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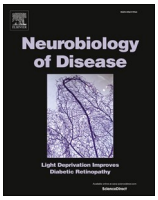

Exposure to anti-seizure medications impact growth of gut bacterial species and subsequent host response

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ABSTRACT

Anti-seizure medications (ASMs) are the first line of treatment for seizure control in children with epilepsy. Cumulative evidence suggests an imbalanced gut microbiota in refractory epilepsy patients. We systematically investigated the differential antimicrobial impacts of nine ASM active ingredients, seven common excipients of ASMs, and four syrup formulations on core early-life gut microbiota strains. Additionally, we evaluated the toxicity and gene expression profiles of HT-29 colon epithelial cells when exposed to active ingredients with or without bacterial supernatants. The physicochemical structure of ASM active ingredients and bacterial phylogeny were found to be related to ASM toxicity. Carbamazepine, lamotrigine, and topiramate reduced the growth of more than ten strains along with syrup excipient propyl-paraben. Various artificial sweeteners present in ASM formulations stimulated the growth of gut bacterial strains. The active ingredients that were more toxic to bacterial strains also exhibited toxicity towards HT-29 cells, yet *Bifidobacterium longum* supernatant reduced cytotoxic effects of carbamazepine and lamotrigine. *Akkermansia muciniphila* or mixed community supernatants reduced the expression of drug resistance genes in HT-29 cell lines. In summary, our results indicate that several ASM active ingredients and their excipients regulate the growth of gut bacterial strains in a species-specific manner. Interactions between ASMs and gut epithelial cells might be modulated by gut microbial metabolites.

1. Introduction

Epilepsy is one of the most prevalent neurological disorders that globally affect more than 70 million people (Thijs et al., 2019). Anti-seizure medications (ASMs) that have various mechanisms of action are used for seizure control as the first line of therapy. However, 30–40% of epilepsy patients do not respond to the minimum of two ASMs and hence, continue to experience seizures and other co-morbidities (Laxer et al., 2014). There has been a growing body of evidence that epilepsy patients also exhibit gastrointestinal dysbiosis (Dahlin and Prast-Nielsen, 2019), a condition that involves perturbations in the commensal gut microbiome. Profiling of the gut microbiome using next-generation sequencing techniques has depicted a peculiar gut microbiome composition in patients with epilepsy in comparison to healthy controls (Gong et al., 2020; Huang et al., 2019; Şafak et al., 2020). The differences were observed in both adult and child cohorts. These microbial disturbances in childhood are especially critical considering the link between gut microbiota and proper immune function and neurodevelopment (Stiemsma and Michels, 2018) at the early stages of life.

Animal models and child cohort studies both showed microbiome-based modulation of the anticonvulsant properties of the ketogenic diet in the resolution of drug-resistant epilepsy (Olson et al., 2018; Lindefeldt et al., 2019). Besides dietary modulation of the microbiome, a few case studies demonstrated the efficacy of antibiotic treatment in seizure control (Ghanizadeh and Berk, 2015; Cheraghmakani et al., 2021). Microbiome fingerprinting studies have also begun to reveal differences in the gut microbiota composition between drug-sensitive and drug-resistant epilepsy patients. For instance, Lee et al. observed a

Abbreviations: ASMs, Anti-seizure medications; API, Active pharmaceutical ingredient; SCFA, short-chain fatty acid; ROS, Reactive oxygen species.

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greater relative abundance of *Bacteroides* and *Ruminococcus* in drug-responsive patients and an overrepresentation of members of the *Negativicutes* group in drug-resistant epilepsy patients (Lee et al., 2021b). A greater alpha-diversity, determined by the number of unique microbial types, was described in drug-resistant patients in comparison to drug-sensitive patients (Peng et al., 2018). The same study reported an overrepresentation of gut commensals including *Akkermansia*, *Dorea*, *Coprococcus*, and *Roseburia* and pathobionts including *Fusobacterium* and *Atopobium* in drug-resistant patients. Similarly, the role of the gut microbiome in ASM efficacy was mechanistically addressed in a mice model (BALB/c AnNHsd male mice) in which intestinal inflammation decreased valproic acid treatment efficacy and short-chain fatty acids (SCFA) treatment reduced the inflammation and hence increased valproic acid efficacy (De Caro et al., 2019). In contrast, valproic acid treatment in a rat model reduced cecal levels of two important SCFAs (propionate and butyrate) along with an increase in the relative abundance of *Clostridia* and *Eubacterium* species (Cussotto et al., 2019).

Advancements in the field of the gut microbiome–drug interactions, as well as high-throughput screening approaches, have revealed an unexpected, yet a wide effect of human-targeted medications on gut microbiome structure and individual bacterial strains (Maier et al., 2018; Maurice et al., 2013). Additionally, common food and drug additives including artificial sweeteners were previously shown to alter gut microbiome composition and function in batch-culture systems (Gerasimidis et al., 2020). These studies revealed that human targeted medications or drug additives may have anti-commensal effects by altering the gut microbiome. In terms of ASMs' impact on the bacterial strains, *in vitro* studies are limited to i) a few drugs at lower than estimated gut concentration on selected bacterial species (Maier et al., 2018), ii) valproic acid on *Lactobacillus rhamnosus* and *Escherichia coli* (Cussotto et al., 2019) and iii) lamotrigine on aerobic strains such as *Staphylococcus aureus* and *Bacillus subtilis* (Qian et al., 2009).

The daily use of ASMs administered orally might induce shifts in the gut microbiota of patients with epilepsy including young children. In the present study, we address the anti-commensal effects of commonly prescribed ASM active ingredients, ASM drug formulations, and their excipients on core bacterial species found in childrens' gut microbiome. Furthermore, we assess host response to the ASMs with or without bacterial metabolites on an intestinal epithelial model. Unwinding the bacterial strain and host response to complex ASM formulation *in vitro* will provide further insights into the ASM mechanisms, their unknown "microbial" side effects, and the potential impact of drug formulation.

2. Material and methods

Species-specific microbial growth impact of ASMs, formulations, and excipients.

2.1. Chemicals considered in the study and experimental set-up

Anti-seizure drugs (valproate, carbamazepine, phenytoin, gabapentin, ethosuximide, lamotrigine, lacosamide, levetiracetam, and topiramate) and the common drug excipients (methyl-paraben, propyl-paraben, sorbitol, maltitol, aspartame, acesulfame K, and polyethylene glycol) were purchased from Sigma Aldrich and Merck (Suppl. Table 1). The following syrups were used: carbamazepine (Tegretol®, Novartis), lacosamide (Vimpat®, UCB Pharma), levetiracetam (Keppra®, UCB Pharma), and valproate (Depakine® 200 mg/mL, Sanofi). Carbamazepine, lacosamide, lamotrigine, topiramate, phenytoin, and parabens were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mM for stock solutions whereas the rest of the chemicals were dissolved in di-water. The experimental set-up was based on Maier et al. with modifications (Maier et al., 2018). The chemicals were subsequently diluted in the modified Gifu Anaerobic Medium (mGAM) at a final concentration of 200 μM, 1000 μM, or 2000 μM and distributed into 96-well plates in triplicates. The final concentration of DMSO was <1%, lower than the previously tested concentration for microbial toxicity and did not influence the growth of the strains. The plates were stored at −20 °C for less than 1 week. Before inoculation with bacterial strains, the plates were thawed and pre-reduced in the anaerobic chamber for >10 h.

Table 1
The primary excipient content of commonly prescribed ASM syrups.

	Tegretol®	*Depakine®*	*Vimpat®*	*Keppra®*
Active Ingredient	Carbamazepine	Valproate	Lacosamide	Levetiracetam
Methyl-paraben	1.2 mg/mL	0.10 mg/mL	2.6 mg/mL	2.7 mg/mL
Propyl-paraben	0.3 mg/mL	0.02 mg/mL	X	0.3 mg/mL
Acesulfame K	X	X	+	+
Maltitol	X	X	X	300 mg/mL
Sorbitol	175 mg/mL	100 mg/mL	187 mg/mL	X
Aspartame	X	X	0.032 mg/mL	X
PEG	X	X	2.14 mg/mL	X

[x]The syrup does not contain the excipient. [+]The syrup contains the excipient; however, the concentration was not reported.

2.2. Bacterial strains, growth conditions, and collection of bacterial supernatants

Twenty-seven bacterial strains were selected to represent the core bacterial diversity of healthy infant and young children gut microbiome [0–6 months, $n = 277$ (Casaburi et al., 2021), 0–6 yrs., $n = 227$ (Stewart et al., 2018), 6–9 yrs. of age, $n = 281$ (Zhong et al., 2019] and 5 strains were selected based on their over-representation in the gut microbiome of patients that resist antiepileptic therapies (Zhang et al., 2018; Peng et al., 2018; Lindefeldt et al., 2019; Lee et al., 2021b) (Suppl. Table 2). The strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and were initially grown in their recommended rich medium and mGAM media. *Veillonella parvula* and *Akkermansia muciniphila* were grown with the addition of sodium lactate and mucin to the mGAM medium, respectively. The frozen stocks of each strain were prepared in 20% glycerol-mGAM and stored at −80 °C. Frozen stocks of selected strains and from a mixed gut microbial community were resuscitated in the mGAM media twice to ensure robust growth using the Hungate method. Mixed community originates from several passages in mGAM media of healthy fecal samples ($n = 3$, <3yo). The strains or community were grown in mGAM media for 24 h at 37 °C and supernatants were centrifuged at 4500 xg for 15 min to pellet the bacterial biomass. The supernatants were filtered through a Nalgene syringe filter with 0.2 μM polyethersulfone membrane, aliquoted immediately, and kept frozen at −20 °C until the experiments.

2.3. Screening of ASM active compound, ASM formulation, and its excipients on microbial growth

Before experimentation, the strains were grown in mGAM media twice and the inoculum density was adjusted to 0.5. Then 8 μL of culture were added to achieve a starting OD of 0.02 in pre-reduced plates that contain ASMs, excipients, or formulation with matching concentration of the active ingredient. The plates were incubated without shaking. The growth of the strains was monitored by measurement of OD at 578 nm with a microplate reader (Infinite® 200 plate reader, TECAN). All experiments were set up under anaerobic conditions at 37 °C in Bactron 600 anaerobic chamber (Coy laboratory products inc.) and performed in triplicates.

2.4. In silico characterization of APIs and bacterial strains

The drug APIs were classified based on their physicochemical

properties using the ChemMine Tools website (https://chemminetools.ucr.edu). The genomic content inferred pathways, and enzymatic families of the strains tested were analyzed using the MetaCyc database (https://metacyc.org).

ASMs and microbial impact on intestinal host response.

2.5. Cell culture of human colonic epithelial cells

The human epithelial cell line HT-29 was obtained from American Type Culture Collection (ATCC, Rockville, MD). The growth and maintenance conditions of the HT-29 cell line were previously described (Marinelli et al., 2019). Briefly, cells were grown in RPMI 1640 GlutaMAX™ media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Eurobio), 50 U/mL penicillin/streptomycin, 1% Hepes, 10 mM nonessential amino acids, 1% pyruvate, and 1% glutamate. The cell culture was maintained in a humidified 5% CO_2 37 °C incubator. The cells were passaged every 2–3 days. MycoAlertTM Mycoplasma Detection Kit (Lonza) was used to check for mycoplasma contamination.

2.6. Cell culture experiment set-up

Before seeding the experimental plates, the cells were passaged twice in antibiotic-free media. For gene expression experiments, cells were seeded in 12-well culture plates at a density of 4.5×10^5 cells per well in antibiotic-free media and incubated overnight before treatment with the ASMs or bacterial supernatants. This optimized density provided 80–90% confluency.

2.7. Gene expression assays

Following the treatment with the ASMs and bacterial supernatants for 24 h, the total RNA was collected and extracted using RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. The in-column DNA depletion was performed using RNase-Free DNase Set (Qiagen). The quality and quantity of the RNA were verified with a nanodrop ND-1000 spectrophotometer (Thermo Scientific) at the wavelength of 260 nm. 1 μg of RNA was used for cDNA synthesis with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instructions. The obtained cDNA samples were subjected to qPCR assays.

2.8. Real-time PCR analysis

The gene expression profiles of HT-29 cells treated with ASM APIs and/or bacterial supernatants were determined using Sybr Green chemistry (PowerSYBR Green Master Mix, Applied Biosystems). Expression patterns of inflammatory markers (*IL1β and TNFα*), oxidative stress and membrane integrity (*sod1* and *ZO1*), and drug transport (*MDR1*, *MVP*, *MRP1*) genes were monitored using the primers reported in Suppl. Table 3. The qPCR recipe included 1× Sybr Green Master Mix, and 10 μM of each forward and reverse primers. The qPCR was performed using QuantStudio 3 (Applied Biosystems) under the following conditions: 5 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 25 s at 60 °C, and 45 s at 72 °C, and then melting curve analysis.

2.9. Cell viability and total reactive oxygen species assays

For toxicity assays, the HT-29 cells were seeded in 96 well plates at a density of 3.5×10^4 cells per well. Cell proliferation was evaluated by MTS measurements using the CellTiter 96 Aqueous One solution (Promega). Cell viability was determined based on the reduction intensity of tetrazolium compounds by the epithelial cells based on absorbance at 490 nm wavelength by spectroscopy (TECAN Infinite 200). The cellular reactive oxygen species assay (ROS) was evaluated with DCFDA/H2DCFDA ROS kit (Abcam). The detection of 2′, 7′-dichlorofluorescein (DCF) was quantified by fluorescence spectroscopy (TECAN) with excitation/emission at 485 nm/535 nm. The HT-29 cells were fixed with ice-cold methanol (96%) for ten minutes, stained with crystal violet (4%) for 10 min, and then washed with water several times prior to imaging with a light microscope (Olympus) at 4× magnification. The screenings were performed in triplicates.

2.10. Statistical analysis

All the statistical analyses were performed with GraphPad Prism v9. For normally distributed data, Student's *t*-test and ANOVA with Tukey's multiple comparisons and non-normally distributed data, Mann-Whitney's *U* test were performed. Principal coordinate analysis was performed based on Euclidean distances among the bacterial communities. Pearson's correlation coefficient was calculated to estimate the strength of the relationship among the variables.

3. Results

3.1. Anti-seizure medications modulate the growth of key members of the gut microbiome in a species-specific manner

We systematically monitored the growth of 32 representative bacterial strains of children's microbiota that were grown in the presence of active pharmaceutical ingredients (API) of the ASMs at three selected concentrations (200, 1000, and 2000 μM). The strains all grew in the mGAM media at various maximum growth rates that were categorized as low, medium, and high based on average growth and 25th quartiles (Suppl. Table 4). The concentration 200 μM did not induce any detectable proliferation or inhibition on the growth of the strains, whereas the impact was more prominent with increasing concentration (1000 and 2000 μM) (Supp. Fig. 1, Suppl. Table 5). As shown in Fig. 1A, the vehicle DMSO for topiramate, carbamazepine, lamotrigine, lacosamide, and phenytoin solubilization did not impact the growth of strains at the final volume administered (<1%). The APIs carbamazepine, lamotrigine, and phenytoin inhibited at least 25% of the growth of 10, 19, and 12 strains, respectively at 2000 μM. Even though APIs reduce the stationary phase growth of the majority of the strains, *Staphylococcus caprae*, *Dorea longicatena*, *E. coli*, and *Klebsiella aerogenes* exhibited higher growth with topiramate, carbamazepine, lacosamide, and phenytoin in comparison to the control.

Based on physicochemical properties, hydrophilic APIs (levetiracetam, gabapentin, valproate, and ethosuximide) and hydrophobic APIs (topiramate, carbamazepine, lamotrigine, lacosamide, and phenytoin) formed two clusters. Hydrophobic APIs collectively reduced the growth of strains in comparison to hydrophilic APIs (Student's *t*-test $P = 0.0002$) (Fig. 1B). Among hydrophobic APIs, lamotrigine exhibited toxicity on a greater number of strains followed by carbamazepine in comparison to other tested APIs.

As the toxicity of antimicrobials on microorganisms often relies on bacterial membrane properties, we subsequently investigated whether APIs had selectivity based on bacterial cell wall constituents defined by Gram staining. As depicted in Fig. 1C, carbamazepine and lamotrigine had opposing effects on the growth of Gram (+) and Gram (−) strains; Gram (−) strains grew significantly less than Gram (+) strains in the presence of carbamazepine (Student's *t*-test $P = 0.03$). In contrast, lamotrigine had greater toxicity on both Gram (+) and Gram (−) strains than carbamazepine; however, the impact was more prominent on Gram (+) strains.

Considering that cell wall differentiation involves evolutionary processes, we clustered the growth patterns of microorganisms based on their phylogeny. Principal component analysis shows the Euclidean distances among the strains based on their growth rates in the presence of 9 APIs of the ASMs (Fig. 1D). Strains belonging to Bacteroidetes phylum that were mainly Gram (−) were significantly distant from the strains from Firmicutes phylum that are mainly Gram (+) on PC2 (Student's t-test $P = 0.0017$) that explained the second-highest variation

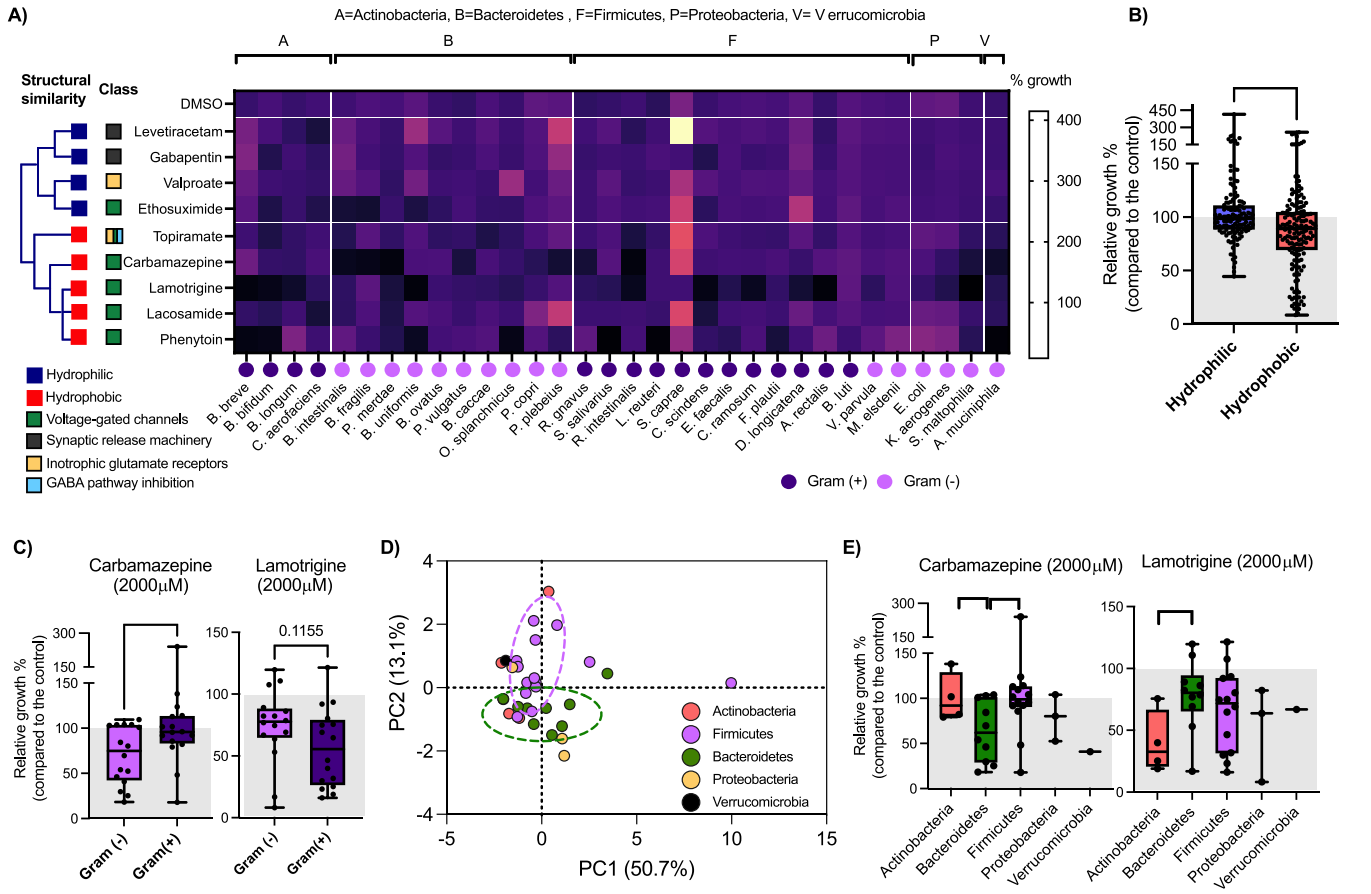

Fig. 1. Active ingredients of ASMs altered the growth of common gut commensals strains. A) Heatmap of 32 gut isolates growth patterns when treated with 2000 μM of ASMs in comparison to the control. Chemical structure of ASMs determined inhibition or proliferation of strains. B) Bacterial strains had lower growth when incubated with hydrophobic ASMs (topiramate, carbamazepine, lamotrigine, lacosamide, and phenytoin) in comparison to hydrophilic ASMs. ***Student's t-test $P = 0.0002$. C) The inhibitory impact of carbamazepine and lamotrigine on microbial strains dissected by cell wall properties described by Gram staining. D) Principal Component Analysis (PCA) of the strains exposed to the ASMs color-coded based on phylum level phylogeny. E) Relative growth of strains grouped based on phylum level under treatment with carbamazepine and lamotrigine. Hydrophobic and hydrophilic ASMs ae defined as poor and excellent solubility in water, respectively.

within the dataset. Similar to the results observed by Gram staining properties, carbamazepine had a greater inhibitory impact on Bacteroidetes strains than on Firmicutes and Actinobacteria strains (Student's t-test $P = 0.024$ and $P = 0.011$). Interestingly, lamotrigine inhibited Actinobacteria more than strains of Bacteroidetes (Student's *t*-test $P = 0.026$).

Besides the cellular membrane and wall properties, several known and unknown mechanisms may contribute to the influence of APIs on microbial growth. To decipher the potential role of microbial genetic makeup on growth under physicochemical constraints including compounds with antimicrobial properties, we extracted the reactions, pathways, and metabolism deduced from full sequences present at the MetaCyc database (Suppl. Table 6). The number of genes involved in detoxification, transport, cell structure biosynthesis, and inorganic nutrient metabolism showed a moderate correlation with growth in the presence of lamotrigine, lacosamide, and phenytoin (Suppl. Fig. 2) (Spearman's correlation coefficient 0.3–0.4, $P < 0.05$).

Overall, the APIs of ASMs had a species-specific effect on microbial growth, however, phylogeny including the cell wall structure in combination with physicochemical properties of the APIs had a confounding impact on the growth response of the strains.

3.2. *Drug formulations induce API-independent response on microbial growth*

ASMs are mainly prescribed to children in syrup formulations. We comparatively evaluated the growth of microbial strains exposed to the most commonly prescribed four ASMs (Tegretol®, Depakine®, Keppra®, and Vimpat®) for children relative to their APIs at the same concentration (2000 μM). Based on Pearson's correlation coefficient, microbial growth patterns weakly correlated with their APIs. The correlation among the APIs was significant and moderate to high (Pearson's correlation coefficient, 0.5. – 0.8; $P < 0.05$) (Fig. 2A). As shown in Fig. 2B, the majority of the strains exhibited different growth rates when exposed to syrups or APIs. In the case of Tegretol®, the strains grew at a greater OD with the active ingredient carbamazepine. In contrast, many tested strains grew at a higher density with Keppra® than its API, levetiracetam. However, the difference was less than 5% on average. In comparison to Keppra®, a smaller number of strains ($n = 12$) overgrew with Vimpat®, yet, the magnitude was greater for those strains. Among the syrups, Depakine® had the least variation from its active ingredient valproate which did not induce a significant overgrowth or undergrowth for the majority of the strains.

Given that phylogeny had a considerable effect on API-mediated growth, we analyzed the phylum-level distribution of strains under each syrup treatment. Tegretol inhibited strains from every phylum, though the impact was more drastic for Actinobacteria strains (Fig. 2C). Vimpat slightly inhibited Proteobacteria strains whereas it promoted the growth of the majority of Bacteroidetes and Firmicutes strains. Keppra® increased the growth of all phyla, the impact was greater for Firmicutes and Bacteroidetes.

At the species level, the inhibitory effect of Tegretol® formulation

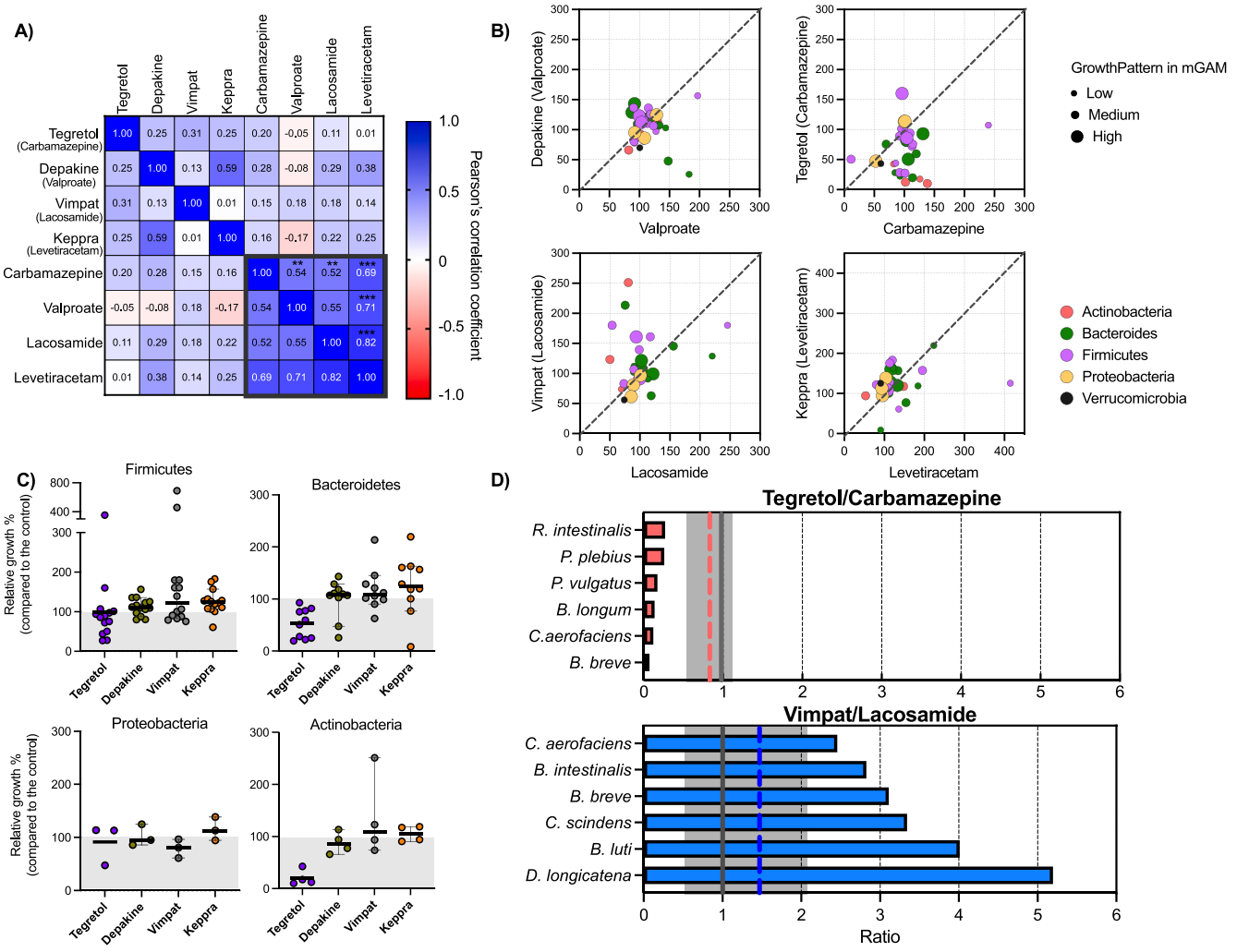

Fig. 2. Drug formulations had diverse and varying effects on growth on gut commensals. A) Dissimilarities between drug formulation and active ingredients based on Pearson's correlation coefficients showed active ingredients had a more similar impact on microbial growth than relative to their formulated versions. B) Scatter plot analysis of active ingredients (valproate, carbamazepine, lacosamide, and levetiracetam) *versus* their formulations showed that Tegretol® had more inhibitory effect than its active ingredient carbamazepine whereas Vimpat®, Keppra®, and Depakine® formulations stimulated the growth of various strains from Actinobacteria and Firmicutes phylum compared to their active ingredients. C) The impact of drug formulations presented by phylum. Tegretol® was more inhibitory to the members of Actinobacteria than the other phylum. Proteobacteria was the least impacted by drug formulations. Depakine®, Vimpat®, and Keppra® had growth stimulatory impacts on Bacteroidetes and Firmicutes strains. D) The ratio of growth in Tegretol® *versus* carbamazepine and Vimpat® *versus* lacosamide for the strains that were impacted most. The syrup Tegretol® was particularly toxic to Actinobacteria strains (*Bifidobacterium longum*, *Collinsella aerofaciens*, and *B. breve*). Syrup Vimpat® had an excessive stimulatory impact on *Dorea longicatena* and *Blautia luti* followed by *Clostridium scindens, B. breve*, *Bacteroides intestinalis*, and *C. aerofaciens*.

was greatest for *Roseburia intestinalis*, *Phocaeicola plebeius*, *Phocaeicola vulgatus*, *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Bifidobacterium breve* in comparison to API carbamazepine. The average ratio of Tegretol® to carbamazepine was 0.8 (± 0.3) when all strains were considered and for the strains shown in Fig. 2D, the ratio varied from 0.07 to 0.270 corresponding to 3.7-to-13.8-fold reduction. The growth-promoting impact of Vimpat® was independent of its API lacosamide and was more prominent for *Dorea longicatena*, *Blautia luti*, *Clostridium scindens*, *B. breve*, *Bacteroides intestinalis*, and *C. aerofaciens* with a range of 2.4-to-5.2-fold increase in growth. The average Vimpat® to lacosamide ratio was 1.4 (±1.0), slightly over one which would indicate equal growth. *D. longicatena* grew best with Vimpat®, 6.8-fold greater than the control and 5.2-fold more in comparison to the API lacosamide. Our results indicate that ASM syrups distinctly modulate the growth of gut bacteria that might exacerbate or dampen the impact of their APIs.

3.3. Excipients present in common ASM syrups have a dose-dependent growth effect

The ASM syrup formulations contain various excipients that are considered to be inert and are to increase the shelf-life, palatability, and stability of the products. Given that we observed differences in microbial growth with ASM syrups in comparison to their APIs, we examined the inhibitory or enhancing role of common individual ASM syrup excipients on our bacterial library. As shown in Table 1, methyl-paraben and propyl-paraben were present in all the syrups besides propyl-paraben was absent in Vimpat®. The syrups also contained a variety of artificial sweeteners including acesulfame K, maltitol, sorbitol, aspartame. Among the syrups, Vimpat® contained three sweeteners (acesulfame K, sorbitol, and aspartame), Keppra® contained two, Tegretol® and Depakine® each contained one.

Among the parabens, methyl-paraben had a smaller antimicrobial effect on the strains in comparison to propyl-paraben. At 2000 μM, propyl-paraben inhibited the growth of 29 strains out of 32 by at least

25% (19.5% ± 26.45). At the same concentration, methyl-paraben inhibited 15 strains by at least 25% (76.4% ± 49.80) (Fig. 3A). When the strains were subjected to 10 times lower concentrations of the parabens, propyl-paraben inhibited 12 strains and methyl-paraben inhibited 9 strains by at least 25%. *A. muciniphila*, *Odoribacter splanchnicus*, *Prevotella copri*, *Flavonifractor plautii*, *Roseburia intestinalis,* and *Parabacteroides merdae* were more susceptible to parabens than the other strains (Fig. 3B). They exhibited limited growth in comparison to the control even at 200 μM. The growth of those strains was also inhibited by Tegretol®, the syrup with the highest antimicrobial activity.

Besides parabens, the syrup formulations contained several artificial sweeteners. Unlike the case of parabens, the growth patterns of the strains were similar when the strains were subjected to 200 μM or 2000 μM of artificial sweeteners as shown in Fig. 3C. The growth of strains was enhanced especially with sorbitol at 2000 μM concentration. Several strains including *D. longicatena*, *Bacteroides intestinalis*, *S. caprae*, and *Megasphaera elsdenii* exhibited greater growth at 2000 μM of the sweeteners in comparison to control or 200 μM preparations. The growth of these strains showed a similar trend to Vimpat® which contained a greater number of sweeteners and also stimulated the growth of the abovementioned strains (Fig. 3D).

3.4. Carbamazepine and lamotrigine reduced cell proliferation and induced the expression of drug resistance genes in human colon epithelial cells

We evaluated the potential impact of ASM APIs on colon epithelial cell proliferation by exposing the HT-29 cells to 9 APIs for 24 h and quantifying the cellular response using the MTS assay. As shown in Fig. 4A, lamotrigine and carbamazepine significantly reduced cell proliferation in comparison to vehicle control; lamotrigine had a more potent impact than carbamazepine. In contrast, gabapentin and phenytoin increased cell proliferation in comparison to the control. Crystal violet staining of the cells following incubation with the APIs confirmed the toxic impact of carbamazepine and lamotrigine on the epithelial cell model (Fig. 4B). Since many APIs induce oxidative stress on cells, we then investigated whether ASM APIs led to the release of reactive oxygen species (ROS). We tracked the generation of ROS when the cells were exposed to the APIs. Among the APIs, topiramate and lacosamide significantly induced the generation of ROS (1.5-to-2-fold increase in comparison to the control). Lamotrigine that exhibited cytotoxic activity also increased ROS production, however not at a level that was significantly different than the control. Overall, the cytotoxicity and ROS generation did not correlate, although cell proliferation assays were based on 24 h and ROS assays were done with six-hour of exposure due to the limitations of the assay.

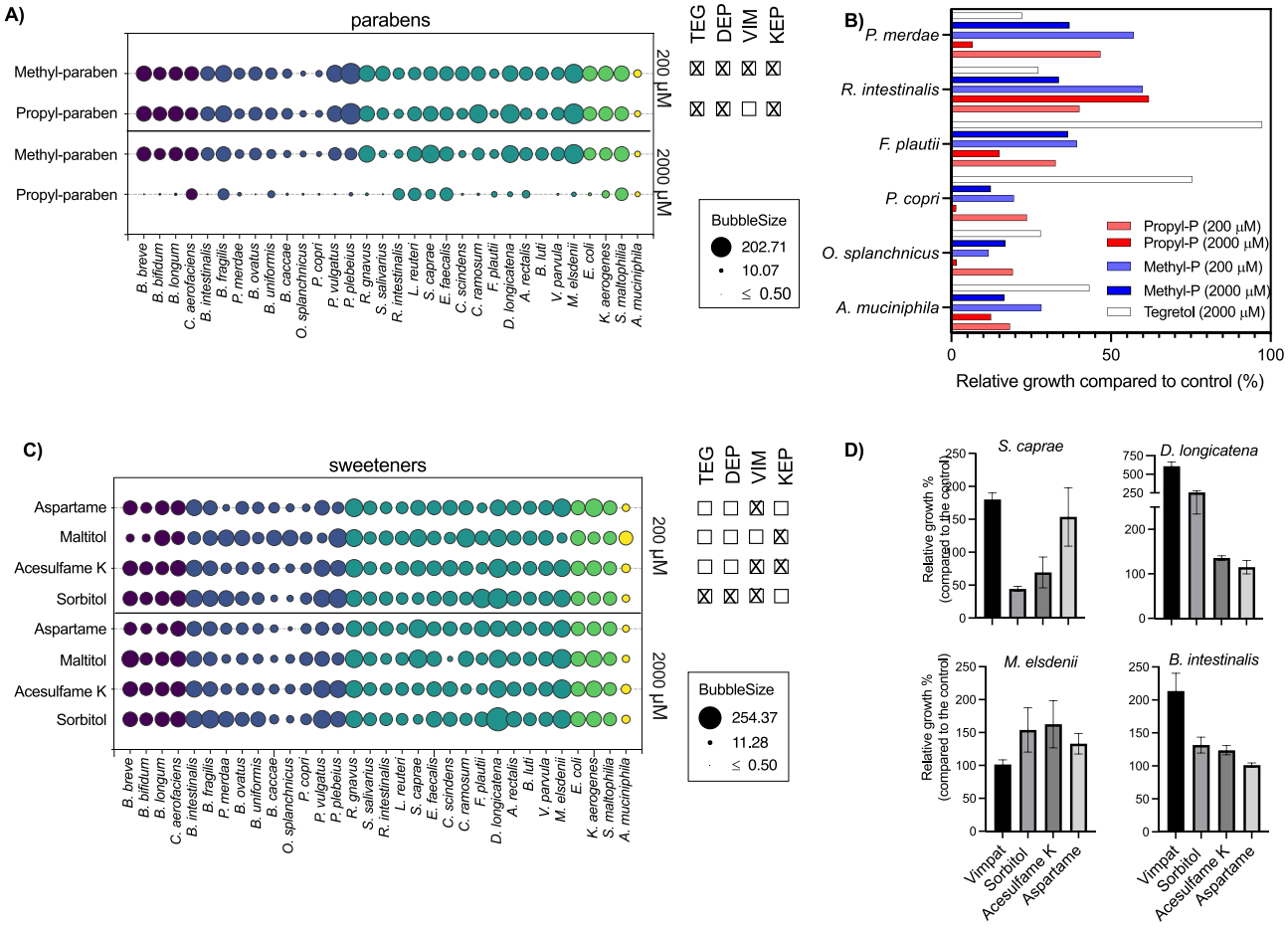

Fig. 3. Common ASM syrup excipients including parabens and sweeteners had inhibitory and growth-stimulating effects, respectively. A) Among the parabens tested, methyl-paraben was less anti-microbial than the propyl-paraben at both 200 and 2000 μM concentrations. Methyl-paraben = methyl 4-hydroxybenzoate. B) The relative growth of the strains that were more susceptible to propyl-paraben was plotted in comparison to methyl-paraben and the most inhibitory syrup formulation Tegretol®. C) Artificial sweeteners sorbitol, acesulfame K; maltitol and aspartame had growth-limiting effects at 200 and 2000 μM concentrations on less than 20% of the strains. They stimulated the growth of many Firmicutes strains. (DEP: Depakine®, KEP: Keppra®, TEG: Tegretol®, VIM: Vimpat®) D) Relative growth of *Dorea longicatena*, *Bacteroides intestinalis*, *Staphylococcus caprae*, and *Megasphaera elsdenii* in the presence of Vimpat® along with its three sweeteners.

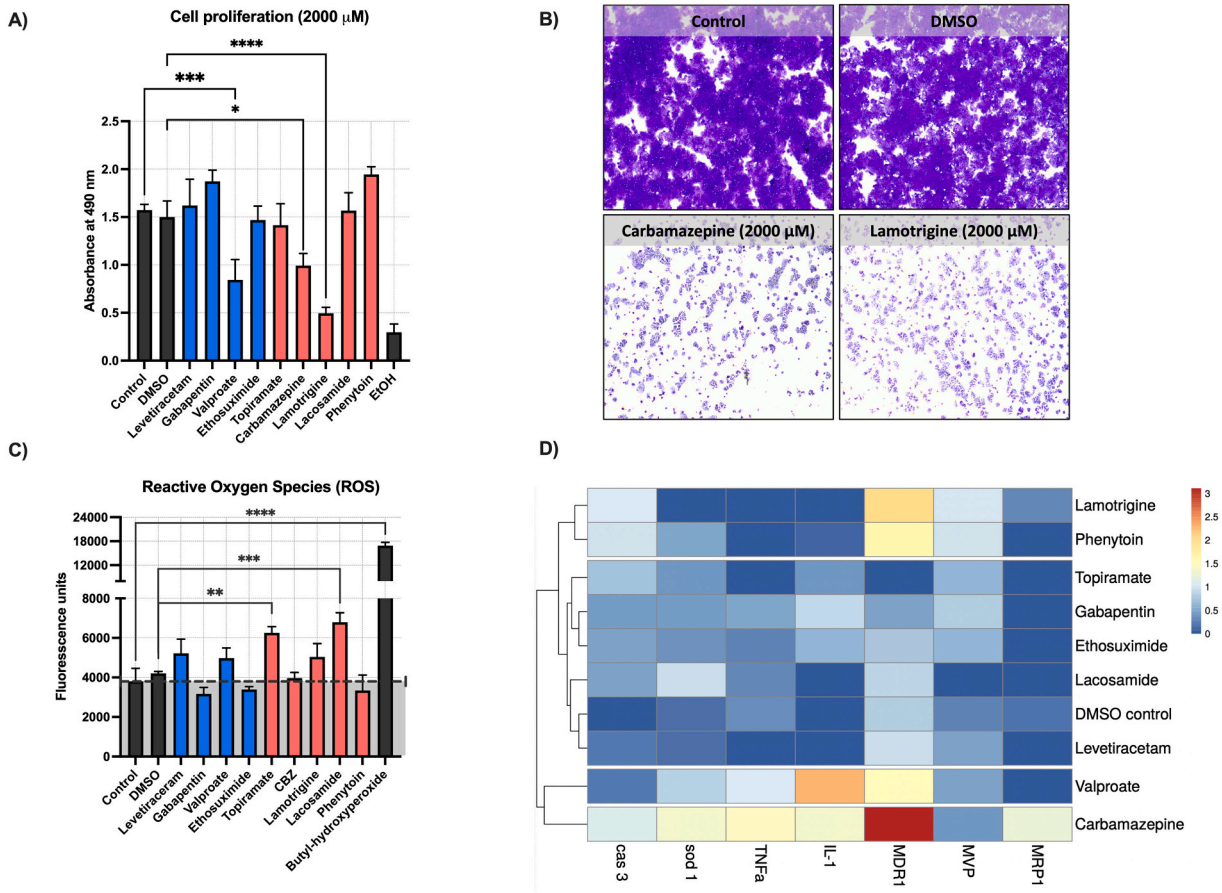

Fig. 4. ASMs valproate, carbamazepine, and lamotrigine negatively impacted the cellular proliferation of HT-29 colon cells whereas topiramate, phenytoin, and lacosamide induced the release of reactive oxygen species. A) Based on the MTS assay, cellular proliferation was impacted prominently with 2000 μM of ASM APIs following 24 h of incubation. B) Crystal violet staining of the HT-29 cells treated with 2000 μM carbamazepine or lamotrigine in comparison to DMSO or PBS treated controls after 24 h of incubation. C). Reactive Oxygen Species assay showed both hydrophobic and hydrophilic ASMs induce oxidative stress including lacosamide and topiramate that did not affect cellular proliferation. D) Gene expression profiles of HT-29 epithelial cells treated with ASM APIs normalized to PBS treated control and GAPDH expression levels and log10 transformed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We also tracked the gene expression profiles of the HT-29 cells when exposed to 2000 μM of the APIs. As presented in Fig. 4D, the expression profiles of the selected genes formed three clusters. Two of the clusters included either carbamazepine or valproate which had more distinct expression profiles than the remaining seven APIs. Lamotrigine and phenytoin treatments formed a subcluster whereas APIs along with vehicle-DMSO control formed another subcluster. Since lamotrigine and carbamazepine exhibited cytotoxicity, we evaluated *cas3* gene expression that encodes for caspase 3. Carbamazepine, lamotrigine, and phenytoin induced the expression of *cas3* more than two-fold. The expression of *sod1* (superoxide dismutase) as a stress response was also elevated for carbamazepine, and lacosamide, compounds that showed either high cytotoxicity or ROS generation, respectively. Proinflammatory cytokines *IL-1* and *TNF-α* were overexpressed under carbamazepine treatment along with valproate and gabapentin.

Finally, we measured the expression levels of genes that are involved in drug efflux or transport. An important target for drug resistance, the *MDR1* gene that encodes for P-glycoprotein 1 was overexpressed with carbamazepine, lamotrigine, phenytoin, and valproate in descending order. Another ABC transporter MRP1 expression was greater in carbamazepine, lamotrigine, and DMSO control in comparison to other treatments. The expression of non-ABC transporter MVP was not significantly modified with any of the APIs.

3.5. *Bacterial supernatants rescued epithelial cells from carbamazepine and lamotrigine toxicity and drug resistance*

We concurrently exposed the HT-29 cell lines to carbamazepine or lamotrigine with or without bacterial supernatants. Cell proliferation assay showed that an addition of 10% by volume filtered supernatants of *Akkermansia muciniphila* or *Bifidobacterium longum* did not significantly alter the viability of the cells even though both strains increased cell proliferation (Fig. 5A). When the cells were co-incubated with the supernatants and carbamazepine (2000 μM), proliferation was greater than 28% and 36% of carbamazepine (2000 μM) alone (Tukey's adjusted $P = 0.0008$ and 0.0001, respectively). Then, we repeated the same assay but this time with 2000 μM lamotrigine which had more cytotoxicity on HT-29 cells than carbamazepine at the given concentration (Fig. 5B). The effect size was smaller with lamotrigine than carbamazepine due to the greater toxicity of lamotrigine at the same concentration. However, *B. longum* supernatant significantly increased the cell proliferation in comparison to lamotrigine alone (Tukey's adjusted $P = 0.02$).

Finally, we evaluated whether bacterial supernatants have an impact on the expression of drug resistance genes. As shown in Fig. 5C, *A. muciniphila* supernatant reduced the MDR1 expression that was associated with carbamazepine exposure, however, this observation was not statistically significant. A similar dampening impact was also observed when cells were incubated with supernatant of a mixed culture

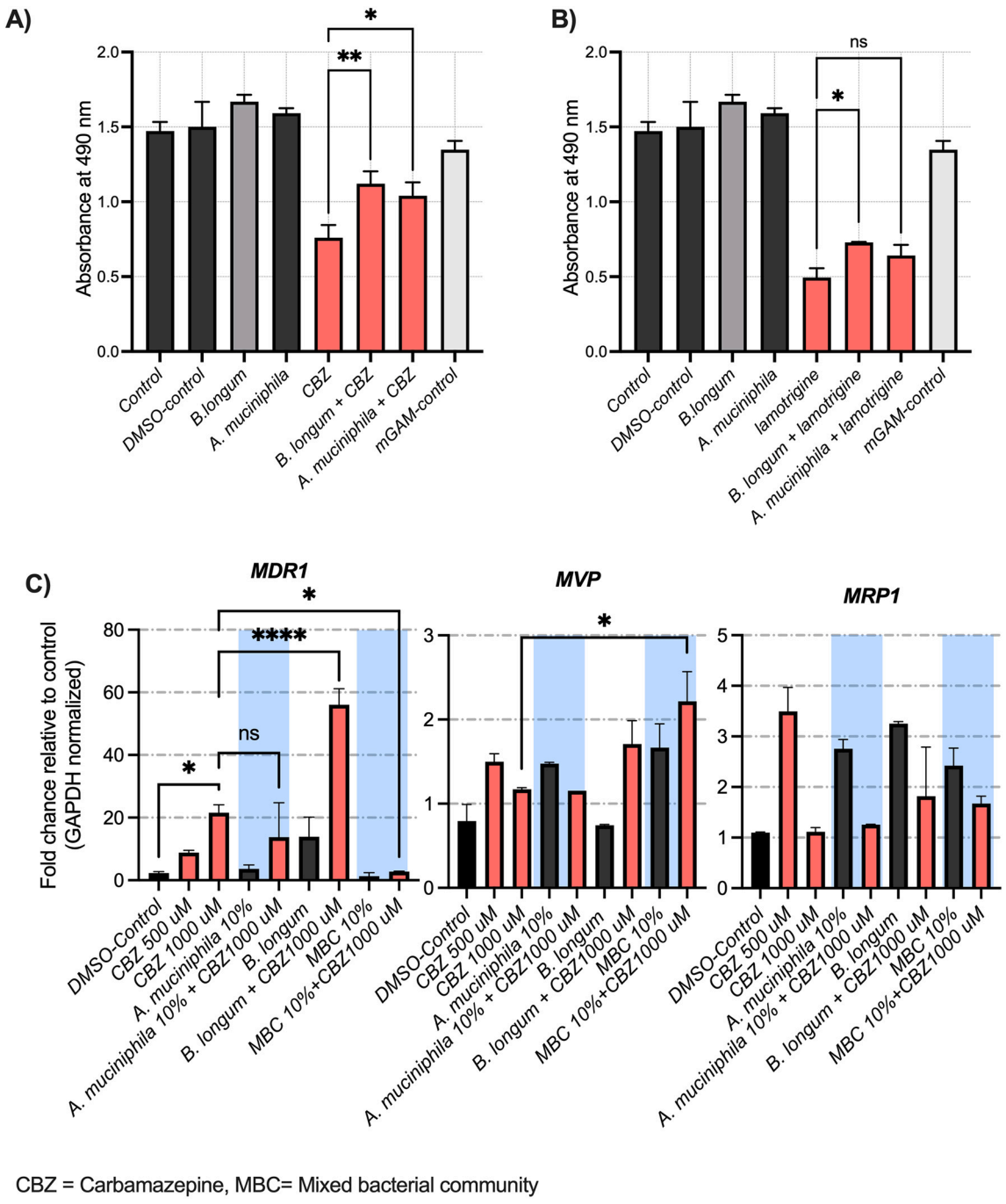

Fig. 5. Bacterial supernatants impacted cytotoxicity and expression of drug resistance genes in the HT-29 cell model. Cytotoxicity estimated by MTS assay showed that *Bifidobacterium longum* and *Akkermansia muciniphila* supernatants reduce cytotoxicity induced by A) carbamazepine and B) lamotrigine. C) Gene expression profiles of MDR1, MVP, and MRP1 were differentially modulated by carbamazepine in the presence of bacterial supernatants.

that derived from a fecal sample (Tukey's $P = 0.03$). In contrast, *B. longum* supernatant increased the expression of *MDR1* alone. When the cells were co-incubated with carbamazepine and *B. longum* supernatant, the expression levels were three times greater than if the cells were incubated with carbamazepine alone. We observed a similar trend with the *MVP* gene yet, the overall expression levels were low. All supernatants reduced the expression level of the *MRP1* gene although the fold difference in comparison to the control was not significantly higher.

4. Discussion

Anti-seizure medications (ASM) are a group of drugs that are extensively used, mainly for the management of epileptic seizures but also for various neurological or psychiatric disorders. Although there are more than 30 ASMs with different mechanisms of action, 30–40% of the patients with epilepsy do not respond to the treatment suffering from drug-resistant epilepsy (Laxer et al., 2014). Human cohort studies have shown that microbiome composition and diversity of drug-resistant patients were distinct from drug-sensitive patients or healthy individuals (Lee et al., 2021a; Peng et al., 2018). Here, we systemically monitored several ASMs along with their APIs, formulations, and excipients to comprehensively address their differential effect on human gut bacterial strains and host epithelial response.

While a limited number of studies documented the microbiome

composition of patients with drug-resistant epilepsy, there are even fewer studies documenting the interactions between ASMs and gut bacterial species. Maier et al. reported that 27% of 1197 non-antibiotic human targeted drugs inhibited the growth of at least one of the 40 bacterial strains tested at a concentration of 20 μM (Maier et al., 2018). Another recent report showed that under *in vitro* conditions, valproate did not impact the growth patterns of two gut commensals, *Lactobacillus rhamnosus* and *Escherichia coli,* at a concentration up to 600 μg/mL (Cussotto et al., 2019). Our comprehensive screening confirmed the results for *E. coli* and another *Lactobacillus* species, *Lactobacillus reuteri* as well as many other gut commensals. Qian et al. estimated minimum inhibitory concentration of lamotrigine for aerobic or facultative aerobic strains including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *E. coli*, and *Enterobacter cloacae* and observed a level of antibacterial activity against Gram-positive species (*B. subtilis*, *S. aureus*, and *S. faecalis*) (Qian et al., 2009). In the current study, we expanded this observation to 32 anaerobic gut strains and validated the finding that lamotrigine induces a stronger anti-commensal activity against Gram (+) strains. Moreover, our analysis also documented an opposite trend for another commonly used ASM API carbamazepine being more anti-microbial towards Gram (−) bacteria, even though both ASM APIs have poor to none water solubility.

ASMs might inhibit bacterial growth through their action on cellular walls or membranes as our study revealed that the impact of carbamazepine and lamotrigine was Gram staining specific and APIs that have low water solubility inhibited bacterial growth most. First-generation ASM carbamazepine and second-generation ASM lamotrigine had greater antimicrobial activity than the others. Another class of human-targeted drugs, serotonin uptake inhibitors and piperazine derivatives, are known to negatively impact the growth of *E. coli* by modulating efflux pumps (Bohnert et al., 2016; Bohnert et al., 2011). Additionally, in *E. coli*, lamotrigine was reported to inhibit ribosomal biogenesis (Stokes et al., 2014), a mechanism that might explain the wide range of phylogeny independent growth inhibition patterns we observed. Carbamazepine, an anthropogenetic biomarker due to its vast presence in the wastewater, was found to inhibit the reproduction of many aquatic microorganisms and also inhibit the sporulation of fungi (Hai et al., 2018). Future studies are warranted to explore the specific mode of action of ASMs on gut microbial strains to better understand the mechanistic impact of the ASM APIs on gut health and their potential side effects.

Another feature of our study relies on a particular focus on core child microbiome gut bacteria. An environmental insult that can be induced by xenobiotics, including pharmaceuticals, during the first 1000 days of life may have a life-long impact on the host due to the irreplaceable role played by the gut microbiome in the immune, endocrine, metabolic and neurologic development (Robertson et al., 2019). Therefore, there is a need to characterize the impact of chronically used ASMs on children's microbiome species. For example, severe inhibition of *Bifidobacterium* species by phenytoin and lamotrigine might have clinical implications since those strains are abundantly found in infants and support infant development by inducing a variety of cytokine production, allergy prevention, and extraction of nutrition from the breast milk (Lawson et al., 2020; Solís et al., 2010). Moreover, the selective inhibition of early life associated *Parabacteroides* species (Noble et al., 2021) with antiseizure effects (Olson et al., 2018) by carbamazepine as well as inhibition of early colonizing butyrate producers including *Ruminococcus gnavus*, *Clostridium ramosum*, and *Roseburia intestinalis* by carbamazepine or lamotrigine highlight a potential dysbiosis in gut health and neurodevelopment.

Since oral suspensions are the common drug delivery method in young children, we also took into consideration the formulation of ASMs. Studies on drug additives are limited; however, a recent report by Gerasimidis et al. showed that food additives including artificial sweeteners impact the composition of the gut microbiome and its fiber degradation capacity in fecal enrichment cultures (Gerasimidis et al., 2020). They observed that aspartame and benzoate increase the relative abundance of *Bifidobacterium* in these mixed cultures. Our study tested four sweeteners and two benzoates as they are also commonly found in syrup formulation of ASM. We reported a concentration-dependent impact of these chemicals; an increase in toxicity or proliferation with increasing concentration for parabens and sweeteners, respectively. *Bifidobacterium* species were negatively impacted by propyl-paraben but not methyl-paraben at high concentration (2000 μM) but not 10 times lower concentration (200 μM). Crovetto et al. previously reported a greater toxic effect of propyl- and butyl-parabens in comparison to methyl-paraben on *E. coli* and *Staphylococcus aureus* under aerobic conditions (Crovetto et al., 2017), an observation consistent with our results under anaerobic conditions with an extended bacteria library.

A goal of our study was to explore the connection between gut commensals, ASMs, and the host intestinal epithelial response. We first evaluated the cytotoxicity of ASM APIs on colonic epithelial cells. Cell proliferation was significantly diminished with carbamazepine along with an increased expression of the *cas3* gene in agreement with a recent report that carbamazepine induces apoptosis (Sohaib and Ezhilarasan, 2020). We also observed a reduction in cell viability with valproate which was previously described (Hajikazemi et al., 2018). An interesting aspect of our study included the co-incubation of ASMs with bacterial supernatants. We demonstrated that co-incubation of the cells with *A. muciniphila* or *B. longum* supernatants reduced the cytotoxic effects of carbamazepine and lamotrigine on HT-29 cells. *In vivo* models demonstrated a protective role of *A. muciniphila* administration against hepatic injury by inhibition of apoptosis (Wu et al., 2017). Additionally, *A. muciniphila* was previously shown to exert a beneficial impact on colon health by reinforcing intestinal integrity (Hiippala et al., 2018). Zhou et al. reported an improved proliferation of intestinal organoids with *B. longum* administration (Zhou et al., 2020). *Bifidobacterium* secretions have long been shown to protect intestinal barrier function *via* reducing intestinal permeability and inflammation (Guo et al., 2017) and in our study, we demonstrated this protective impact on a cytotoxic ASM API carbamazepine.

One of the proposed mechanisms of refractory epilepsy is the overexpression of drug resistance genes (mainly *MDR1* encoding for G-protein) (Tishler et al., 1995), especially in the brain-blood barrier. Indeed, animal models clearly showed overexpression of the *MDR1* gene (Ma et al., 2013; Rizzi et al., 2002). Additionally, in a drug-resistant epilepsy cohort, increased *MDR1* gene expression was associated with reduced plasma concentrations of ASMs (Skalski et al., 2017). Additionally, pharmaco-resistance also occurs due to several ASMs being a substrate for the P-gp efflux pump, hence ASMs may contribute to the pharmaco-resistance issue (Wang-Tilz et al., 2006). We evaluated the expression of these genes in the HT-29 epithelial model as carbamazepine is an oral drug and the reduced bioavailability of the drug may also stem from reduced intestinal absorption. We observed that carbamazepine can induce *MDR1* expression in the intestinal epithelia and this expression can be reduced by the presence of bacterial supernatants (*A. muciniphila* or mixed culture) or further elevated (by *B. longum*). An increase in MDR1 expression translates into a lower intracellular drug concentration but also reduced drug-induced toxicity (Mercado-Lubo and McCormick, 2010).

Toxicity or gene expression profiles of gut bacteria or intestinal epithelial cells depend on the administered dose. A limitation of our study can be attributed to unknown intestinal or fecal concentrations of the ASMs, especially in children. ASMs are chronic use drugs and their dosage in children is determined by age and body weight, hence making it more difficult to determine physiologically relevant concentrations. Additionally, plasma concentrations of these medications vary depending on the time the samples were collected following treatment. For example, the plasma concentration of carbamazepine, lamotrigine, and phenytoin varied greatly 2 to 8 h of 1 mg/kg oral administration (Hotta et al., 2021). However, Maier et al. inferred intestinal concentrations of a few ASMs along with their comprehensive drug library from published

plasma concentrations. According to their analysis, ASMs can exist in the intestines from 130 μM (Topiramate) to 1223 μM (Levetiracetam) (Maier et al., 2018). Our range of 200–2000 μM is within the estimated intestinal concentration of ASM APIs, hence potentially providing a physiologically relevant dose-effect response both on gut microbial strains and host intestinal epithelial barrier level.

5. Conclusions

The gut microbiome has become a key target for the modulation of drug efficacy and toxicity due to its multi-faceted interactions with drugs and the host organisms. However, many host-targeted drugs have been shown to influence gut microbial dynamics, hence impacting host health in unprecedented ways. Similar to many drugs, ASMs are not inert towards gut microbial strains and epithelial cells. While our observations need to be confirmed *in vivo*, the putative antagonist role of microbial metabolites on carbamazepine and lamotrigine-associated toxicity on epithelial cells suggests a role of the gut microbiome in ASM response.

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Authors' contributions

ZEI, PL, and SA: Conceptualization - ZEI and VB: formal analysis, investigation, methodology, visualization - PL, NL, and SA: supervision and funding acquisition - ZEI and PL: writing the original draft and final manuscript - VB, NL, and SA review and editing. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2022.105664.

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