

# Gut microbiome-depleting antibiotic regimens are not tolerated by all mouse strains: learn from (our) bitter experience

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## ABSTRACT

Why the gut microbiome is critical for the success of checkpoint inhibitor cancer therapy is a question that remains unanswered, but progress has slowed. We argue that this lack of advancement is due to an unappreciated biological detail. Here, we show that the antibiotic cocktail used in seminal publications—all of which have used the C57BL/6 mouse strain—are bitter and not tolerated by other common mouse strains (ie, BALB/c and DBA/2). We write to alert readers of this important biological limitation that must be considered when planning cancer experiments investigating the gut microbiota, to prevent the unnecessary dehydration of experimental animals, and to save our colleagues valuable experimental time and resources.

## REPORT

The importance of commensal gut bacteria on the effectiveness of cancer immunotherapies has been well established in both mouse models<sup>1–3</sup> and patients.<sup>2–4</sup> Though these papers have fully established the gut microbiome's importance, none have adequately explained how the gut microbes influence therapeutic outcome. We have proposed a model dependent on bacteria translocating out of the colon and infecting the host, eventually colonizing a solid tumor, and promoting an adjuvant effect.<sup>5</sup> Others have highlighted the role of bacterial by-products, such as inosine and butyrate, which can modulate systemic immunity and improve the anti-cancer immune response.<sup>6–7</sup> Understanding this mechanism is critical for improving the success rate of checkpoint inhibitor therapy in patients with cancer. A viable mouse model that is amenable to having its gut flora modified is required to investigate these hypotheses and study the complex interactions between commensals, their host, and therapeutic outcome. We have attempted to make such a model but have encountered an unexpected experimental difficulty, and so wanted to publish our experience in this editorial to inform other researchers and encourage

further investigation into this fascinating topic.

Broad-spectrum antibiotics are the most accessible tool to modulate gut microbes. Several published papers have used this method, and a common solution has been a mix of 1 mg/mL colistin, 1 mg/mL ampicillin, and 5 mg/mL of streptomycin.<sup>2–7–8</sup> Our attempt to replicate these experiments failed with BALB/c mice because they refused to drink the antibiotic water and became severely dehydrated. We tried to overcome their aversion by flavoring the antibiotic-water but failed. We had some success at preventing dehydration by wetting food pellets with the antibiotic solution. However, we were ultimately obliged to end the experiment early due to ethical concerns for the animals' well-being.

To understand why we were unable to replicate previously published studies, we first questioned the published concentrations of the antibiotics. However, two independent laboratories had successfully used the same antibiotic solution to deplete the gut flora of their mice.<sup>2–7–9</sup> An alternative hypothesis was that different mouse strains may have different tolerances for bitter compounds. This was supported by finding literature describing how C57BL/6 mice had a higher tolerance for bitter compounds than both DBA/2 mice and BALB/c mice<sup>10–11</sup> and that all of the studies we were trying to replicate were indeed feeding their antibiotic solution *ad libitum* to only C57BL/6 mice. Tasting of the antibiotic water by one of the authors (expendable PhD student; without swallowing) confirmed the antibiotic water was extremely bitter.

To quickly evaluate whether there was a difference in tolerance to the antibiotic solution between C57BL/6 mice and BALB/c mice, we first compared tolerances for the individual antibiotic components at either



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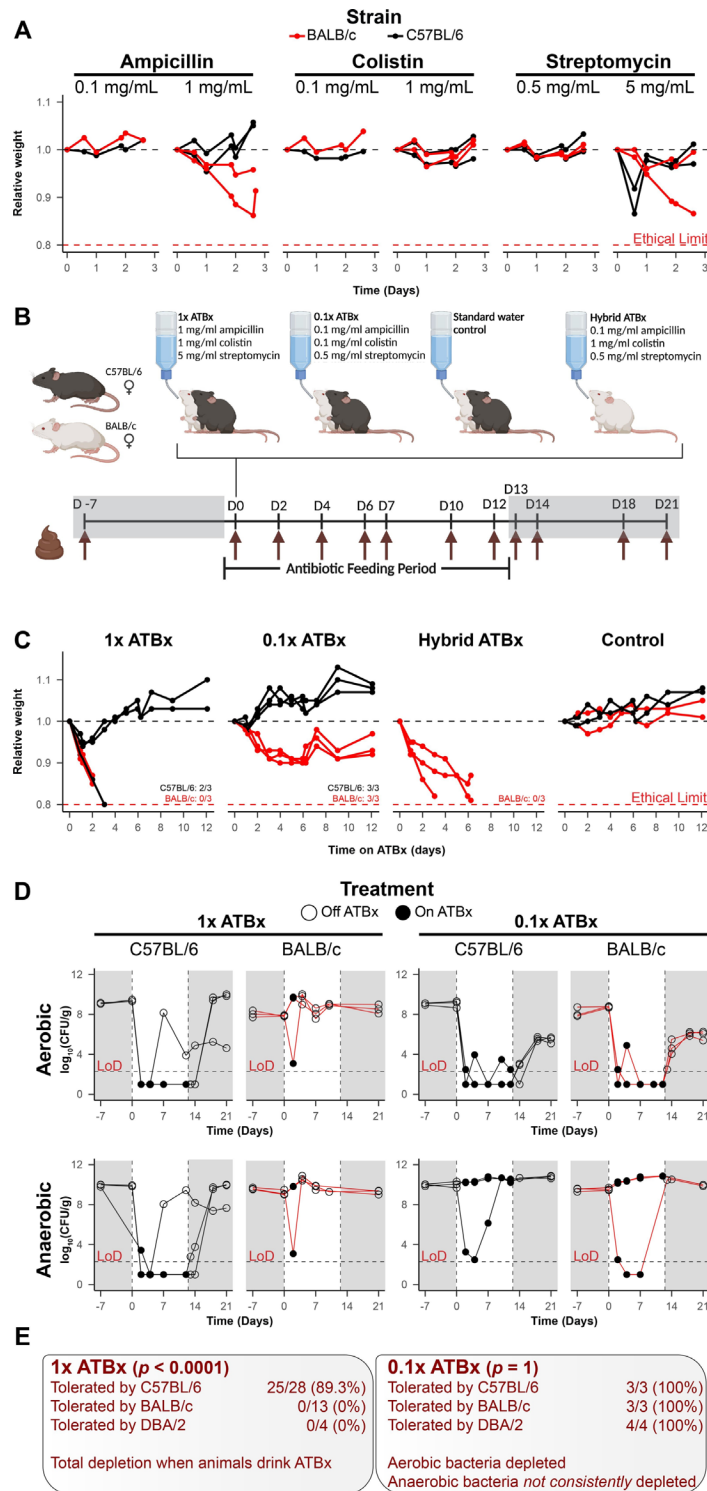
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**Figure 1** Antibiotic tolerance was tested in both BALB/c and C57BL/6 mice. (A, C) Relative weights of mice (red – BALB/c, black – C57BL/6), as a measure of antibiotic (ATBx) water intake. (A) Animals were fed individual antibiotics ad libitum at both the published and 1/10th of published concentrations. (B) Experimental design for C and D. (C) Effects of different antibiotic formulations fed ad libitum on weights of age-matched and sex-matched BALB/c and C57BL/6 mice ( $n=3$ , each group). Results in A and C are from two representative experiments. (D) Aerobic and anaerobic bacterial counts from fecal pellets collected from the same mice shown in C and plated on horse blood agar. Samples were collected when mice were fed standard water (open circles) and ATBx (closed circles). Linear mixed effects models of  $\log_{10}$ (CFA/g) with strain, day and bacteria type as fixed effects and mouse as random effect were fit for each antibiotic dose (1x and 0.1x). P values: 1x ATBx ( $p=0.006$ ) and 0.1x ATBx ( $p=0.95$ ) indicate a statistically significant effect of mouse strain on bacterial depletion only at the higher antibiotic dose. (E) A summary of our experience with feeding antibiotics to several common laboratory mouse strains, summary of five independent experiments. Fisher's exact test was used to compare strains ( $p < 0.0001$  and 1 at higher and lower antibiotic concentrations, respectively). Figure 1B was created in BioRender.com. CFU, colony forming unit; LoD; limit of detection,.

the published concentrations or at 1/10<sup>th</sup> the concentration over a 2.5-day period. We quickly observed that BALB/c mice did not reliably drink the published ampicillin and streptomycin concentrations but could tolerate 1/10th concentrations. Both strains could tolerate the published colistin concentration (figure 1A).

Building on the first experiment, we designed a second experiment with the aim of establishing whether the mice can tolerate all three antibiotics mixed at their individual maximum tolerable doses. Furthermore, we wanted to determine if these solutions were still effective at depleting the gut flora. Age-matched and sex-matched BALB/c and C57BL/6 mice were fed antibiotics over a 12-day period. To estimate the microbial load of each mouse, fecal samples were collected, diluted, and plated onto horse blood agar plates, incubated at 37°C in aerobic and anaerobic conditions, and the resulting colonies counted. These values were used to calculate the colony-forming units per gram of feces. Animals that approached or reached 80% of their initial bodyweight were removed to a separate cage with standard water. These animals regained all their lost weight within 24 hours.

As expected, the BALB/c mice needed to be removed from the published antibiotic concentrations within 48 hours, while two of three C57BL/6 mice reliably drank the solution (figure 1C). All animals tolerated the 1/10th concentration solution, but the colony counts showed the antibiotics were not of sufficient strength to deplete the anaerobic gut flora. A hybrid solution with the full dose of colistin but 1/10th of the published dose for ampicillin and streptomycin was tested on only BALB/c mice, but it was also not tolerated (figure 1C). We also conducted a series of similar experiments with DBA/2 mice and obtained comparable results in tolerances and bactericidal activity as observed in the BALB/c mice (data not shown).

From the experiments described above, it became apparent to us that C57BL/6 mice are best suited for feeding a broad-spectrum antibiotic solution containing 1 mg/mL ampicillin, 1 mg/mL colistin, and 5 mg/mL of streptomycin. Of the concentrations tested, it is the most effective at completely depleting the gut microbiome, but of the three strains we studied, C57BL/6 mice were the only strain tolerant of the bitter-tasting solution. It is worth noting that streptomycin is poorly absorbed by the gastrointestinal tract, thus oral administration is the optimal route for the effective depletion of gut microbes (colistin and ampicillin can be injected to achieve the same effect). Therefore, researchers should consider oral gavage or mixing antibiotics in the food if planning to use this antibiotic cocktail with BALB/c or DBA/2 mice in their gut microbiome studies.

Individual mouse strain differences have also been shown when the aim of antibiotic administration is to modulate rather than deplete intestinal bacteria.<sup>12–14</sup> Depending on the strain of mouse and antibiotics used, the effects on the gut microbiome can be distinct; the

same antibiotics alter the gut microbiomes of individual mouse strains differently, with proportions of some bacterial genera increasing in C57BL/6 mice while decreasing or remaining unchanged in BALB/c mice.<sup>12 13</sup> The reason for this is unclear, but may be due to the bias C57BL/6 mice have toward a Th1-type immune response, and BALB/c and DBA/2 mice have toward a Th2-style immune response.<sup>12 15 16</sup>

Elucidating the mechanism by which gut microbes impact cancer immunotherapies (and chemotherapies<sup>9 17</sup>) is critical for the improved treatment of patients with cancer. However, many important murine cancer models are not on the C57BL/6 background. Where the aim is complete depletion of the gut microbiome, C57BL/6 will tolerate the commonly used combination of ampicillin, colistin and streptomycin in their drinking water, while DBA/2 and BALB/c mice will not. We write to alert readers of this important technical limitation, to prevent the unnecessary dehydration of experimental animals, and to save our colleagues valuable experimental time and resources.

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