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The impact of different antimicrobial exposures on the gut microbiome in the ARMORD observational study

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eLife Assessment

This study offers a **valuable** assessment of the impact of antibiotics on the human gut microbiota across diverse observational cohorts. The findings presented are **convincing**, despite the observational design and residual confounding that may still contribute to discrepancies between the cross-sectional and longitudinal data. The work is relevant for researchers and clinicians interested in antimicrobial resistance and the impact of antibiotics on the host.

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Abstract

Background

Better metrics to compare the impact of different antimicrobials on the gut microbiome would aid efforts to control antimicrobial resistance (AMR).

Methods

The Antibiotic Resistance in the Microbiome – Oxford (ARMORD) study recruited inpatients, outpatients and healthy volunteers in Oxfordshire, UK, who provided stool samples for metagenomic sequencing. Data on previous antimicrobial use and potential confounders were recorded. Exposures to each antimicrobial were considered as factors in a multivariable linear regression, also adjusted for demographics, with separate analyses for those contributing samples cross-sectionally or longitudinally. Outcomes were Shannon diversity and relative abundance of specific bacterial taxa (*Enterobacteriaceae*, *Enterococcus*, and

major anaerobic groups) and antimicrobial resistance genes (targeting beta-lactams, tetracyclines, aminoglycosides, macrolides, and glycopeptides).

Results

225 adults were included in the cross-sectional analysis, and a subset of 79 patients undergoing haematopoietic stem cell transplant provided serial samples for longitudinal analysis. Results were largely consistent between the two sampling frames. Recent use of piperacillin-tazobactam, meropenem, intravenous co-amoxiclav and clindamycin were associated with large reductions in microbiome diversity and reduced abundance of anaerobes. Exposure to piperacillin-tazobactam and meropenem were associated with a decreased abundance of *Enterobacteriaceae*, and an increased abundance of *Enterococcus* and major AMR genes, but there was no evidence that these antibiotics had a greater impact on microbiome diversity than iv co-amoxiclav or oral clindamycin. In contrast, co-trimoxazole, doxycycline, antifungals and antivirals had less impact on microbiome diversity and selection of AMR genes.

Conclusion

Simultaneous estimation of the impact of over 20 antimicrobials on the gut microbiome and AMR gene abundance highlighted important differences between individual drugs. Some drugs in the WHO Access group (co-amoxiclav, clindamycin) had similar magnitude impact on microbiome diversity to those in the Watch group (meropenem, piperacillin-tazobactam) with potential implications for acquisition of resistant organisms. Metagenomic sequencing can be used to compare the impact of different antimicrobial agents and treatment strategies on the commensal flora.

Introduction

Effective antimicrobial stewardship is necessary to limit the emergence and spread of antimicrobial resistance (AMR),[1] and this includes the preferential use of agents least likely to select for drug resistant pathogens among the commensal flora. This typically consists of avoiding “broad” spectrum antimicrobials, as well as those to which resistance is currently rare, and these principles underlie the World Health Organisation AWaRe classification (Access, Watch and Reserve).[2] AWaRe is reflected in the current UK National Health Service standard contract, which requires hospitals to increase the proportion of “Access” antibiotics used, replacing previous requirements to reduce overall antibiotic use.[3] However, these classifications are largely based on activity against pathogens rather than direct measures of AMR gene selection or microbiome disruption, and stewardship could be improved if such measures were available. For example, two antibiotics being considered for use may have similar spectra against a target pathogen, but very different impacts on AMR selection, either because the amounts reaching the commensal flora differ, or because they have differing spectra against non-pathogenic commensals that protect against colonisation with drug-resistant pathogens. Metagenomic sequencing provides a direct measure of AMR genes and microbiome composition in a sample, and its increasing availability in recent years is starting to allow the fuller impacts of antimicrobials to be measured.

The large intestine contains the vast majority of human commensal bacteria and is the primary reservoir for several clinically important commensal pathogens, particularly the *Enterobacteriaceae* (including *Escherichia coli* and *Klebsiella pneumonia*) and *Enterococcus faecium*. Increasing multi-drug resistance in these organisms represents a major global public health challenge.[4,5] Existing evidence linking antibiotic exposure to individual-level selection for AMR in the gut flora comes predominantly from small, healthy volunteer studies, which have shown that antibiotics can cause rapid microbiome disruption, but provide limited comparative data between antibiotics. They also may also have limited applicability to real patients, who often have recent exposure to several different agents and are at high risk of colonisation with drug resistant organisms. Randomised trials are a robust method to assess the impacts of different treatment approaches, but few have reported microbiome outcomes.[6–9] Another approach is to exploit variation in routine use of antibiotics in groups of patients at high risk of AMR to understand the nature and extent of differences between agents which cannot easily be achieved in other designs. Here we report results from a prospective observational study assessing the impact of antimicrobial use on the gut microbiome and selection of AMR genes. This study included two different sampling frames to produce independent and complimentary estimates, one cross-sectional, analysing a single stool sample from each participant, and one longitudinal, analysing changes in serial samples taken from the same participant admitted for haematopoietic stem cell transplant to enrich for broad spectrum antimicrobial exposure.

Methods

Study design and participants

The Antibiotic Resistance in the Microbiome – Oxford (ARMORD) study was an observational study that recruited healthy individuals living in Oxfordshire, and patients at the Oxford University Hospitals NHS Foundation Trust (OUH). The study was coordinated by the Nuffield Department of Medicine, University of Oxford, and was approved by the East Midlands-Leicester Central Research Ethics Committee (15/EM/0270).

The study involved two sampling strategies:

- *Cross-sectional sampling*. Participants provided a single stool sample, and measures of the microbiome and AMR gene abundance were related to exposures recorded at the time of sampling.
- *Longitudinal sampling*. Participants provided serial stool samples, and changes in the microbiome and AMR gene abundance between serial samples from the same individual were related to exposures between samples. Longitudinal sampling was only performed in patients admitted to the OUH haematology ward for haematopoietic cell transplant (HCT). The initial sample collected from these patients was also used in the cross-sectional analysis.

Participants were eligible if they were ≥18 years old, had no stoma or active inflammatory bowel disease, and were able to give informed consent and provide a history of recent antibiotic use. General medical inpatients and outpatients at OUH were approached by a member of the study team during routine care (without regard to the reason for admission or attendance), and healthy individuals responded to articles in local media. After providing written informed consent, participants were interviewed and their medical notes were reviewed to collect information about antimicrobial exposures in the past year, recent travel, diet, alcohol and tobacco use, animal exposures, healthcare exposure and use of specific drugs (case report form in Appendix p8). Electronic patient records available at OUH included i) inpatient, emergency department and outpatient attendances, ii) inpatient and discharge antimicrobial prescriptions iii) microbiology,

haematology and biochemistry results, iv) inpatient clinical observations (used to calculate the National Early Warning Score 2 [NEWS2] summary score of physiological abnormality),[10] and v) discharge coding (including Charlson co-morbidity index).

Sample collection

In the cross-sectional stratum, participants were asked to provide the first stool sample passed after recruitment. This was stored at ambient temperature for a maximum of 24 hours before being frozen at −80°C. In the longitudinal stratum, participants were asked to provide a stool sample every other day until discharge. These were stored at 4-8°C for up to 72 hours before being frozen at −80°C.

DNA extraction, sequencing and bioinformatic analysis

DNA extraction was performed by bead beating in Lysing Matrix E tubes (MP Biomedicals) followed by QIAGEN Fast DNA Stool MiniKit (QIAGEN) (details in Appendix p1). Samples were sequenced in batches of 56-114 at the Oxford Centre for Genomics, and had automated normalisation and library preparation using either NEBNext Ultra or NEBNext Ultra II FS kits. All samples from the same batch were pooled and sequenced across 2-8 Illumina HiSeq4000 lanes using 150bp paired-end sequencing. Following human read removal all samples were subsampled to a depth of 3.5 million paired reads, and samples with fewer reads were excluded. Taxonomic classification was performed with MetaPhlAn2 (for diversity indices) and Kraken2 (for abundance of specific taxa). AMR gene detection in metagenomic sequence data was performed with the ARIBA software package, using the CARD database and ontology (v3.0.2) (details in Appendix p2).

Outcomes

Sequence data was used to derive three types of outcome (further details in Appendix p3):

- *Shannon diversity index* - a single metric of diversity for each sample (calculated as the sum of p*ln(p) for all species, where p is proportional abundance). This index incorporates relative abundance and the number of species detected (richness).
- *Log relative abundance of specific bacterial taxa*. The taxa of interest were two major groups of opportunistic pathogens (family *Enterobacteriaceae* and genus *Enterococcus*), and three major groups of anaerobes that make up the majority of the gut microbiome in most people but are largely non-pathogenic, so may be important for colonisation resistance (phyla Bacteroidetes and Actinobacteria, and class Clostridia). Relative abundance was the proportion of reads in the sample that mapped to a group. If a particular taxon was not detected, its relative abundance was imputed as 10^{-6} (i.e. a pseudocount at the approximate lower limit of detection).
- *Log relative abundance of specific classes of AMR genes*. Five classes of clinically important resistance mechanisms were of interest: Clinically significant beta-lactamases (CTX-M, OXA, TEM, SHV), tetracycline ribosomal protection proteins, aminoglycoside transferases (AAC, ANT, APH), macrolide/clindamycin resistance genes (erm and mef), and the vanA vancomycin resistance gene. If a particular gene was not detected its relative abundance was imputed as 10^{-5} (i.e. a pseudocount at the approximate lower limit of detection).

Statistical analysis

A separate model was fitted for each of the eleven outcomes (Shannon diversity, five bacterial taxa, and five classes of AMR genes, as above). Model results are presented side-by-side for the five bacterial taxa, and for the five AMR gene classes. For bacterial taxa and AMR genes the outcomes are relative (not absolute) abundance, so the effects of antimicrobials on different taxa/genes are

not independent (e.g. if the only effect of an antimicrobial was to eradicate one major taxon and leave the absolute abundances of others unchanged, then the relative abundances of these other taxa would increase).

Analysis was performed in R v4.2.3.

Cross-sectional sampling frame

Multivariable linear regression was used to estimate the effects of specific antimicrobial exposures on the outcomes above. Covariates were: age, sex, participant category (healthy, general medical, autologous stem cell transplant, allogeneic stem cell transplant), days of chemotherapy received (0 for non-HCT participants, truncated at 14), maximum Charlson comorbidity score in the year before sampling (identified from electronic health records), and the following physiological abnormalities in the fortnight prior to sample collection; maximum NEWS2 score, C-reactive protein (CRP) >50 mg/dL, white cell count (WCC) >11 $x10^9$/L, and WCC <0.5 $x10^9$/L. Healthy volunteers lived in the OUH catchment area but most had no previous activity at OUH, and in this case normal values were imputed (i.e. Charlson Index 0, NEWS2 score 0, CRP <50 mg/dL, WCC <11 and >0.5 $x10^9$/L). Other covariates such as diet and travel were not included in the final model, as they were not significantly associated with Shannon diversity in multivariable analysis and their inclusion did not materially affect other estimates. We did not make a formal adjustment for multiple testing, and present unadjusted p-values which should be interpreted in the light of the number of outcomes (11) and antimicrobials (21) considered. Many of the antibiotic exposures were correlated or reflected iv/oral administrations of antimicrobials from the same class, and we considered relative abundances as described above, meaning that it is not straightforward to correct for multiple testing without being overly conservative. We therefore chose to interpret the findings as exploratory in the context of the supporting level of evidence, the number of comparisons performed and consistency across outcomes.

All antimicrobial exposures observed in >5 participants were included in the model. Individual agents were categorised separately if given by different administration routes (e.g. oral vancomycin and intravenous vancomycin), but route of administration was ignored in analyses of antimicrobial class (e.g. glycopeptides). In categorising antimicrobial class, beta-lactams were divided into 'narrow spectrum' (defined as penicillin, amoxicillin, flucloxacillin and first generation cephalosporins) and 'broad spectrum' (all others).

Exposure to each antimicrobial was included as a separate variable on a scale of 0 (no exposure) to 1 (full exposure). In order to reflect both recency and total duration of antimicrobial use, this exposure was modelled as the area under an exponential decay curve of the form $y = 2^{-x/\lambda}$, in which λ is the microbiome disruption half-life, and x is time before sample collection. A single value of λ was used for all analyses, chosen as the common value across all antimicrobial exposures with the lowest Akaike Information Criterion across 1 to 14 days in the cross-sectional model for microbial diversity (6 days; Supplementary Table 1). The disruption half-life of 6 days means that after 6 days of an antibiotic course a patient would have an exposure of 0.5 to that agent, and after 12 days they would have an exposure of 0.75. Details of the exposure calculation, including graphical depictions are in the Appendix (p3 and Supplementary Figures 1-2).

Longitudinal sampling frame

Serial samples collected from participants undergoing HCT were used for the longitudinal analysis. The unit of analysis was a pair of consecutive samples collected from the same individual. For patients with >2 samples, each consecutive pair was used (i.e. sample 2 in pair 1 was sample 1 in pair 2, and so on, so the total number of pairs per participant is one less than the number of samples). Only pairs of samples collected within 2-30 days of each other were used. A multivariable linear regression model was used that was analogous to the cross-sectional model

	Healthy volunteers (n=33)	General medical patients (n=91)	HCT patients (n=101)	All participants (n=225)
Age, years (median, IQR)	37 (31-49)	76 (67-83)	58 (50-66)	64 (50-73)
Sex (n, %)				
Male	7 (21%)	53 (58%)	60 (59%)	120 (53%)
Female	26 (79%)	38 (42%)	41 (41%)	105 (47%)
Recent antibiotic use (n, %)				
Receiving antibiotics at time of sampling	0 (0%)	55 (60%)	42 (42%)	97 (43%)
Use in past month (but not at time of sampling)	3 (9%)	26 (29%)	22 (22%)	51 (24%)
Use in past year (but not in past month)	4 (12%)	6 (7%)	33 (33%)	43 (19%)
No antibiotics in past year	26 (79%)	4 (4%)	4 (4%)	34 (15%)
Max Charlson index in past year (median, IQR)*	0 (0-0)	4 (0-13)	0 (0-8)	0 (0-8)
Maximum values in past 14 days (median, IQR)				
NEWS2*	0 (0-0)	5 (2-8)	3 (2-4)	3 (1-5)
C-reactive protein†	0.2 (0.2-0.2)	63 (22-163)	10 (3-69)	21 (2-81)
White cell count‡	7.5 (7.5-7.5)	11.5 (8.4-14.4)	7.6 (5.8-10.9)	8.4 (7.4-12.4)
Days of chemotherapy at time of sampling (median, IQR)	0 (0-0)	0 (0-0)	3.0 (1.4-7.2)	0 (0-2.8)

* National Early Warning Score 2. Imputed as 0 if no observations recorded, see Methods.
† Imputed as 0.2 if no result recorded
‡ Imputed as 7.5 if no result recorded

Characteristics of participants in cross-sectional analysis

Table 1.

Characteristics of participants in cross-sectional analysis

described above, except that the outcome was the *change* between the first and second samples in a pair, rather than absolute values (i.e. change in Shannon diversity, or change in log relative abundance of bacterial taxa or AMR genes).

Because pairs of samples from the same participant may not be independent of one another, robust standard errors were used to allow for possible clustering.

Covariates were: age, sex, type of transplant (allogeneic or autologous), days of chemotherapy received at collection of sample 1 (0 in non-HCT patients, and truncated at 14), number of days between sample 1 and sample 2, change in NEWS2 score between sample 1 and sample 2, and the presence of the following new physiological abnormalities recorded after collection of sample 1 and before collection of sample 2; CRP >50 mg/dL, WCC >11 $x10^9$/L, WCC<0.5 $x10^9$/L. For each outcome, the value for the first sample in the pair was also included as a covariate (i.e. baseline diversity or log relative abundance of taxa/AMR genes).

Antimicrobial exposures were calculated for samples as in the cross-sectional analysis above, and the exposure for each pair was the difference between the first and second samples. This has the following implications: i) if a patient starts an antimicrobial after the first sample is collected then the exposure for that pair is the same as for sample 2, ii) if a patient is on long-term antimicrobial treatment then the exposure for a pair is zero (as one would not expect this to lead to a difference between samples), iii) if a patient stops antimicrobial treatment shortly after sample 1 then the exposure to that agent will be negative (as one expects gut microbiome diversity to increase after stopping an antimicrobial). Truncating the small number of negative values at zero had little impact on results (data not shown).

Results

Between July 2015 and November 2018, 225 participants were recruited and had at least one sequenced stool sample, all of whom were included in the cross-sectional analysis (**Table 1** and Supplementary Figure 3 [CONSORT diagram]). Thirty-three (15%) were healthy volunteers, 91 (40%) were general medical patients (84 acute admissions and 7 attending general outpatient clinics), and 101 (45%) were HCT patients. The healthy volunteers were on average younger, more likely to be female, and rarely had recent antibiotic exposure. In contrast, 184/192 (96%) of medical and haematology patients had received antibiotics in the past year, and 97 (51%) of these were receiving antibiotics at the time of sampling. Of those with antibiotic exposure in the past month, 99/148 (67%) had received >1 type of antimicrobial. Of 101 HCT participants in the cross-sectional analysis, 79 had >1 sequenced sample and so also contributed to the longitudinal analysis, and 173 sample pairs were included in this analysis. Bacteroidetes and Clostridia were predominant in most sequenced samples, and along with Actinobacteria, *Enterobacteriaceae* and *Enterococcus* accounted for a median 91% of classified organisms in baseline samples (Supplementary table 2). All these taxa were detected in all samples, apart from three samples with no detectable *Enterobacteriaceae*. The clinically significant AMR mechanisms included in this analysis were frequently detected: beta-lactamases in baseline samples from 67 (30%) patients, tetracycline AMR genes in 214 (95%), aminoglycoside transferases in 166 (74%), macrolide/clindamycin AMR genes in 210 (93%) and vanA in 31 (14%). Microbiome and resistome profiles of all samples are included in the Supplementary Data.

Impact of antimicrobial exposures on gut microbiome diversity

A half-life of 6 days was identified as the best fit for the antimicrobial exposure in the cross-sectional model of microbiome diversity, and this value was used for all subsequent analyses (Supplementary table 1). The independent effects of antimicrobial exposure on gut microbiome diversity in the cross-sectional and longitudinal models are shown in **Figure 1**. The cross-

sectional model (**Figure 1A**) provided more precise estimates of microbiome disruption than the longitudinal model, and four antimicrobial exposures were associated with a significant reduction in gut Shannon diversity: iv co-amoxiclav (−3.0 [95% Confidence Interval −4.7 to −1.4; p = 0.0005), iv piperacillin-tazobactam (−2.6, [−3.4 to −1.8]; p $<10^{-9}$), oral clindamycin (−2.2 [−3.5 to −0.8]; p = 0.002), and iv meropenem (−1.6 [−2.6 to −0.5]; p = 0.003). There was no evidence that the impact of iv co-amoxiclav was greater than iv meropenem (p=0.13). In contrast, co-trimoxazole and doxycycline were associated with minimal reductions in gut microbiome diversity, as were azole antifungals and acyclovir (lower 95% CI above −1.0). In the longitudinal analysis (**Figure 1B**), only five exposures had data from more than 20 sample pairs, and these were consistent with the cross-sectional results, including large reductions in diversity associated with exposure to piperacillin-tazobactam (−3.9 [−5.0 to −2.9]; p $<10^{-10}$) and meropenem (−3.0 [−4.1 to −1.9]; p $<10^{-6}$) (data on iv co-amoxiclav and oral clindamycin insufficient for comparison). In the longitudinal model, gentamicin was associated with marginally increased gut microbiome diversity (+6.6 [+1.3 to +11.8]; p = 0.02).

Analyses by antimicrobial class were also largely consistent between cross-sectional and longitudinal analyses (Supplementary Figure 4). In both analyses narrow spectrum beta-lactams were associated with substantially lower microbiome disruption than broad spectrum beta-lactams. In the cross-sectional analysis, several other classes of antibiotics also had little to no impact on the microbiome diversity; antifolates, macrolides, aminoglycosides and tetracyclines (lower 95% CI above −1.0).

Effects of antimicrobials on the abundance of specific taxa and AMR genes

Piperacillin-tazobactam, meropenem, and ciprofloxacin were associated with significant decreases in the relative abundance of *Enterobacteriaceae* in both models, as were ceftriaxone and ceftazidime in the cross-sectional model (>1,000 fold decreased relative abundance, p<0.01, **Figure 2** & Supplementary Figure 5). There was no evidence of effects of iv co-amoxiclav (p=0.90) or oral clindamycin (p=0.31) on relative abundance of *Enterobacteriaceae*. Piperacillin-tazobactam and meropenem were also associated with large increases in the relative abundance of *Enterococcus* in both models (>100 fold increased relative abundance, p<0.01), as were iv co-amoxiclav (p=0.04), iv ceftriaxone (p=0.02) and iv vancomycin (p=0.03) in the cross-sectional model. Many antibiotics were associated with large reductions in the relative abundance of major anaerobe groups, particularly Actinobacteria (reductions in which were associated with exposure to ceftazidime, oral ciprofloxacin, oral clindamycin, iv co-amoxiclav, meropenem, piperacillin-tazobactam, and iv vancomycin). When analysed by antimicrobial class, broad spectrum beta-lactams were associated with reductions all anaerobe groups (Supplementary Figure 5).

Several antimicrobial exposures were also associated with increases in the relative abundance of AMR genes (**Figure 3**, Supplementary Figure 6). In the cross-sectional analysis, iv piperacillin-tazobactam and meropenem were both associated with increases in several AMR mechanisms, including aminoglycoside transferases and macrolide resistance genes (all p<0.02) but there was no evidence that these antibiotics had any effect on overall relative abundance of beta-lactamases (p≥0.14), potentially reflecting lower abundance of relevant species (see above) but greater AMR gene carriage in those surviving. In the cross-sectional model, exposure to several classes of antimicrobials were associated with an increase in the abundance of corresponding resistance genes, including glycopeptides, tetracyclines, macrolides and clindamycin (all p≤0.02). Broad-spectrum beta-lactam use was not associated with an increase in the abundance of beta-lactamases, but it was associated with an increase in the abundance of glycopeptide and aminoglycoside AMR genes (both p≤0.0001). Increased aminoglycoside AMR gene abundance was also associated with clindamycin and glycopeptide use (both p≤0.02), but not aminoglycoside use.

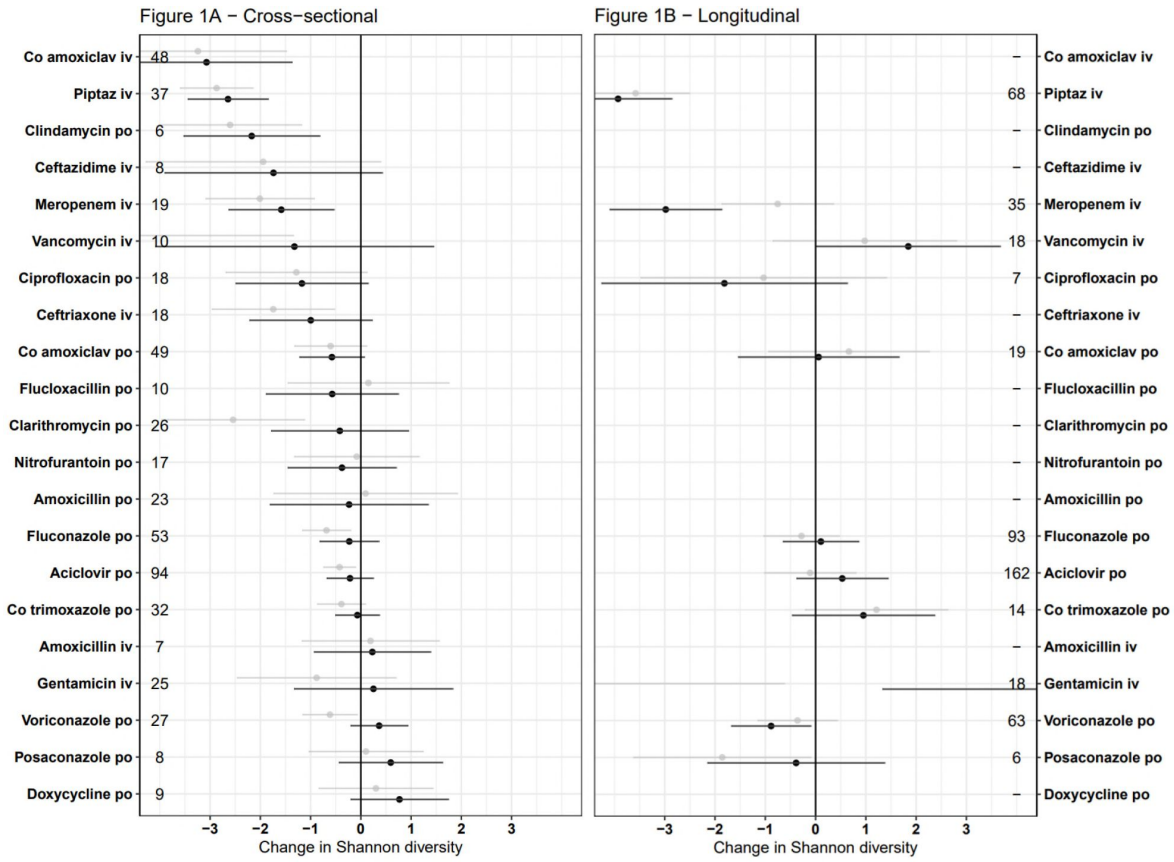

Figure 1

Independent effect of specific antimicrobial exposures on Shannon diversity in A) cross-sectional, and B) longitudinal analyses.

Multivariable estimates are in black, univariable (unadjusted) estimates in grey. Error bars represent 95% confidence intervals. Non-antimicrobial covariates are not shown but were included in the model and can be found in supplementary data. Results are not plotted for 4 antimicrobials (n = 6-12) that had standard errors <3 and did not differ significantly from zero. Estimates represent the impact of prolonged use, when exposure ≈ 1 (approximately 42 days, see Supplementary Figure 2).

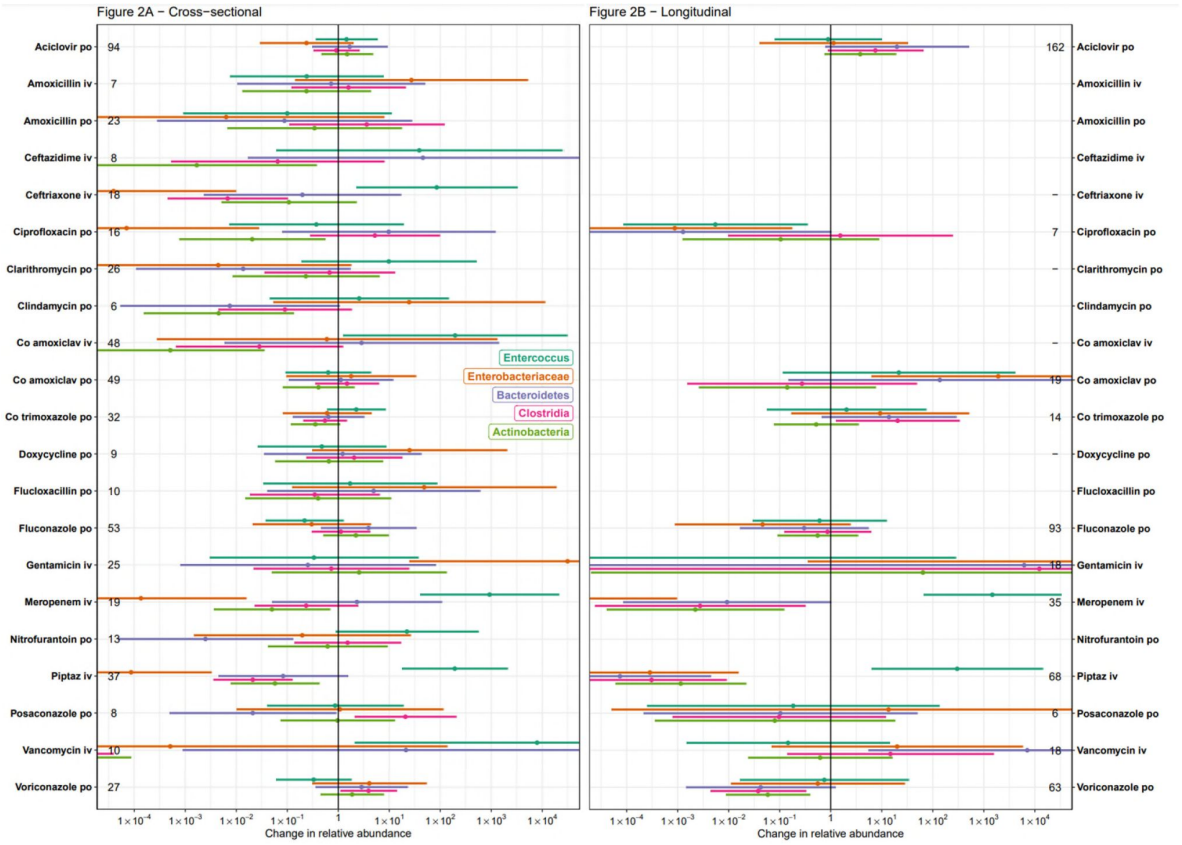

Figure 2

Independent effects of specific antimicrobial exposures on relative abundance of selected taxa in A) cross-sectional, and B) longitudinal multivariable analyses.

Error bars represent 95% confidence intervals. Non-antimicrobial covariates are not shown but were included in the model and can be found in supplementary data. Estimates represent the impact of prolonged use, when exposure ≈ 1 (approximately 42 days, see Supplementary Figure 2).

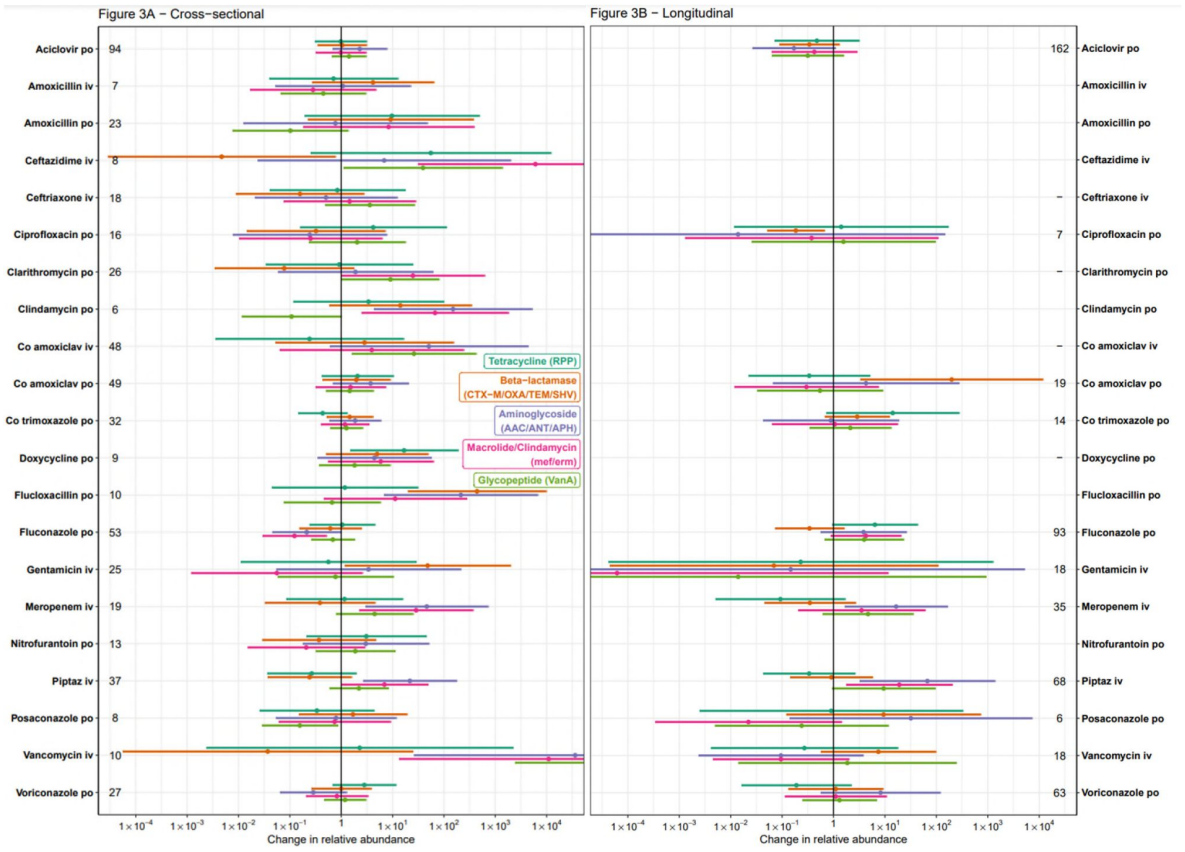

Figure 3

Independent effects of specific antimicrobial exposures on relative abundance of selected AMR genes in A) cross-sectional, and B) longitudinal multivariable analyses.

Error bars represent 95% confidence intervals. Non-antimicrobial covariates are not shown but were included in the model and can be found in supplementary data. Estimates represent the impact of prolonged use, when exposure ≈ 1 (approximately 42 days, see Supplementary Figure 2).

Discussion

Our findings demonstrate that simultaneous modelling of multiple antimicrobial exposures in a heterogeneous and heavily antibiotic exposed population can produce direct, quantitative comparisons of the impact of different agents on multiple aspects of the gut microbiome. Several broad-spectrum antibiotics were associated with large and rapid reductions in gut microbiome diversity, including decreased abundance of several major anaerobe groups, and increased abundance of *Enterococcus* species, along with glycopeptide and aminoglycoside resistance mechanisms often found in *Enterococcus faecium*.[5] The plausibility of these results is reinforced by the consistency between the two independent analysis frameworks that were used; cross-sectional and longitudinal.

Microbiome disruption was clearest with clindamycin and broad spectrum beta-lactams including iv co-amoxiclav, piperacillin-tazobactam, and meropenem, consistent with some previous microbiome studies of these drugs.[11–14] This is also in keeping with estimates of risk of *Clostridioides difficile* infection (CDI) which is highest with clindamycin, fluoroquinolones, carbapenems and third-generation cephalosporins.[15,16] The limited impact of doxycycline on diversity is also consistent with the lower risk of CDI observed with tetracyclines. The decreased relative abundance of *Enterobacteriaceae* seen in this study with piperacillin-tazobactam, ceftriaxone, meropenem, and ciprofloxacin correspond with reductions seen in culture based studies.[17] The absence of any clear effect of beta-lactams on the abundance of beta-lactamase gene abundance may be because there were too few patients in this study to create robust categories of beta-lactamase by resistance spectrum, so opposite impacts on different beta-lactamase genes would have been combined.

Observational studies of the gut microbiome allow large numbers of patients to be recruited much more easily than interventional studies such as clinical trials. However, using data from these to inform antibiotic usage is complicated by potential biases from confounding, and because of difficulties in accurately modelling multiple exposures in patients receiving many different antibiotics. In ARMORD, the apparent impact of vancomycin and clarithromycin on diversity was substantially reduced when adjusting for other antibiotic exposures (**Figure 1A**), in keeping with the fact that these drugs are often given alongside broad-spectrum beta-lactams in the UK. Nevertheless, the complexity of antibiotic exposure captured in observational data also more closely reflects real life, whereas clinical trials generally manipulate one single antibiotic, and restrict background antibiotic exposure to produce a cleaner, but potentially less generalisable, comparison.

The large degree of inter-individual variation in the gut microbiome introduces an additional source of variation to cross-sectional studies compared to longitudinal sampling. Despite this, cross-sectional sampling in ARMORD generally gave more precise estimates than longitudinal sampling, despite using a similar number of samples. Recruiting any hospitalised patients for longitudinal sampling before they have received antibiotics is challenging, and this was true in ARMORD, even among patients admitted electively for HCT. In ARMORD the quasi-experimental approach of longitudinal sampling starting before antimicrobial exposure had no advantage over cross-sectional sampling, and complicated recruitment.

The ARMORD study has important limitations. Short-read sequencing was used, meaning the genetic context of AMR genes is uncertain, for example if they are associated with mobile genetic elements or present in opportunistic pathogens.[23] Along with age and sex, several markers of acute illness and comorbidity were included in the model to adjust for potential confounding, but there may be residual and unmeasured confounding related to factors not adequately represented in the model. This could explain some inconsistencies in the effect of specific exposures between

the cross-sectional and longitudinal estimates, such as the impact of vancomycin on microbiome composition. The microbiome disruption half-life used was 6 days, as this value best fitted the overall data, which implies that the majority of the disruption and recovery of the bowel flora diversity occurs rapidly after starting and stopping antimicrobials (Supplementary Figure 2). This is in keeping with interventional studies in volunteers,[13,18] but is a simplification that does not account for some longer term impacts of antibiotic use that can be detected months after treatment, in particular the presence or absence of individual species or AMR genes.[19–21] A larger study would allow more complex exposure models to be explored, as well as more granular analyses at the level of individual species or resistance genes. Also, because the study was observational, no data were available on drugs that are not commonly used at OUH, including imipenem, cefepime and aztreonam, and few data were available for some other drugs, such as clindamycin, limiting the reliability of these estimates. The study focussed on participants with healthcare exposure, as it is this setting where antimicrobials are mostly likely to be used and where microbiome disruption or AMR selection are of most consequence. However, this limited its ability to assess the impact of some potentially relevant exposures that were uncommon in our population such as recent foreign travel, which is associated with the acquisition of AMR genes.[22] Finally, although we estimate the independent effects of multiple exposures, the uncertainty is still large making it challenging to use these results to adequately inform clinical use, as this would require clear distinction between antibiotics that might be given for the same indication. For example, the cross-sectional data are consistent with an identical average reduction in diversity with piperacillin-tazobactam, meropenem, ceftazidime, ceftriaxone, intravenous co-amoxiclav, ciprofloxacin and clindamycin, but they are also consistent with important differences between these drugs. A larger study is required to identify or rule out such differences.

Overall, however, simultaneous estimation of the impact of over 20 antimicrobials on the gut microbiome and AMR gene abundance highlighted important differences between individual drugs, and that some drugs in the WHO Access group (co-amoxiclav, clindamycin) had similar magnitude impact on microbiome diversity to those in the Watch group (meropenem, piperacillin-tazobactam) with potential implications for acquisition of other resistant organisms. The consistency of the ARMORD results between sampling frames and with previous studies supports the wider use of observational metagenomic studies to compare the impact of antimicrobials on the gut microbiome. Although some challenges remain, such as identifying an optimal measure of antimicrobial exposure, this is a practical approach to inform future research and stewardship.

Availability of data and code

The data and code used to produce this manuscript are available in the supplementary material, including processed microbiome data, and pseudonymised patient metadata. The sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB86785.

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Additional files

Supplementary appendix

Supplementary data

ARMORD R project

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Reviewer #2 (Public review):

Summary:

In this manuscript by Peto et al., the authors describe the impact of different antimicrobials on gut microbiota in a prospective observational study of 225 participants (healthy volunteers, inpatients and outpatients). Both cross-sectional data (all participants) and longitudinal data (subset of 79 haematopoietic cell transplant patients) were used. Using metagenomic sequencing, they estimated the impact of antibiotic exposure on gut microbiota composition and resistance genes. In their models, the authors aim to correct for potential confounders (e.g. demographics, non-antimicrobial exposures and physiological abnormalities), and for differences in the recency and total duration of antibiotic exposure. I consider these comprehensive models an important strength of this observational study. Yet, the underlying assumptions of such models may have impacted the study findings and residual confounding is likely. Other strengths include the presence of both cross-sectional and longitudinal exposure data and presence of both healthy volunteers and patients. Together, these observational findings expand on previous studies (both observational and RCTs) describing the impact of antimicrobials on gut microbiota.

Weaknesses:

(1) The main weaknesses result from the observational design. This hampers causal interpretation and makes correction for potential confounding necessary. While the authors have used comprehensive models to correct for potential confounders and for differences between participants in duration of antibiotic exposure and time between exposure and sample collection, I believe residual confounding is likely (which is mentioned as a limitation in the discussion).
For their models, the authors found a disruption half-life of 6 days to be the best fit based on Shannon diversity. Yet, the disruption caused by antimicrobials may be longer than represented in this model - as highlighted in the discussion.

(2) Another consequence of the observational design of this study is the relatively small number of participants available for some comparisons (e.g. oral clindamycin was only used by 6 participants). Care should be taken when drawing any conclusions from such small numbers.

Comments on revisions:

The authors have adequately addressed all of my comments.

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Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, the authors provide a study among healthy individuals, general medical patients and patients receiving haematopoietic cell transplants (HCT) to study the gut microbiome through shotgun metagenomic sequencing of stool samples. The first two groups were sampled once, while the patients receiving HCT were sampled*

> *longitudinally. A range of metadata (including current and previous (up to 1 year before sampling) antibiotic use) was recorded for all sampled individuals. The authors then performed shotgun metagenomic sequencing (using the Illumina platform) and performed bioinformatic analyses on these data to determine the composition and diversity of the gut microbiota and the antibiotic resistance genes therein. The authors conclude, on the basis of these analyses, that some antibiotics had a large impact on gut microbiota diversity, and could select opportunistic pathogens and/or antibiotic resistance genes in the gut microbiota.*
>
> *Strengths:*
>
> *The major strength of this study is the considerable achievement of performing this observational study in a large cohort of individuals. Studies into the impact of antibiotic therapy on the gut microbiota are difficult to organise, perform and interpret, and this work follows state-of-the-art methodologies to achieve its goals. The authors have achieved their objectives and the conclusion they draw on the impact of different antibiotics and their impact on the gut microbiota and its antibiotic resistance genes (the 'resistome', in short), are supported by the data presented in this work.*
>
> *Weaknesses:*
>
> *The weaknesses are the lack of information on the different resistance genes that have been identified and which could have been supplied as Supplementary Data.*

We have now supplied a list of individual resistance genes as supplementary data.

> *In addition, no attempt is made to assess whether the identified resistance genes are associated with mobile genetic elements and/or (opportunistic) pathogens in the gut. While this is challenging with short-read data, alternative approaches like long-read metagenomics, Hi-C and/or culture-based profiling of bacterial communities could have been employed to further strengthen this work.*

We agree this is a limitation, and we now refer to this in the discussion. Unfortunately we did not have funding to perform additional profiling of the samples that would have provided more information about the genetic context of the AMR genes identified.

> *Unfortunately, the authors have not attempted to perform corrections for multiple testing because many antibiotic exposures were correlated.*

The reviewer is correct that we did not perform formal correction for multiple testing. This was because correlation between antimicrobial exposures meant we could not determine what correction would be appropriate and not overly conservative. We now describe this more clearly in the statistical analysis section.

> *Impact:*
>
> *The work may impact policies on the use of antibiotics, as those drugs that have major impacts on the diversity of the gut microbiota and select for antibiotic resistance genes in the gut are better avoided. However, the primary rationale for antibiotic therapy will remain the clinical effectiveness of antimicrobial drugs, and the impact on the gut microbiota and resistome will be secondary to these considerations.*

We agree that the primary consideration guiding antimicrobial therapy will usually be clinical effectiveness. However antimicrobial stewardship to minimise microbiome disruption and AMR selection is an increasingly important consideration, particularly as

choices can often be made between different antibiotics that are likely to be equally clinically effective.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In this manuscript by Peto et al., the authors describe the impact of different antimicrobials on gut microbiota in a prospective observational study of 225 participants (healthy volunteers, inpatients and outpatients). Both cross-sectional data (all participants) and longitudinal data (a subset of 79 haematopoietic cell transplant patients) were used. Using metagenomic sequencing, they estimated the impact of antibiotic exposure on gut microbiota composition and resistance genes. In their models, the authors aim to correct for potential confounders (e.g. demographics, non-antimicrobial exposures and physiological abnormalities), and for differences in the recency and total duration of antibiotic exposure. I consider these comprehensive models an important strength of this observational study. Yet, the underlying assumptions of such models may have impacted the study findings (detailed below). Other strengths include the presence of both cross-sectional and longitudinal exposure data and the presence of both healthy volunteers and patients. Together, these observational findings expand on previous studies (both observational and RCTs) describing the impact of antimicrobials on gut microbiota.*
>
> *Weaknesses:*
>
> *(1) The main weaknesses result from the observational design. This hampers causal interpretation and corrects for potential confounding necessary. The authors have used comprehensive models to correct for potential confounders and for differences between participants in duration of antibiotic exposure and time between exposure and sample collection. I wonder if some of the choices made by the authors did affect these findings. For example, the authors did not include travel in the final model, but travel (most importantly, south Asia) may result in the acquisition of AMR genes [Worby et al., Lancet Microbe 2023; PMID 37716364). Moreover, non-antimicrobial drugs (such as proton pump inhibitors) were not included but these have a well-known impact on gut microbiota and might be linked with exposure to antimicrobial drugs. Residual confounding may underlie some of the unexplained discrepancies between the cross-sectional and longitudinal data (e.g. for vancomycin).*

We agree that the observational design means there is the potential for confounding, which, as the reviewer notes, we attempt to account for as far as possible in the multivariable models presented. We cannot exclude the possibility of residual confounding, and we highlight this as a limitation in the discussion. We have expanded on this limitation, and mention it as a possible explanation for inconsistencies between longitudinal and cross sectional models. Conducting randomised trials to assess the impacts of multiple antimicrobials in sick, hospitalised patients would be exceptionally difficult, and so it is hard to avoid reliance on observational data in these settings.

We did record participants' foreign travel and diet, but these exposures were not included in our models as they were not independently associated with an impact on the microbiome and their inclusion did not materially affect other estimates. However, because most participants were recruited from a healthcare setting, few had recent foreign travel and so this study was not well powered to assess the effects of travel on AMR carriage. We have added this as a limitation.

> *In addition, the authors found a disruption half-life of 6 days to be the best fit based on Shannon diversity. If I'm understanding correctly, this results in a near-zero modelled*

> *exposure of a 14-day-course after 70 days (purple line; Supplementary Figure 2). However, it has been described that microbiota composition and resistome (not Shannon diversity!) remain altered for longer periods of time after (certain) antibiotic exposures (e.g. Anthony et al., Cell Reports 2022; PMID 35417701). The authors did not assess whether extending the disruption half-life would alter their conclusions.*

The reviewer is correct that the best fit disruption half-life of 6 days means the model assumes near-zero exposure by 70 days. We appreciate that antimicrobials can cause longer-term disruption than is represented in our model, and we refer to this in the discussion (we had cited two papers supporting this, and we are grateful for the additional reference above, which we have added). We agree that it is useful to clarify that the longer term effects may be seen in individual components of the microbiome or AMR genes, but not in overall measures of diversity, so have added this to the discussion.

> *(2) Another consequence of the observational design of this study is the relatively small number of participants available for some comparisons (e.g. oral clindamycin was only used by 6 participants). Care should be taken when drawing any conclusions from such small numbers.*

We agree. Although our participants received a large number of different antimicrobial exposures, these were dependent on routine clinical practice at our centre and we lack data on many potentially important exposures. We had mentioned this in relation to antimicrobials not used at our centre, and have now clarified in the discussion that this also limits reliability of estimates for antimicrobials that were rarely used in study participants.

> *(3) The authors assessed log-transformed relative abundances of specific bacteria after subsampling to 3.5 million reads. While I agree that some kind of data transformation is probably preferable, these methods do not address the compositional data of microbiome data and using a pseudocount (10-6) is necessary for absent (i.e. undetected) taxa [Gloor et al., Front Microbiol 2017; PMID 29187837]. Given the centrality of these relative abundances to their conclusions, a sensitivity analysis using compositionally-aware methods (such as a centred log-ratio (clr) transformation) would have added robustness to their findings.*

We agree that using a pseudocount is necessary for undetected taxa, which we have done assuming undetected taxa had an abundance of 10^{-6} (based on the lower limit of detection at the depth we sequenced). We refer to this as truncation in the methods section, but for clarity we have now also described this as a pseudocount. Because our analysis focusses on major taxa that are almost ubiquitous in the human gut microbiome, a pseudocount was only used for 3 samples that had no detectable Enterobacteriaciae.

We are aware that compositionally-aware methods are often used with microbiome data, and for some analyses these are necessary to avoid introducing spurious correlations. However the flaws in non-compositional analyses outlined in Gloor et al do not affect the analyses in this paper:

(1) The problems related to differing sequence depths or inadequate normalisation do not apply to our dataset, as we took a random subset of 3.5 million reads from all samples (Gloor et al correctly point out that this method has the drawback of losing some information, but it avoids problems related to variable sequencing depth)

(2) The remainder Gloor et al critiques multivariate analyses that assess correlations between multiple microbiome measurements made on the same sample, starting with a dissimilarity matrix. With compositional data these can lead to spurious correlations, as measurements on an individual sample are not independent of other measurements made on the same sample.

In contrast, our analyses do not use a dissimilarity matrix, but evaluate the association of multiple non-microbiome covariates (e.g. antibiotic exposures, age) with single microbiome measures. We use a separate model for each of 11 specified microbiome components, and display these results side-by side. This does not lead to the same problem of spurious correlation as analyses of dissimilarity matrices. However, it does mean that estimates of effects on each taxa outcome have to be interpreted in the context of estimates on the other taxa. Specifically, in our models, the associations of antimicrobial exposure with different taxa/AMR genes are not necessarily independent of each other (e.g. if an antimicrobial eradicated only one taxon then it would be associated with an increase in others). This is not a spurious correlation, and makes intuitive sense when using relative abundance as outcome. However, we agree this should be made more explicit.

For these reasons, at this stage we would prefer not to increase the complexity of the manuscript by adding a sensitivity analysis.

> *(4) An overall description of gut microbiota composition and resistome of the included participants is missing. This makes it difficult to compare the current study population to other studies. In addition, for correct interpretation of the findings, it would have been helpful if the reasons for hospital visits of the general medical patients were provided.*

We have added a summary of microbiome and resistome composition in the results section and new supplementary table 2), and we also now include microbiome and resistome profiles of all samples in the supplementary data. We also provide some more detail about the types of general medical patients included. We are not able to provide a breakdown of the initial reason for admission as this was not collected.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) Provide a supplementary table with information on the abundance of individual genes in the samples.*

This supplementary data is now included.

> *(2) Engage with an expert in statistics to discuss how statistical analyses can be improved.*

A experienced biostatistician has been involved in this study since its conception, and was involved in planning the analysis and in the responses to these comments.

> *(3) Typos and other minor corrections:*
>
> *Methods: it is my understanding that litre should be abbreviated with a lowercase l.*

Different journals have different house styles: we are happy to follow Editorial guidance.

> *p. 9: abuindance should be corrected to abundance.*

Corrected

> *p. 9: relative species should be relevant species?*

Yes, corrected. Thank you.

> *p. 9 - 10: can the apparent lack of effect of beta-lactams on beta-lactamase gene abundance be explained by the focus on a small number of beta-lactamase resistance genes that are found in Enterobacteriaceae and which are not particularly prevalent, while other classes of resistance genes (e.g. Bacteroidal beta-lactamases) were excluded?*

It is possible that including other beta-lactamases would have led to different results, but as a small number of beta-lactamases in Enterobacteriaceae are of major clinical importance we decided to focus on these (already justified in the Methods). A full list of AMR genes identified is now provided in the supplementary data.

> *p. 10: beta-lactamse should be beta-lactamase*

Corrected

> *Figure 3A: could the data shown for tetracycline resistance genes be skewed by tetQ, which is probably one of the most abundant resistance genes in the human gut and acts through ribosome protection?*

TetQ was included, but only accounted for 23% of reads assigned to tetracycline resistance genes so is unlikely to have skewed the overall result. We limited the analysis to a few major categories of AMR genes and, other than VanA, have avoided presenting results for single genes to limit the degree of multiple testing. We now include the resistome profile for each sample in the supplementary data so that readers can explore the data if desired.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) Given the importance of obligate anaerobic gut microbiota for human health, it might be interesting to divide antibiotics into categories based on their anti-anaerobic activity and assess whether these antibiotics differ in their effects on gut microbiota.*

The large majority of antibiotics used in clinical practice have activity against aerobic bacteria and anaerobic bacteria, so it is not possible to easily categorise them this way. There are two main exceptions (metronidazole and aminoglycosides) but there was insufficient use of these drugs to clearly detect or rule out a difference between them, even when categorising antimicrobials by class, so we prefer not to frame the results in these terms. Also see our comments on this categorisation below.

> *(2) For estimating the abundance of anaerobic bacteria, three major groups were assessed: Bacteroidetes, Actinobacteria and Clostridia. To me, this seems a bit aspecific. For example, the phylum Bacteroidetes contains some aerobic bacteria (e.g. Flavobacteriia). Would it be possible to provide a more accurate estimation of anaerobic bacteria?*

We think that an emphasis on a binary aerobic/anaerobic classification is less biologically meaningful that the more granular genetic classification we use, and its use largely reflects the previous reliance on culture-based methods for bacterial identification. Although some important opportunistic human pathogens are aerobic, it is not clear that the benefit or harm of most gut commensals relates to their oxygen tolerance, and all luminal bacteria exist in an anaerobic environment. As such we prefer not to perform an additional analysis using this category. We are also not sure that this could be done reliably, as many of the taxa are characterised poorly, or not at all.

We appreciate that Bacteroidetes, Actinobacteria and Clostridia are diverse taxa that include many different species, so may seem non-specific, but these were chosen because:

i) they are non-overlapping with Enterobacteriaceae and Enterococcus, the major opportunistic pathogens of clinical relevance, so could be used in parallel, and

ii) they make up the large majority of the gut microbiome in most people and most species are of low pathogenicity, so it is plausible that their disruption might drive colonisation with more pathogenic organisms (or those carrying important AMR genes).

We have more clearly stated this rationale.

> *(3) A statement on the availability of data and code for analysis is missing. I would highly recommend public sharing of raw sequence data and R code for analysis. If possible, it would be very valuable if processed microbiome data and patient metadata could be shared.*

We agree, and these have been submitted as supplementary data. We have added the following statement “The data and code used to produce this manuscript are available in the supplementary material, including processed microbiome data, and pseudonymised patient metadata. The sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB86785.”

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