modeling predicts mechanistic links between pre-treatment microbiome composition and metronidazole efficacy in bacterial vaginosis

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Bacterial vaginosis is a condition associated with adverse reproductive outcomes and characterized by a shift from a Lactobacillus-dominant vaginal microbiota to a polymicrobial microbiota, consistently colonized by strains of Gardnerella vaginalis. Metronidazole is the first-line treatment; however, treatment failure and recurrence rates remain high. To understand complex interactions between Gardnerella vaginalis and Lactobacillus involved in efficacy, here we develop an ordinary differential equation model that predicts bacterial growth as a function of metronidazole uptake, sensitivity, and metabolism. The model shows that a critical factor in efficacy is Lactobacillus sequestration of metronidazole, and efficacy decreases when the relative abundance of Lactobacillus is higher pre-treatment. We validate results in Gardnerella and Lactobacillus co-cultures, and in two clinical cohorts, finding women with recurrence have significantly higher pre-treatment levels of Lactobacillus relative to bacterial vaginosis-associated bacteria. Overall results provide mechanistic insight into how personalized differences in microbial communities influence vaginal antibiotic efficacy.
**Results**

**Model predicts *Lactobacillus* MNZ sequestration influences efficacy.** To determine how MNZ treatment efficacy can be altered by bacterial-mediated interactions in vitro, we created an ODE model to predict growth of *Gv* and *Li* upon co-culture and treatment with MNZ (Fig. 1a). Parameters for each bacterial species were obtained by least-squares fitting of in vitro kinetic data and dose–response curves for MNZ exposure with each species in monoculture (Supplementary Figs. 1, 2, Supplementary Table 1), before the ODE model was used to predict co-culture conditions with *Gv* and *Li* both interacting with extracellular MNZ. The model assumes that *Gv* and *Li* internalize or sequester MNZ at rates $k_{\text{int-Gv}}$ and $k_{\text{int-Li}}$, respectively, and *Gv* can convert MNZ to the stable metabolite, acetamide, and unknown metabolites at rate $k_{\text{met}}$.

The model additionally assumes logistic growth at rates $k_{\text{grow-Gv}}$ and $k_{\text{grow-Li}}$, with carrying capacities of $K_{\text{Gv}}$ and $K_{\text{Li}}$, and growth inhibition by MNZ toxicity at rates $k_{\text{kill-Gv}}$ and $k_{\text{kill-Li}}$, in a dose-dependent manner based on 50% effective concentrations of MNZ on *Gv* and *Li* ($EC_{50\text{-Gv}}$, $EC_{50\text{-Li}}$).

Since MNZ is a pro-drug that is activated when internalized by anaerobic bacteria, the cytotoxicity of MNZ in the model is dependent on the intracellular concentration of MNZ rather than extracellular MNZ concentration; however, we used the external MNZ concentration as the basis for $EC_{50}$ of internalized MNZ, as experimentally determining the intracellular level of MNZ per cell was challenging and the main goal was to capture the relative sensitivity between *Gv* and *Li*.

To identify model parameters that were most critical for decreasing *Gv* growth, we performed a 1-dimensional (1D) sensitivity analysis by altering each parameter three orders of magnitude above and below baseline and evaluated *Gv* growth (Fig. 1b, c). Growth was scaled relative to the predicted growth in an unperturbed (no MNZ) co-culture based on the time point and initial population sizes evaluated and is referred to as percent maximum growth. The sensitivity analysis identified *Gv* growth as highly dependent on the MNZ internalization/sequestration rate into *Li* ($k_{\text{int-Li}}$). A 50-fold increase in this rate increased the growth of *Gv* from 7.42 to 69.5% its maximal growth upon 48 h treatment with MNZ (Fig. 1c). Likewise, changing the MNZ internalization rate into *Gv* ($k_{\text{int-Gv}}$) has similar effects on *Li* where increasing this rate 50-fold resulted in 89.7% *Li*’s maximal growth (Supplementary Fig. 3a).

Overall, our findings highlight the importance of leveraging quantitative models that evaluate interactions of target bacteria and non-target *Lactobacillus* spp. with MNZ in improving insight into personalized differences in BV recurrence and treatment failure.
Varying the initial Gv:Li ratios in the presence of 500 μg/ml MNZ experimentally in vitro by Model validation in Gv initially.

Growth with 500 μg/ml (Fig. 2a, b)39,40. Doses below 100 μg/ml had no effect on Gv or Li growth and doses above 1600 μg/ml exhibited near-complete cell killing for both bacterial strains (Fig. 2a, b); these data are in agreement with experimentally determined effective concentrations of MNZ on Gv and Li cultured individually (Supplementary Fig. 2c, d). However, for doses between 100 and 1600 μg/ml there were significant differences depending on the initial Gv:Li ratio, where MNZ was most efficacious in eliminating Gv when more Gv than Li was present initially.

**Model validation in Gv and Li co-cultures.** We validated these counterintuitive model predictions experimentally in vitro by varying the initial Gv:Li ratios in the presence of 500 μg/ml MNZ and tracking growth for 48 h (Fig. 2c, d). Experimental measurements confirmed model predictions that MNZ efficacy for inhibiting Gv growth decreased when Li was initially dominant (p = 4.67 × 10^{-8}), and were not significantly different than model predictions (0.001× Gv:Li, p = 0.430; 1000× Gv:Li ratio, p = 0.689, Fig. 3c), with Gv exhibiting a predicted 30.3% and experimental 41.4% ± 13.3% maximal growth after treatment when Li was initially dominant compared to a predicted 2.1% and experimental 9.4% ± 13.8% maximal growth when Gv initially was dominant. Li growth in the presence of 500 μg/ml MNZ was also dependent on the initial Gv:Li ratio, where MNZ inhibited Li growth the most when Li was initially dominant, 7.2% ± 3.9% maximal growth compared to when Gv was initially dominant, 70.5% ± 33.8% (p = 3.29 × 10^{-9}, Fig. 2d). Notably, the model over-predicted the growth of the Li population when Li was initially dominant (0.001× Gv:Li), where the model prediction of 23.9% maximal growth was over threefold higher than experimentally observed, 7.19% ± 3.91% growth (0.001× Gv:Li experiment vs simulation, p = 1.43 × 10^{-5}), suggesting efficacy dependence on a high pre-treatment Gv:Li ratio may be even greater than that predicted by the model. Experimental and model predictions of Li growth were not significantly different when Gv was initially dominant (1000× Gv:Li, p = 0.726).

**Fig. 1 Model schematic for bacterial growth dynamics in BV with MNZ treatment.** a MNZ is internalized by both G. vaginalis (Gv) and L. iners (Li) at rates k_{int-GV} and k_{int-LI}. Cells are proliferating at k_{grow-GV} and k_{grow-LI} and MNZ inhibits growth by k_{kill-GV} and k_{kill-LI}. For Gv, a potential mechanism of MNZ resistance is the bacterial-mediated interactions to the drug leading to the formation of metabolites (k_{met}). b Sensitivity of Gv growth with 500 μg/ml MNZ when parameters directly related to Gv growth are varied 0.001× to 1000× baseline values. Percent maximal growth refers to the final cell count compared to the carrying capacity of the culture, or the maximum cell density the unperturbed culture can reach at 48 h based on initial cell density. c Sensitivity of Gv growth with 500 μg/ml MNZ when parameters related to Li survival are varied 0.001× to 1000× baseline values. d Percent maximal growth of Gv (left) and Li (right) when the initial ratio of Gv to Li is varied with 500 μg/ml MNZ treatment. e Percent maximal growth of Gv when MNZ internalization rate of Li is varied at three different population compositions with 500 μg/ml MNZ treatment.
Likewise, model predictions of MNZ and MNZ metabolite concentrations were not significantly different from experimental results in cultures starting with a 0.001× Gv:Li ratio (extracellular MNZ: \( p = 0.255 \), intracellular MNZ: \( p = 0.336 \), acetamide: \( p = 0.877 \)), but predictions for extracellular MNZ, intracellular MNZ, and acetamide concentrations in cultures with a 1000× Gv:Li ratio did vary significantly from experimental data (Supplementary Fig. 4). The deviation of model predictions when Gv is initially dominant suggests that experimental investigation of detailed mechanisms of Gv interactions with MNZ is warranted (for example, the potential ability of Gv to externally degrade MNZ). Despite some deviation of peripheral model predictions from experimental measurements, the Gv:Li ratio-dependent trends were reproduced by the model. The dependency on initial culture ratios of Gv to Li on growth suggests that non-target bacteria that sequester MNZ could significantly alter drug efficacy.

We observed some variation in the sensitivity (EC50) of Li to MNZ. Variability in minimum inhibitory concentrations (MIC) estimations have been reported, as changes in culture conditions including incubation length and the inoculum effect can influence the apparent sensitivity of bacteria to antibiotic. In addition, the sensitivity of Lactobacillus spp. and Gv to MNZ and their MICs are reported to range from 500 to 4000 µg/ml and 0.75 to 17; respectively. To ascertain whether our results would be influenced by variation in Li sensitivity to MNZ, we repeated the simulations over a range of EC50 values. To represent reported resistance of Lactobacillus spp. in vitro, we increased the EC50 value of Li to be 10-fold higher than Gv (EC50 Li = 4200 µg/ml). MNZ efficacy in inhibiting Gv growth was similarly decreased at low Gv:Li ratios (36.5% max growth at 0.001× Gv:Li) compared to high Gv:Li ratios (3.96% max growth at 1000× Gv:Li; Supplementary Fig. 5a). Li had little to no susceptibility over the range of MNZ concentrations tested (Supplementary Fig. 5b). In addition, these EC50 values replicated trends in experimental data for growth kinetics (Supplementary Fig. 2d, h versus Supplementary Fig. 5c, d). These results support that the initial Gv:Li ratio-dependent trends in MNZ efficacy for inhibiting Gv growth are independent of Li's sensitivity to MNZ.

**Fig. 2** A higher initial Gv:Li ratio improves MNZ treatment efficacy. a Surface plot to illustrate predicted percent maximal growth of Gv (z-axis) when concentration of MNZ (x-axis) and the ratio of Gv:Li (y-axis) are varied in simultaneously. Arrows indicate the concentration of MNZ and ratios of Gv:Li used for model validation. b Percent maximal growth of Li after simultaneous variation of MNZ dose and Gv:Li ratio. c, d Comparison of model simulations to experimental data for 500 µg/ml MNZ at 1000× and 0.001× Gv:Li. Gv percent maximal growth 0.001× initial Gv:Li ratio and 1000× initial Gv:Li ratio experimental vs simulation, and experimental vs experimental p-values were \( p = 0.430 \), \( t = 0.809 \), df = 17; \( p = 0.680 \), \( t = 0.420 \), df = 17; \( p = 4.67 \times 10^{-8} \), \( t = 6.99 \), df = 34, respectively. Li percent maximal growth 0.001× initial Gv:Li ratio and 1000× initial Gv:Li ratio experimental vs simulation, and experimental vs experimental p-values were \( p = 1.43 \times 10^{-5} \), \( t = 6.00 \), df = 17; \( p = 0.726 \), \( t = 0.357 \), df = 17; \( p = 3.29 \times 10^{-9} \), \( t = 7.91 \), df = 34, respectively. Data are presented as mean ± SD, \( n = 18 \) independent, biological replicates for each initial ratio, asterisks indicate significance as: ‘*’ \( p < 0.05 \), ‘**’ \( p < 0.01 \), ‘***’ \( p < 0.001 \) without adjustment for multiple comparisons, unpaired two-sided t-test. Source data are provided as a Source data file.
Optimal MNZ doses are dependent on pre-treatment microbiome. We next used the model to determine specific combinations of MNZ concentrations and initial Gv:Li ratios that resulted in optimal final Li proportion after 48 h MNZ exposure. The initial Gv:Li ratio was highly associated with the final Gv:Li ratio for doses of MNZ >250 µg/ml (Fig. 3a). Interestingly, cultures that were initially Li dominant (0.001× Gv:Li), were nearly insensitive to any dose of MNZ, resulting consistently with >50% Gv (Fig. 3a). This result carries the surprising implication that women with Li-dominant vaginal microbiomes at treatment initiation are likely to undergo recurrence, regardless of MNZ dose. Of note, cultures that were originally Gv dominant (Gv:Li >1) were the most likely to be Li dominated after 48 h exposure to MNZ. Experimental data supported these trends, as the simulation predictions were not significantly different for the final proportion of Li at 500 µg/ml for 1000× (p = 0.680, t = 0.420, df = 17). The model did overestimate the final proportion of Li at the 0.001× Gv:Li ratio (predicting a 44.1% proportion of Li compared to 14.2% ± 7.16% obtained experimentally); however, this result suggests an even more significant reduction in Li proportion when Gv is initially dominant (p = 0.008, t = 4.06, df = 17).

A phase diagram of MNZ therapy outcomes at 48 h was created to characterize both Li and Gv endpoint growth dynamics, which depict either an increase/expansion or decrease in population size relative to the initial population. The optimal growth dynamics would depict the expansion of only the Li population and the least optimal growth dynamics would be the expansion of only Gv. A decrease in both populations is additionally not optimal, as lower levels of beneficial microbiota are often associated with opportunistic infections or overgrowth of non-optimal species. We observed that higher initial Li:Gv ratios in conjunction with MNZ concentrations over 250 µg/mL were more likely to result in optimal final growth dynamics where the Li bacterial population was the only population expanding (Fig. 3b). Likewise, it was possible for only the Gv population to grow and the Li population to decrease when the initial Li:Gv ratio was <1×. Interestingly, the diagram predicts that it is possible that both Gv and Li populations would decrease for intermediate ratios of Gv:Li, which expand to include a wider range of ratios as the dose of MNZ is increased. Overall, in vitro co-culture experimental data supported the model predictions for endpoint growth dynamics, with 15 of 18 samples agreeing with the dynamics predicted by the phase diagram for the 1000× Gv:Li, 500 µg/ml group and for all 18 samples agreeing with the predictions for the 0.001× Gv:Li ratio, 500 µg/ml group (Fig. 3b, right). This result reinforces the importance of pre-treatment Gv: Li ratio on post-treatment bacterial community composition.

Initial composition influences efficacy in more complex models. While our model results emphasize the importance of pre-treatment Gv:Li ratios in MNZ efficacy in co-cultures, BV in women is more complex, and involves interspecies interactions and strain variability across many different bacterial species. To evaluate the above results in more complex settings that include multiple species, interspecies interactions, and strain variability, we created three additional model structures (Fig. 4a–d). In Model B and Model D, we account for potential interspecies interactions, such as amensalism between Lactobacillus spp. and BV-associated bacteria and commensal or mutualistic behavior within BV-associated bacteria subpopulations and Lactobacillus spp. (Fig. 4a, d). In Models C and D we add additional representative species; a second BV-associated species and second Lactobacillus spp. (BV:LB ratio) as associated parameters, we randomly selected parameter values from physiologically relevant ranges determined from previously published studies (Supplementary Tables 2 and 3). Notably, across all four model structures we found that higher initial relative amounts of BV-associated bacteria to Lactobacillus spp. had higher relative post-antibiotic levels of Lactobacillus spp. (BV: LB ratio, Fig. 4e, f). Supplementary Fig. 6, p < 1E–6, p < 1E–6, p < 1E–6, p < 1E–6). This result held for a range of ratios (0.6× BV:LB and 100× BV:LB), chosen to reflect the observed relative abundance of Lactobacillus spp. in BV positive women (60–1.0%)52. Moreover, for each of these model structures, the global sensitivity analyses consistently selected the MNZ internalization/sequestration parameter (k_int) and the initial relative abundance of BV-associated bacteria to Lactobacillus spp. (BV:LB ratio) as significantly sensitive parameters in post-antibiotic treatment Lactobacillus spp. relative abundance. Variability in Gv sensitivity
to MNZ (EC50) and growth rate were also selected as critical parameters in dictating response to MNZ treatment, which are of interest as there is significant variability across Gv subclasses in terms of resistance to MNZ, and metabolism. Furthermore, when models were modified such that Lactobacillus spp. could not internalize/sequester MNZ, the ratio-dependent effect was abrogated, and was independent of sensitivity of Lactobacillus spp. to MNZ (Supplementary Fig. 7a, b). Altogether, this provides additional quantitative evidence that Lactobacillus spp. sequestration of MNZ may contribute to BV recurrence in more complex microbial environments.

**Pre-treatment composition is associated with clinical outcome.**

We next evaluated whether the influence of initial BV:LB ratio on MNZ efficacy is observed clinically. We compared the pre-treatment ratio of BV-associated bacteria to Lactobacillus spp. (BV:LB ratio) in vaginal samples collected from women who underwent MNZ treatment for BV and were cured or experienced recurrence, in two clinical studies; the UMB-HMP study (n = 11) and CONRAD BV study (n = 33). We chose to evaluate each study separately to minimize the effects of differences in sample collection and in methods of microbial species measurements. In the UMB-HMP cohort, 11 women were observed over 12 weeks, of which 10 were observed over a second episode of BV during the 10-week period (Supplementary Table 4). Results resonated with model predictions where...
individuals who experienced recurrence had higher amounts of Lactobacillus spp. relative to BV-associated bacteria (lower BV:LB ratios, \( p = 0.0366 \)) at treatment initiation and tended to have higher abundances of Lactobacillus spp., particularly Li, but abundance of individual species were not statistically significant after adjustment for multiple comparisons (\( p = 0.201 \), Fig. 5a, Supplementary Fig. 8a). In addition, Gv relative abundance was not significantly different between groups (\( p = 0.984 \), Supplementary Fig. 8b). Furthermore, when we analyzed the specific species in the original two-species model, we also observed similar results where cured women had significantly higher ratios of Gv to Li (\( p = 0.0497 \), Fig. 5b). It is important to note that since the Gv:Li ratio comparison was a selective analysis, we did not correct for multiple comparisons based on individual species in the original data set (over 190 species measured). These results support both the in vitro experimental data and model results that predicted a lower efficacy of MNZ treatment when a lower ratio of Gv to Li was present pre-treatment.

We also evaluated model findings in a second clinical cohort, the CONRAD BV study, which consisted of 33 women whose vaginal microbiome was sampled at enrollment in the study, 1 week after MNZ treatment and 1 month after MNZ treatment. Relative abundances were determined by sequencing of the V4 region of the 16S rRNA. Women were excluded from this subset analysis if they failed to finish the antibiotic regimen, contracted a secondary vaginal infection, did not respond or had delayed response of treatment. Of the 33 women, 21 met inclusion criteria (n = 10 individuals for the cured group, n = 11 individuals for the recurrent group) describing a log base 10 transform of initial BV-associated bacteria relative abundance to Lactobacillus spp. relative abundance, \( p = 0.0366, t = 2.678, df = 6 \). Clinical results for the CONRAD BV cohort (n = 10 individuals for the cured group, n = 11 individuals for the recurrent group) showing a log base 10 transform of initial BV-associated bacteria relative abundance to Lactobacillus spp. relative abundance, \( p = 0.242, t = 2.449, df = 19 \). Initial Gv:Li ratio, \( p = 0.0338, t = 2.287, df = 19 \). Data are presented as median, 25th and 75th quartiles, statistical analysis was completed with unpaired, two-sided t-tests that were not adjusted for multiple comparisons. Source data are provided as a Source data file.

Discussion

Here we show a personalized tolerance mechanism that may contribute to BV recurrence and treatment failure. Our model illustrates how non-target bacteria, such as Li or other Lactobacillus spp., may sequester antibiotic and lower the amount of MNZ available to target bacteria like Gv. This model result implies that MNZ efficacy may be dependent on highly variable pre-treatment relative abundances of Lactobacillus spp. such as Li to BV-associated bacteria populations (BV:LB ratios) and raises the question of whether patients with higher levels of Lactobacillus spp. are more susceptible to recurrent BV than those with higher degrees of dysbiosis. Importantly, results from the model, in vitro experiments, and clinical data all point to a higher pre-treatment BV-associated bacteria population relative to Lactobacillus spp. as a driver of MNZ efficacy in inhibiting Gv growth and facilitating post-treatment Lactobacillus dominance. This study complements ongoing work in the search for drivers of BV treatment efficacy, in which experimental studies are often limited to delineating the role of individual bacteria, and it is challenging to assess the importance of numerous clinical and microbial variables that are associated with treatment outcomes.

The potential for non-antibiotic-target bacterial populations to act as a sink for MNZ and alter efficacy is similar to a concept that has been previously explored in bacterial ecology, termed the inoculum effect (IE), which describes an increase in antibiotic MICs due to increased bacterial load and decreased per cell antibiotic concentration. While the IE and the ability of bacterial species to influence MNZ bioavailability has been previously reported, to our knowledge its role in BV recurrence has not yet been considered. Furthermore, the ODE model used here was essential for determining the critical importance of MNZ sequestration by Lactobacillus spp. across multiple interactions that have the potential to influence efficacy and recurrence, including metabolism, proliferation, and susceptibility to MNZ of both target and non-target species. The model was also necessary...
for translating the importance of this parameter to microbial communities with varying compositions and with different MNZ dosing regimens. Though the proposed MNZ sequestration mechanisms were not experimentally validated in this study, the model predictions for associated relationships between pre-treatment microbial composition and BV recurrence were recapitulated in both co-cultures and in cervicovaginal samples, providing an additional mechanism for recurrence that has not previously been considered.

Recent studies evaluating pre-treatment vaginal microbiota composition on MNZ efficacy have reported inconsistent results, likely due to differences in patient exclusion criteria, time point of treatment outcome assessment, drug regimen, and methods to collect and quantify the vaginal microbiota. One study that employed a similar drug regimen (oral MNZ) and sample collection methods to the clinical cohorts evaluated here supported our results, finding higher pre-treatment loads of antibiotic-target species, Gv and A. vaginae, associated with BV treatment efficacy.19 Other studies that used different sample collection methods and antibiotic regimens did not explicitly evaluate the pre-treatment ratio of BV-associated bacteria to Lactobacillus spp.; generally suggested there was an association between total Lactobacillus relative abundance and successful treatment.15,55,56 Notably, some of these studies focused on analyzing treatment outcome immediately after antibiotic therapy was completed, and in some cases treatment failure was due to no response to therapy. We propose that recurrence and failure to respond to therapy likely arise from different factors, where recurrence is due to a collective bacterial population’s resilience to antibiotic therapy and failure to respond is due to inherent resistance of BV-associated bacteria. Studies that have associated higher Gv loads with treatment failure correspond with the latter and could be due to the formation of biofilms or other resistance mechanisms.86 As our model predicts immediate post-therapy Lactobacillus spp. relative abundance, no response to treatment would be equivalent to predicting no change or low Lactobacillus spp. relative abundance at 48 h. An additional limitation of our model is that it does not appear to be applicable to cases of MNZ treatment failure in women who initially had very low levels of Lactobacillus spp. (<1%), which our model would predict should promote MNZ efficacy.34 However, we propose that treatment failure in this case may be a result of the Allee effect, which can be caused by a variety of mechanisms that lead to decreased fitness at low population densities, suggesting these women have Lactobacillus abundances that are too low to recolonize the vagina and may be associated with more precisely modeling interspecies interactions. Moreover, since Li is the only Lactobacillus spp. observed to date to significantly sequester MNZ, it will be important to characterize how other vaginal bacterial species interact with MNZ to further explore the role of non-target bacterial species on MNZ efficacy. Altogether, conflicting results in clinical studies of pre-treatment vaginal microbiota composition support the need for the development of quantitative platforms to evaluate the interplay between multiple microbial species, clinical variables, and dosing regimens that contribute to personalized differences in treatment failure.

Models presented here are only simple reconstructions of the minimal possible interactions between bacterial species and an antibiotic that have been established as key species by the existing literature, with a time-scale that was limited by in vitro co-culture conditions. While the model provided useful insight into how non-target bacterial species may influence BV recurrence after MNZ treatment, predicting regrowth of Lactobacillus spp., and the full quantitative mechanisms underlying responses to treatment are likely more nuanced. More complex model frameworks did suggest key results would hold true in microbial communities with additional microbial species, interspecies interactions, and strain variability, though we were not able to validate this experimentally. Interspecies interactions in our models were incorporated with generalized Lotka–Volterra equations which simplifies relationships to a single term, but represent a good starting point for recapitulating ecosystem-level complexities.59–63 Specific metabolic interactions that dictate survival and elimination of bacterial species in the vagina could be included with greater mechanistic detail in the future. In instances where parameters are unknown or difficult to measure experimentally, this work demonstrates the value of a global computational sensitivity analysis for understanding the relative importance of strain-level differences in antibiotic uptake, metabolism, or sensitivity. Predictive simulations can be run across multiple possible parameter ranges to determine the effects of variation prior to costly experimental measurements. This tool will be valuable in isolating the role of individual parameters in making a bacterial population or community more tolerant to antibiotic therapy.

In this study, we demonstrated that ODE models can provide insights into antibiotic–microbe interactions pertinent to understanding BV treatment efficacy. Our work highlights that it is possible for BV treatment to fail, even if target bacteria are not resistant to MNZ as vaginal bacterial populations as a whole can be resilient to antibiotic, resulting in recurrent BV. While our clinical analysis is limited in sample size and therefore should be considered preliminary, future extensions of this work could be used to inform clinical decision-making regarding personalized therapy options. More generally, we envision that the use of quantitative models such as this will provide a framework for integrating knowledge of interactions between multiple bacterial species and drug treatments in mucosal tissues to give insight into the diverse responses observed in infectious disease and other syndromes of the female reproductive tract.

Methods

Bacterial strains and culture conditions. Lactobacillus iners ATCC 55195 and Gardnerella vaginalis ATCC 14018 (group C) were obtained from the American Type Culture Collection (ATCC) and maintained on Human Bilayer Tween Agar (BD) plates and New York City III (NYCIII) medium according to the manufacturer’s instructions. Agar plates and liquid cultures were incubated at 37 °C with anaerobic gas mixture, 80% N_2, 10% CO_2, and 10% H_2. Frozen stocks of strains were stored at −80 °C in 40% (v/v) glycerol.

Metronidazole quantification by tandem mass spectrometry. MNZ concentrations were determined by validated LC-MS/MS assays. Sample aliquots were centrifuged at 3000 × g and divided between supernatant and cell pellet. Extracellular MNZ was extracted from supernatant via protein precipitation using acetonitrile. For intracellular concentration measurements, cell pellets were lysed using sonication and re-suspended in 100 μL of sterile water. Samples were subjected to positive electrospray ionization (ESI) and detected via multiple reaction monitoring (MRM) using a LC-MS/MS system (Agilent Technologies 6460 QQQ/MSMassHunter). Calibration standards were prepared with an inter- and intra-day precision and accuracy of 5% with an r^2 value of 0.9988 ± 0.0009. Quantification was performed using MRM of the transitions of m/z 172.2 → 128.2 and 176.2 → 128.2 for MNZ and MNZ-d4 respectively. Each transition was monitored with a 100-ns dwell time. Stock solutions of MNZ and MNZ-d4 were prepared at 1 mg/mL in acetonitrile-water and stored at −20 °C. Mobile phase A is 0.1% acetic acid in H_2O and mobile phase B is 0.1% acetic acid in ACN, and chromatographic separation was achieved using a gradient elution with a Chromolith Performance RP-18e C_18 column maintained at 35 °C from 0 to 6.6 min, 8% 0–100, with 0.5 μL/min flow. During pre-study validation, calibration curves were defined in multiple runs on the basis of triplicate assays of spiked media samples as well as QC samples. This method was validated for its sensitivity, selectivity, accuracy, precision, matrix effects, recovery, and stability. Replicates of reference samples were included every six samples and evenly distributed throughout the MS analysis to monitor consistency and performance and to utilize for downstream normalization.

Bacterial quantification. Bacterial quantification determined via turbidimetry was completed by measuring the optical density at each time point, 100 μL of sample inoculum was read at O.D. 600 nm using a SpectraMax Plus 384 UV
spectrophotometer. Time points were recorded within 5 min of sampling and stored at −80 °C. Bacterial quantification using plate counting was done by doing a 10-fold dilution using sterile water and aliquoting 10 μL spread evenly onto BD agar plates. Cultures were incubated at 37 °C. Plating was done in triplicates and was counted manually. Prior optimization ensured the dilution would result in no more than 300 colonies making quantification as accurate as possible.

Bacteria–MNZ experiments. For the MNZ experiments, 50 μL MNZ was added at appropriate concentrations to 5 mL of NCYI media. Samples equilibrated at 37 °C for 1 h prior to the addition of 50 μL of bacterial inoculum (2 x 10^6 CFU/mL). One hundred and fifty micro liters of aliquot was taken for time-point readings for MNZ and bacterial quantification (as described above). Samples were incubated at 37 °C under constant mixing and only removed for time-point measurements. For the co-culture experiments, Gv:Li ratios were added at appropriate experimental conditions in a likewise manner. For each varying ratio sample within each experiment, a side-by-side duplicate was performed without MNZ as a negative control. The negative control was assessed only for bacterial quantification to ensure that no growth condition or external stimuli promoted the growth of one over another. Negative control experiments demonstrated bacterial proliferation that mirrored growth of each individual bacteria cultured alone without confirming any changes in growth seen in our bacteria–MNZ experiments were the result of the addition of MNZ.

ODE models. The model equations were constructed assuming both Li and Gv internalize MNZ at rates k_{Li} and k_{Gv}, MNZ toxicity to Li and Gv occurred at rates dependent on the maximum rates k_{Gv-Li} and k_{Li-Gv}, and the concentration of internalized MNZ where growth inhibition increased as internalized MNZ exceeded a threshold as described by 50% effective concentrations, EC50Gv and EC50Li. The growth of Li and Gv was assumed to be logistic in behavior at rates k_{grow-Li} and k_{grow-Gv}, with distinct carrying capacities for each bacterium, K_{Li} and K_{Gv}. The parameters for k_{grow-Li}, k_{grow-Gv}, K_{Li}, and K_{Gv} were determined by nonlinear least-squares fitting of the logistic function to growth curves for Li and Gv grown in separate cultures (Supplementary Fig. 2a, b)74. The k_{Li-Li}, k_{Gv-Gv}, EC50Li, and EC50Gv were determined by fitting the Hill equation to kill curves for Li and Gv cultured in isolation (Supplementary Fig. 2c, d). Internalization rates, k_{int-Li} and k_{int-Gv} and metabolism rates, k_{met-Li} and k_{met-Gv} were determined from fitting the ODE model to time course metabolic spectrometry data for external MNZ, internal MNZ and acetamide and cell densities (optical density) using a multistart local optimization strategy (Multistart) with the local solver Iqcurvefit.

Model simulations and validation. Unless otherwise noted, all simulations were completed at MNZ concentration of 500 μg/ml over the course of 48 h. Growth outputs were normalized to the maximal growth density (K_{Li} and K_{Gv}) for comparison across simulations and to experimental data. External, internal MNZ, and acetamide concentrations were relative to the total volume of cellular pellets. Sensitivity analyses were completed by perturbing a single model parameter while keeping the rest of the parameters constant over 1000×−0.001× the original value. Surfaces were generated over three orders of magnitude for MNZ concentration (10−1500 μg/ml) and eight orders of magnitude for ratio of Gv:Li (1.6 x 10−4−1.6 x 104) at 1225 combinations of MNZ concentration and Gv:Li ratio. Model validation was completed by comparing the experimental co-culture data to model predictions using unpaired t-tests.

Generalized models and global sensitivity analysis. To incorporate intraspecies and interspecies we developed three additional model structures and ran simulations with randomized parameter sets to determine if the influence of initial Gv:Li ratio, or the more generalized BV:LB ratio, on endpoint Lactobacillus spp. composition is consistently observed across these model structures. For capturing intraspecies variation, we used Latin Hypercube Sampling of parameter ranges for each parameter to create 100 parameter sets. We derived these parameter ranges from the literature and a summary of these ranges can be found in Supplementary Table 1 and 2. The Latin Hypercube Sampling methods were used for the global sensitivity and uncertainty analysis, which analyzed the partial rank correlation coefficient with 2000 randomly generated parameter sets with endpoint (48h, 500 μg/ml MNZ) Lactobacillus spp. relative abundance75. For capturing interspecies interactions and microbial interactions like cross-feeding, we developed a four species model that includes two representative BV-associated bacteria, and two Lactobacillus species, L. iners and a second species representing L. crispatus, L. jensenii, or L. gasseri.

**Interspecies interaction terms.** Gause76 noted the calculation for interaction terms for a generalized Lotka–Volterra model describing competitive exclusion (Eqs. (1) and (2)). In our model, we generalized the interaction terms further to be able to capture many different interactions, specifically amensal behavior where Lactobacillus spp. can inhibit BV-associated bacterial growth with no effect of BV-associated bacteria on Lactobacillus species growth (−0/0) as well as mutualistic (both species benefit from the other −/+1) and commensal behaviors (one species benefits 0/−) between BV-associated bacteria or within the Lactobacillus population. The amensal behavior between Lactobacillus species has been documented experimentally in co-culture77 and we calculated the interaction term for many different species and strains of Lactobacillus on G. vaginalis and A. vaginae from Atassi et al.99. It is largely believed that D-lactic acid produced by many Lactobacillus species inhibits the growth of BV-associated bacteria; however, L. iners does not produce this isomer of lactic acid and is the reasoning behind not including an interaction term between L. iners and the BV-associated bacteria78. It is not clear how BV-associated bacteria metabolize Lactobacillus acid in the cross of feeding, so we allowed the model to simulate this behavior51. In addition, L. vaginus is associated with promoting the growth of other BV-associated bacteria like A. vaginae79. Calculations were completed assuming the reported mono and co-cultures were at steady state to derive Eqs. (3) and (4).

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Equations (3) and (4) relate to the parameters in Supplementary Table 3 by Eqs. (5) and (6), which generalizes the reported interaction strength from the literature to be able to be adjusted for varying carrying capacities modeled in the simulation that do not equal the carrying capacities from the literature.

\[
\frac{dN}{dt} = r_N N - \frac{N + s_{NP} P}{K_N} \quad \text{(1)}
\]

\[
\frac{dP}{dt} = r_P P - \frac{P + s_{PN} N}{K_P} \quad \text{(2)}
\]

\[
s_{NP} = \frac{K_N - N}{P} \quad \text{(3)}
\]

\[
s_{PN} = \frac{K_P - P}{N} \quad \text{(4)}
\]

\[
s_{NP} = \frac{K_N - s_{NP} K_N}{s_{PN} f_{NP}} \quad \text{(5)}
\]

\[
s_{PN} = \frac{K_P - s_{PN} K_P}{s_{NP} f_{PN}} \quad \text{(6)}
\]

Software. Parameterization, ODE modeling, and sensitivity analyses were completed using Matlab® 2018b (Matlab, Natick, MA). Statistical analyses were performed using PRISM 8, exact p-values <1e-6 were calculated in Matlab.

Clinical data and study population

The UMB-HMP cohort. The study results and associated clinical data were previously published and all data provided were de-identified to this study. The UMB-HMP study was not an interventional study, but an observational study at the general level.

Analysis of clinical outcomes. In the Human Microbiome Project cohort, patients were defined as cured or recurrent based on whether after initial MNZ treatment patients exhibited Lactobacillus dominance at both 1 week and 1 month after treatment were considered cured, and that exhibited Lactobacillus dominance at week 1 and not at 1 month were considered recurrent. The statistical analysis followed the same methodology as the HMP cohort.

Data availability

The source data are provided with this paper for Figs. 2–5, Supplementary Figs. 1, 2, 4 and 8, includes a detail of the simulation, model parameterization, and clinical validation. For the clinical studies, the UMB-HMP cohort study sequence data andmetadata were deposited in the Sequence Read Archive (SRA) and are available upon request; however, we have included an abbreviated version of this data set that includes all the data necessary for the reproduction of the source data. The sequence data and metadata for the CONRAD BV cohort are available in dbGAP BioProject PRJNA208535. In this study, the vaginal microbiota composition data from 11 women who experienced BV and were treated with MNZ during remaining of the 10-week observation period. The CONRAD BV cohort. The study results and associated clinical data were previously published and all data provided were de-identified to this study. The original clinical study protocol was approved by the Institutional Review Board of the University of Alabama at Birmingham and the University of Maryland School of Medicine. Written informed consent was appropriately obtained from all participants, who also provided consent for storage and use in future research studies related to women's health.

Women self-collected cervicovaginal swabs for 10 weeks. Vaginal microbiota data were generated by sequencing the V3-V4 regions of the 16S rRNA gene and is available in dbGAP BioProject PRJNA208535. In this study, the vaginal microbiota composition data from 11 women who experienced BV and were treated with MNZ within the UMB-HMP study were analyzed. Any participants who failed to complete the CONRAD regimen, who did not have BV according to Nugent scoring at the time of MNZ treatment, or who did not have follow-up data available were excluded from the analysis. The initial relative abundances were averaged across the week before starting MNZ treatment. Patients were classified to have recurrent BV if they exhibited a second episode of BV based on Nugent scoring (7–10) during the 10-week observation period.

The source data are provided with this paper for Figs. 2–5, Supplementary Figs. 1, 2, 4 and 8, includes a detail of the simulation, model parameterization, and clinical validation. For the clinical studies, the UMB-HMP cohort study sequence data and metadata were deposited in the Sequence Read Archive (SRA) and are available upon request; however, we have included an abbreviated version of this data set that includes all the data necessary for the reproduction of the source data. The sequence data and metadata for the CONRAD BV cohort are available in dbGAP BioProject PRJNA208535. In this study, the vaginal microbiota composition data from 11 women who experienced BV and were treated with MNZ during remaining of the 10-week observation period. The CONRAD BV cohort. The study results and associated clinical data were previously published and all data provided were de-identified to this study. The original clinical study protocol was approved by the Chesapeake Institutional Review Board (IRB) (Pro #0008122) with a waiver of oversight from the Eastern Virginia Medical School (EVMS) and registered in ClinicalTrials.gov (NCT01347632). A total of 69 women were screened from symptomatic discharge and 35 women were enrolled in the study. Vaginal microbiota data were generated by sequencing the V4 region of the 16S rRNA gene, providing taxonomic resolution at the general level.

Thirty-three women completed all three visits. BV was evaluated by vaginal microbiota compositional data (molecular-BV)76. After biological samples were obtained at visit 1 (V1), women with BV were prescribed twice daily, 500-mg MNZ for 7 days. Participants returned for visit 2 (V2) 7–10 days after completing the course of MNZ therapy and visit 3 (V3) 28–32 days after completing treatment. At all three visits, samples were obtained to evaluate vaginal semen (ABACard, West Hills, CA), vaginal pH, gram stain for Nugent score and semiquantitative vaginal flora culture. CVLs were collected, followed by vaginal swabs and three full-thickness biopsies.

Analysis of clinical outcomes. In the Human Microbiome Project cohort, patients were defined as cured or recurrent based on whether after initial MNZ treatment the patient suffered an additional episode of BV (Nugent 7–10) during the 10-week course of data collection. For analysis, initial flora relative abundances were averaged across the 7 days prior to reported treatment start date. To analyze the relative ratio between BV-associated bacteria and Lactobacillus spp., we combined the relative abundances for the top 20 BV-associated bacteria and all Lactobacillus spp. The genera BV-associated bacteria included were Gardnerella, Atopobium, Megasphaera, BVAB1-3, Streptococcus, Prevotella, Leptotrichia, Anaerococcus, Peptostreptococcus, Eubacterium, Veillonella, Sneathia, Mobiluncus, Corynebacterium, Ureaplasma, Eubacterium, Porphyromonas, Dialister, Peptostreptococcus, Bacteroides, Fusobacterium, Actinomyces, and Riboflabibacterium. Before statistical analysis, the BV:LB ratio was log-transformed, and the relative abundances of L. iners, G. vaginalis were center-log ratio (CLR) transformed, with pseudocounts added to taxonomic units with relative abundances equal to zero. Statistical analysis of the BV:LB ratio and GeoLi ratio was completed using two-sided unpaired Student’s t-tests and analysis of the CLR-transformed single species abundances was completed using two-sided unpaired Student’s t-tests and were corrected using the FDR method of Benjamini and Hochberg (PRISM 8).

For the CONRAD BV cohort, treatment outcome was defined based on Lactobacillus dominance evaluated at enrollment, 7 days after treatment and 28–32 days after treatment. Patients that exhibited Lactobacillus dominance at both 1 week and 1 month after treatment were considered cured, and patients that exhibited Lactobacillus dominance only at week 1 and not at 1 month were considered recurrent. The statistical analysis followed the same methodology as the HMP cohort.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Acknowledgements

This work was in part funded by startup funds from the University of Miami to N.R.K., funds from NIH/NIDDK grant RO1DK112254 to N.R.K., and NIH/NIAID grant R01AI138718 subcontract to N.R.K., and startup funds from the University of Michigan to K.B.A. The UMB-HMP study, J.R. and M.F. were supported by the National Institute for Allergy and Infectious Diseases of the National Institutes of Health under award numbers UH2AI083264 and R01NR015495. The CONRAD BV Study was funded by an intra-agency agreement between the Centers for Disease Control and Prevention (CDC), United States Aid and International Development (USAID) and CONRAD (GPO-A-00-08-00005-00). The views expressed by the authors do not necessarily reflect those of the funding agency or CONRAD.

Author contributions

C.Y.L., R.K.C., M.M.L., N.R.K., and K.B.A. conceived and designed the study. C.Y.L. completed the computational analysis and analyzed the clinical data. R.K.C., N.R.K., and B.H. designed and conducted monoculture and co-culture kinetic experiments. A.G., M.F., and J.R. curated data from the UMB-HMP cohort. J.R. led the UMB-HMP study and data collection. A.T. and G.D. provided CONRAD BV protocol development, patient care, and data analysis. C.Y.L., R.K.C., K.B.A., and N.R.K. wrote the manuscript and all authors read and revised the manuscript.

Competing interests

J.R. is co-founder of LUCA Biologics, a biotechnology company focusing on translating microbiome research into live biotherapeutic drugs for women’s health. All other authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-19880-w.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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