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Repurposing Drugs as *Bacteroides fragilis* BFT-3 Inhibitors in the Animal Infection Model *Galleria mellonella*

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ABSTRACT

Bacteroides fragilis is a key component of the human gut microbiota, although enterotoxigenic strains (ETBF), which produce *B. fragilis* toxin (BFT), can act as opportunistic pathogens. BFT disrupts intestinal epithelial integrity and contributes to conditions such as inflammatory bowel disease and colorectal cancer. This study aimed to characterize three allosteric inhibitors of BFT-3 (isoform 3 of BFT), previously identified by our group through high-throughput screening of US Food and Drug Administration approved drugs. We evaluated their activities in vitro and in vivo. Using *Galleria mellonella* larvae as a novel infection model for *B. fragilis*, we assessed the antimicrobial and antivirulence potential of these compounds. Among the three tested compounds, MOA4 demonstrated superior efficacy, enhanced bacterial clearance in vivo, and increased larval survival in a dose-dependent manner, with minimal toxicity. Synergy studies have revealed the potential combinatory effects of MOA4 and conventional antibiotics. These findings establish *G. mellonella* as a valuable alternative model for studying *B. fragilis* infections and highlight MOA4 as a promising candidate to be repurposed for the treatment of *B. fragilis*-mediated diseases while preserving commensal microbiota.

1 | Introduction

The largest population of bacteria in the human body is found in the colon, with the majority being anaerobes. Approximately 25% of the anaerobes belong to the genus *Bacteroides*, which are bile-resistant and non-spore-forming gram-negative rods [1]. Under normal conditions, *Bacteroides* spp. are harmless commensals, but in some cases, such as after rupture of the gastrointestinal tract or intestinal surgery, some species can cause severe infections in the intra-abdominal space [1–4]. Following infiltration of the normally sterile peritoneal cavity by gut bacteria, aerobes such as *Escherichia coli* dominate the infection. However, once sufficient oxygen is depleted, *Bacteroides* spp. predominate, resulting in chronic infection [1].

The most pathogenic species of the genus *Bacteroides* is *Bacteroides fragilis*; while it accounts for only 0.5%–2% of the human gut microbiota, it is the most frequently isolated anaerobic pathogen from clinical specimens, among which enterotoxigenic *B. fragilis* (ETBF) strains are considered the most virulent [1, 5–8]. Untreated *B. fragilis* infections have a mortality rate of 60% [5]. Aerotolerance is the main feature explaining the successful colonization of the mucosa by *B. fragilis*. Thus, it can survive in

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the mucosa, where oxygen tension is higher, as well as induce bacteremia in the microenvironment [9, 10].

ETBF strains are highly virulent [1, 5–8]. They produce a toxin called *B. fragilis* enterotoxin (BFT) or fragilysin [8, 11, 12], the only recognized virulence factor specific to ETBF when compared to its nontoxigenic counterparts (NTBF). BFT, encoded by the chromosomal *bft* gene, is synthesized as a 44.4-kDa preproprotein zinc-dependent metallopeptidase (MP) enterotoxin, which is processed and secreted into the culture supernatant by ETBF as the mature 20-kDa protein BFT [1, 13]. This toxin can degrade the *zonula adherens* in the intestinal epithelium by cleaving E-cadherin, thereby causing (1) delocalization of other tight junction proteins, (2) loss of cell adhesion, (3) rearrangement of the actin cytoskeleton, (4) nuclear translocation of β-catenin, (5) secretion of inflammatory signaling molecules, and (6) loss of fluids, which collectively cause diarrhea and other gut-related pathologies. Consequently, patients with ETBF exhibit an increased risk of inflammatory bowel disease and colorectal cancer [11–23].

To treat *Bacteroides* spp. infections, β-lactams (such as cefoxitin or carbapenems) coadministered with β-lactamase inhibitors, clindamycin, and metronidazole are frequently prescribed (the latter two are often in combination with fluoroquinolones). However, many *B. fragilis* strains are intrinsically resistant to several classes of structurally unrelated antibiotics, while still affecting other components of the microbiota. Thus, the specific inhibition of BFT is required to combat ETBF-mediated pathogenicity without disturbing the commensal microbiota [24–36].

In recent studies from our research group, we performed a comprehensive drug discovery approach to target BFT-3, which is one of the three described BFT isoforms sharing >90% sequence identity (BFT-1, -2, and -3) [37–39]. For this purpose, we employed a chemical library of 1120 drugs approved by the US Food and Drug Administration (FDA). Through a combination of biophysical, biochemical, structural, and cellular techniques, we identified three compounds (MOA4, MOA9, and MOA10; see therapeutic information in Table S1) capable of specifically binding to the catalytic domain of the proBFT-3 zymogen and to the BFT-3 mature toxin. In contrast to canonical MP inhibitors, which target the active site of mature enzymes, these effectors bind to a distal allosteric site (exosite) in the proBFT-3 zymogen structure, stabilizing a partially unstructured, inactive, and zinc-free enzyme conformation by shifting the zinc-dependent proBFT-3 conformational equilibrium. This yields proBTF-3 that is incompetent for autoactivation, thus ablating the hydrolytic activity of the mature toxin. Our strategy represents a novel method for the development of highly specific drugs for ETBF-mediated enteropathogenic conditions [40], and a new example of a general strategy for identifying allosteric inhibitors for zinc-dependent proteins, following our proof-of-concept studies on the NS3 protease from the hepatitis C virus [41, 42]. In addition, since these three BFT-3 allosteric inhibitors are FDA-approved drugs, their potential repurposing for treating ETBF infections is straightforward.

Over the past few decades, murine models have been the gold standard for studying microbial infections. However, ethical concerns and high costs have led to the development of alternative host models. Organisms such as *Acanthamoeba castellanii*, *Artemia salina*, *Caenorhabditis elegans*, *Danio rerio* larvae, *Drosophila melanogaster*, and *Galleria mellonella* have been successfully used as models for several diseases [43–47]. In particular, *G. mellonella* has seen a 42% increase in its use since 2016 owing to the similarities of its immune system to that of mammals and other advantages [47–49]. These worms can be incubated at 37°C, allowing for the study of human pathogens at relevant temperatures [47–50]. They are cost-effective, easy to maintain, and are not subject to strict ethical regulations. Despite their advantages, the use of *G. mellonella* can be affected by factors such as a short lifespan and lack of standardization under experimental conditions [51–54]. There are no known studies using *G. mellonella* with *B. fragilis*, highlighting the need for tailored infection methods and assessment of their suitability for studying this opportunistic gut pathogen.

As a natural step forward in our efforts to identify new drugs against *B. fragilis*, it is important to verify the in vitro effects of the identified compounds in more complex scenarios and, in particular, test their activity in in vivo systems, such as bacterial cultures and animal infection models. For this purpose, it is important to evaluate whether the identified compounds can affect the viability and growth of *B. fragilis* in a real gut infection process using an animal infection model, in addition to inhibiting the BFT-3 toxin. Therefore, we extensively characterized *G. mellonella* in relation to *B. fragilis* infection. In this study, we provide sound evidence for the in vivo antibacterial effects of these previously identified compounds.

We would also like to mention that this larval model is not intended to replace mammalian assays in studies related to infection processes or host response mechanisms, but this model could be a potent additional tool to analyze the effect of antimicrobials and compounds against pathogens before advancing to highly demanding studies in mammals (Figure 1).

The aim of this study was to investigate the activity of three BFT-3 inhibitors in in vitro cultures of both ETBF and NTBF strains and in animal infection models. For this purpose, we implemented the *G. mellonella* infection model for the gut pathogen *B. fragilis*, as an alternative, simple, effective, and appropriate in vivo model for studying infections caused by this pathogen.

2 | Materials and Methods

2.1 | *B. fragilis* Strains and Culture Conditions

Two *B. fragilis* strains were used: ETBF (ATCC 43858), which is capable of producing the toxin, and NTBF (EUCAST clinical strain: 617161), which does not produce the toxin. Bacterial stocks (15% glycerol) were preserved frozen in solution or with cryo-beads at –80°C.

Anaerobic chambers and sachets (GasPak EZ Anaerobe Container System, 260678, from BD) were used for *B. fragilis* cultures. *Brucella* broth (BBL *Brucella* Broth, from BD) was employed as culture medium, supplemented with 5% defibrinated sheep blood (SR0051C, from Thermo Scientific), 5 μg/mL hemin (Sigma-Aldrich), and 1 μg/mL vitamin K1 (Sigma-Aldrich). Solid media

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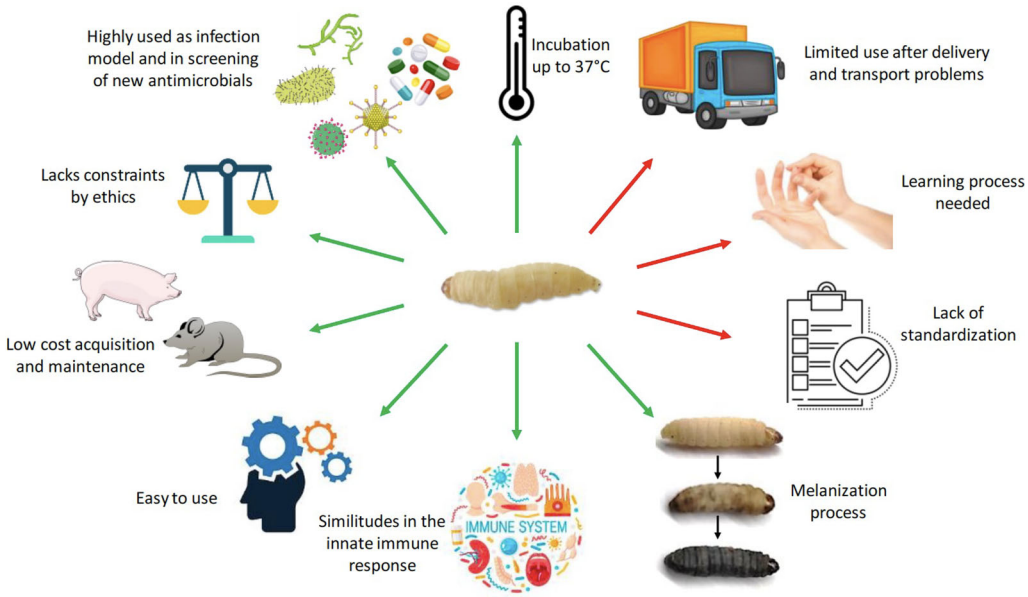

FIGURE 1 | Advantages (green arrows) and disadvantages (red arrows) of *G. mellonella* as an animal infection model.

were prepared by adding 1.5% agar (European Bacteriological Agar, Condalab) to the *Brucella* broth, as described previously. All cultures were performed in Class II Biological Safety Cabinets.

Routine maintenance of *B. fragilis* cultures was performed in liquid medium and subcultured every 48–72 h. We determined that for *B. fragilis*, OD_{600} = 0.88 corresponds to $4{\cdot}10^8$ cells/mL in an Ultrospec 10 Cell Density Meter (Biochrom). In addition, for the MIC_{90}, synergy, and TKA evaluation assays (see Materials and Methods), defibrinated sheep blood was replaced with fetal bovine serum (Pan Biotech) in the same proportion.

2.2 | Compound Information and Maintenance

The compounds were selected in an experimental molecular screening carried out in previous studies by our group [40], in which the Prestwick Chemical Library (Prestwick Chemical), comprising 1120 FDA-approved drugs, was employed. Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) at a final concentration of 4 mM, arranged in 96-well plates, and preserved at −20°C while not in use. The compounds selected for in vitro and in vivo assays were purchased from Sigma-Aldrich, diluted in DMSO to a concentration of 40 mM, and preserved at −20°C while not in use. The chemical absorption, distribution, metabolism, excretion, and toxicity properties, therapeutic indications, toxicity, and safety in humans are available from the manufacturer of Prestwick compounds.

2.3 | Determination of the Antibacterial Activity of the Selected Compounds: Minimum Inhibitory Concentration Assays

The minimum inhibitory concentrations (MICs) of BFT inhibitors against both *B. fragilis* strains were determined in 96-well plates to assess antimicrobial activity [55]. MIC_{90} is defined as the minimum inhibitory concentration of an antibiotic/compound that inhibits the growth of 90% of the cells. Metronidazole CRS (MTZ; European Pharmacopoeia Reference Standard) and cefoxitin sodium CRS (FOX; European Pharmacopoeia Reference Standard), two antimicrobial drugs currently used for the treatment of *B. fragilis* gut infections, were tested in parallel as controls.

Compounds at 2× (maximal concentration 20 mM) were diluted in supplemented *Brucella* broth. One hundred and fifty microliters of the 2× compounds were added to row A of 96-well plates in triplicate. Then, 75 µL of supplemented *Brucella* broth with the appropriate DMSO percentage was added to rows B−H. Serial two-fold dilutions were made by transferring 75 µL from row A to row B, mixing, and continuing the same procedure until row G (discarding 75 µL from row G). Positive controls (75 µL medium + 75 µL bacterial inoculum) and negative controls (150 µL medium with DMSO) were placed in row H.

Briefly, two-fold serial dilutions of the drugs were inoculated with $5{\cdot}10^5$ colony forming units (CFU) per mL (OD_{600} = 0.88 corresponding to $4{\cdot}10^8$ cell/mL) in 96-well plates (final volume of 150 µL) and incubated at 37°C for 48 h. Positive and negative growth controls were included in the assays. After this period, bacterial cell viability was estimated using the MTT assay (see below). All MICs were determined in triplicate in at least two independent experiments.

2.4 | Synergy Assays

We explored the potential synergy (i.e., scenario in which the combined effect of two drugs is greater than the sum of their individual effects [56]) between the BFT inhibitory compound MOA4 and the antibiotics MTZ and FOX on the two strains of *B. fragilis* (ETBF and NTBF) in 96-well flat-bottom plates (Tissue Culture Test Plate 96F, from Sigma-Aldrich).

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Serially diluted concentrations of MOA4 (prepared in 96-well plates, as above) were mixed with three subinhibitory concentrations (corresponding to 1/2 and 1/4 of their MIC_{90}) of either MTZ or FOX, considering that the DMSO concentration in all wells in the plate should be the same as that in the wells with the highest concentration of MOA4. Finally, the plates were inoculated with $5{\cdot}10^5$ cells/mL. Positive and negative controls and incubation conditions were performed as described in the MIC assay (above). Bacterial cell viability was estimated using the MTT assay (see below). Synergy was considered if the MIC_{90} of the combination was reduced at least four-fold with respect to the sum of the MIC_{90} of individual drugs, or if the FICI (calculated as the sum of the FIC of individual drugs, where every FIC is the MIC_{90} of the combination divided by the MIC_{90} of one individual drug) was less than 0.5.

2.5 | Estimation of Bacterial Viability with MTT

The viability of enterotoxigenic or nontoxigenic *B. fragilis* cultures in liquid media (MIC_{90} determinations or synergy tests) was analyzed using the yellowish reagent 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Merck-Sigma), which is metabolized by viable cells into formazan, a purple compound. By measuring the absorbance at 580 nm, it was possible to quantify the degree of MTT conversion, which was proportional to the percentage of viable cells, taking the positive growth control as 100% bacterial viability.

A stock solution of 5 mg/mL MTT in water was protected from light and stored at 4°C. To determine bacterial viability in 96-well plates, 30 μL/well of the working MTT solution (5 mg/mL MTT, 20% Tween-80) was added and incubated at 37°C for 4 h in the dark. The plates were shaken cautiously and equilibrated at room temperature. Then, they were covered with a plastic film, and the absorbance at 580 nm was recorded using a Synergy HTX multimode plate reader (BioTek) and Gen5 Software. Each experiment was performed in duplicate/triplicate, repeated at least twice, and normalized using untreated bacterial cells (positive growth controls) as a reference (100% viability).

2.6 | Time-Kill Kinetic Assays

A time-kill kinetic assay (TKA) was used to study the effect of BFT-3 inhibitors against *B. fragilis* over time to determine their bactericidal or bacteriostatic activity.

For TKA, five assay concentrations (10×, 4×, 1×, 0.25×, and 0.1× MIC_{90}) of antibiotic controls and BFT-3 inhibitors were prepared in 96-well flat-bottom plates with a bacterial inoculum of $5{\cdot}10^5$ cells/well for each strain in *Brucella* broth supplemented medium (final volume of 280 μL). At each time point (t = 0, 2, 5, 8, 24, 48 h), 20 μL of each condition was serially diluted (final volume of 200 μL) with sterile saline solution in 96-well U-bottom plates (U-bottom, tissue culture test plate). Finally, 10 μL of each well from the sterile saline solution plate was transferred to agar-containing square Petri dishes (OmniTray w/Lid, nontreated sterile, polystyrene, from Thermo Scientific). The agar plates were then incubated under an anaerobic atmosphere for 48 h at 37°C. After this period, the cell concentration (cell/mL) was calculated by counting the number of colonies under each condition, assuming a limit of detection of 50 CFU/mL. Each experiment was performed in duplicate or triplicate, repeated at least twice.

Bactericidal activity corresponds to a sustained $3\log_{10}$-fold decrease or greater in CFU (surviving bacteria), compared to the initial inoculum, and was achieved within a specified time (usually 48 h), which is equivalent to killing ≥ 99.9% of the inoculum, while bacteriostatic activity corresponds to growth arrest, compared to the initial inoculum, but cells are not necessarily dead.

2.7 | *G. mellonella* Waxworm Larvae

Waxworms were purchased from Harkito Reptile S.L. (Madrid), a supplier that does not introduce hormones, antibiotics, or other treatments to the larvae, and delivered live specimens.

Upon reception of larvae, each container of worms was checked to ensure that the larvae were viable and healthy, as determined if they were completely submerged in the bedding, mobile, had a slightly yellow/tan coloration, and lacked black pigmentation along the larval body; dead worms with advanced dark pigmentation [57], as well as moths, were discarded. Larvae of approximately 2 cm, with an approximate weight of 180–250 mg (indicative of being between the fifth and sixth stages of development), were selected for in vivo analysis.

Selected larvae were kept in the dark in their own container, at room temperature (20−25°C), and in a ventilated space until used, no later than 2 weeks after arrival, depending on the initial state of the larvae. Whenever possible, to guarantee optimal conditions for the larvae, all experiments (infection with *B. fragilis* and compound treatment, see below) were carried out on the same day of reception or the following day.

In all experiments, we used 10 larvae per condition; they were kept in independent sterile Petri dishes, without bedding and food supply, in order to avoid variability between samples.

2.8 | Preparation of *B. fragilis* Cultures for *G. mellonella* Infections

Worms were manipulated in a Class II Biological Safety Cabinet, and the protocol was based on previously published protocols for other bacterial species in *G. mellonella* infections, including specific adaptations for *B. fragilis* [58].

The two strains of *B. fragilis* were grown in *Brucella* broth for 48 h before each experiment, and 3–5 mL of culture was used to prepare the inoculum for infection. For this, cultures were centrifuged at 4000 rpm for 5 min, supernatants were discarded, and pellets were resuspended in 2–3 mL of 1× PBS. The washing process was repeated at least thrice, after which the bacterial pellet was resuspended by pipetting and transferred to a new tube containing 2 mL of 1× PBS, leaving behind the blood clot at the bottom of the pellet. Then, 1:10/1:100 dilutions in 1× PBS were used in order to estimate the bacterial concentration by OD_{600}.

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2.9 | Infection of *G. mellonella* Larvae with *B. fragilis*

The bottom of sterile 90 mm diameter Petri dishes was covered with filter paper in order to limit the adherence of *G. mellonella* larvae to the surface of the Petri dishes. The filter paper pieces were sprayed with 70% ethanol and placed under UV light on both sides. Then, the syringe was loaded with a disposable needle (30G x 1/2″, 0.3×13 mm, BD Microlance), and washed several times with 100% ethanol and 1× PBS, prior to use and between infections. The suspension of *B. fragilis* for inoculation (see above) was vortexed, 10 µL of undiluted culture (or dilutions in PBS) was loaded into the syringe, and injected into the lower left proleg of a healthy larva.

For each larva, two 10 µL-injections were done in each case: the first injection for producing (with *B. fragilis* cultures) or simulating (with 1× PBS) the infection, and the second injection for treating (with compound) or simulating (with 1× PBS/DMSO) treatment. Thus, performing two injections was instrumental in avoiding systematic errors and reducing variability sources, and ensured that the same damage or stimulation level due to background injection effects was always produced. To prevent bacterial sedimentation, the *B. fragilis* cultures were vortexed every few minutes. Appropriate control larvae were prepared as follows: first injection with *B. fragilis*, and second injection with PBS/DMSO (infection only), and first injection with PBS and second injection with compound (treatment only). Each test condition was assayed using a set of 10 larvae, which were kept after inoculation/treatment in the same Petri dish at 37°C until the end of the assay.

2.10 | Monitoring of *G. mellonella* Larvae Mortality and Survival Rate Analysis

Petri dishes with the larvae were incubated at 37°C for 6 days, and checked every 24 h to monitor the progress of the infection and detect dead worms. Larvae that either had generalized black pigmentation throughout the body, patches, or black spots that were formed at some locations on the body, or showed absence of movement were considered dead. To confirm death, sterile clamps were used to carefully turn the larvae on their backs and gently touch the body. Then, it was recorded whether they were able to turn on their own and regain their natural position, that is, with their legs on the surface, and move as uninfected worms. The absence of response to these stimuli, observed as a lack of movement of the body or legs, as well as the inability to turn over on themselves, was also classified as infected/dead. Larvae that began to develop into moths were included in the analysis as live larvae, but were removed from the Petri dishes. Survival curves for the *G. mellonella* toxicity model were plotted using the Kaplan–Meier estimator in GraphPad Prism 6.

3 | Results

3.1 | Compound Activity Against *B. fragilis* Cultures In Vitro

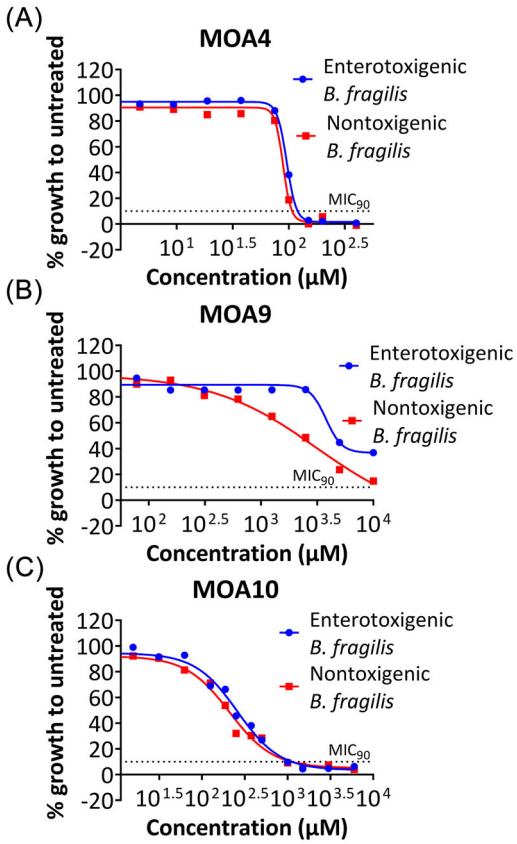

FIGURE 2 | MIC_{90} determination curves for (A) MOA4, (B) MOA9, and (C) MOA10 compounds on both strains of *B. fragilis*. The MIC_{90} values were estimated from the equation of the dose–response curve fitted to the experimental data. Each experiment was performed in triplicate, and the SD is < 10%.

3.1.1 | Antimicrobial Activity of Compounds on *B. fragilis* Cultures

The antimicrobial activities of MOA4, MOA9, and MOA10 compounds were evaluated in both enterotoxigenic and nontoxigenic strains of *B. fragilis*. Compounds MOA4 and MOA10 were found to inhibit bacterial growth at concentrations much lower than those of MOA9. In addition, almost no differences in susceptibility to compounds MOA4 and MOA10 were found between the ETBF and NTBF strains. However, in addition to compound MOA9 being considerably less effective in inhibiting bacterial growth, the nontoxigenic strain was more susceptible to this compound (Figure 2 and Table 1) than the enterotoxigenic strain.

To determine whether the selected compounds had bactericidal or bacteriostatic effects, TKA assays were performed on both strains of *B. fragilis*. Results obtained using MOA4 and MOA10 compounds, as examples, are detailed below (MOA9 not shown).

In the case of MOA4 (Figure 3A,B), at the most active concentrations of 4× and 10× MIC_{90} (i.e., at concentrations 4- and 10-fold higher than the MIC_{90}), bacterial growth was inhibited up to ~2.5$\log_{10}$ in both strains. Based on these data, the compound could not be considered bactericidal *sensu stricto*, because the decrease in CFU/mL was lower than 3$\log_{10}$-fold. In addition, there was also a slight reduction in bacterial growth

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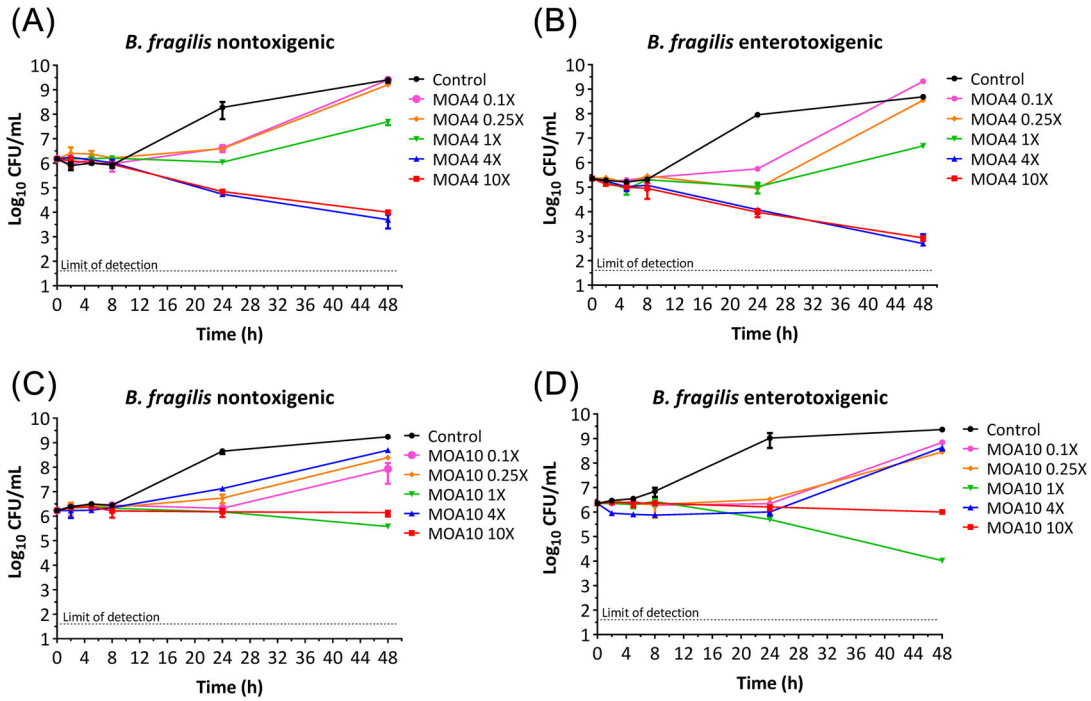

FIGURE 3 | TKA for the antimicrobial characterization of MOA4 and MOA10 on *B. fragilis* cultures: (A) nontoxigenic strain; MOA4, (B) enterotoxigenic strain; MOA4, (C) nontoxigenic strain; MOA10, (D) enterotoxigenic strain; MOA10. In these assays, several concentrations of MOA4 and MOA10 compounds were tested for 48 h. Each experiment was performed in duplicate, and the SD is < 10%. Abbreviation: TKA, time-kill kinetic assay.

TABLE 1 | MIC_{90} determination for the MOA4, MOA9, and MOA10 compounds on *B. fragilis* cultures.

	***B. fragilis*: MIC_{90}**	
	Nontoxigenic strain	**Enterotoxigenic strain**
MOA4	~ 29 μg/mL (~ 110 μM)	~ 31 μg/mL (~ 117 μM)
MOA9	>3074 μg/mL (>10,000 μM)	>3074 μg/mL (>10,000 μM)
MOA10	~ 302 μg/mL (~ 1000 μM)	~ 302 μg/mL (~ 1000 μM)

Note: Each experiment was performed in triplicate.

at a concentration of 1× MIC_{90} of MOA4 after 24 h, hence showing a bacteriostatic effect, although after 48 h, a rebound and an increase of more than $1\log_{10}$ CFU/mL was observed. In the case of subinhibitory concentrations (0.25× MIC_{90} and 0.1× MIC_{90}), delayed bacterial growth was observed at 24 h, but cultures resumed growth and reached levels similar to those of the untreated culture. No significant differences for MOA4 activity were observed between the two bacterial strains. Thus, MOA4 was a dose-dependent compound, and increasing both the dose and time changed its profile from a bacteriostatic compound to a quasi-bactericidal compound.

In the case of MOA10 (Figure 3C,D), at 4× and 10× MIC_{90}, turbidity in the culture medium was observed, which could indicate that the compound was slightly precipitated/aggregated. At 1× MIC_{90}, a bacteriostatic effect was achieved, similar to the 10× MIC_{90}.

The results obtained in both MIC_{90} determination and TKA assays suggested that MOA4 had a greater antimicrobial effect in vitro than MOA9 and MOA10, eliciting a larger reduction in bacterial growth in *B. fragilis* cultures. Furthermore, in these assays, a single initial addition of compound is made, and depending on compound metabolism and other processes, the effective concentration of the compound after 24 h may be different. Therefore, further studies on compound metabolism would be of interest to complement this study.

3.1.2 | Study of Drug Interactions of BFT Inhibitors with Clinically Used Antibiotics

We first determined the susceptibility of *B. fragilis* strains to MTZ and FOX, two antimicrobials already used clinically to treat *B. fragilis* gut infections (Figure 4) [59, 60].

Once the susceptibility to MTZ and FOX was evaluated in both strains of *B. fragilis* used in this study, synergy studies of these two antimicrobials with MOA4 were performed. The results showed that MOA4 in combination with FOX at different concentrations had no synergistic effects (Figure 5). However, the combination of MOA4 and MTZ resulted in reduced bacterial growth (Figure 6).

In these synergy studies, the MIC_{90} of MOA4 in the presence of subinhibitory concentrations of FOX or MTZ (Table 2) and the

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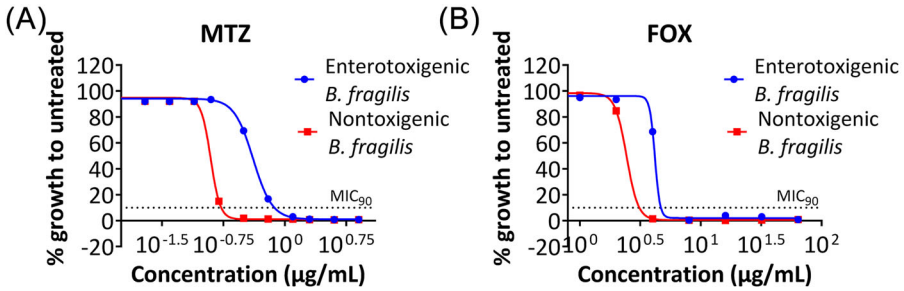

FIGURE 4 | MIC_{90} determination curves for (A) MTZ and (B) FOX antimicrobials on ETBF and NTBF strains of *B. fragilis*. Each experiment was performed in duplicate, and the SD is < 10%. Abbreviations: ETBF, enterotoxigenic *B. fragilis*; NTBF, nontoxigenic *B. fragilis*.

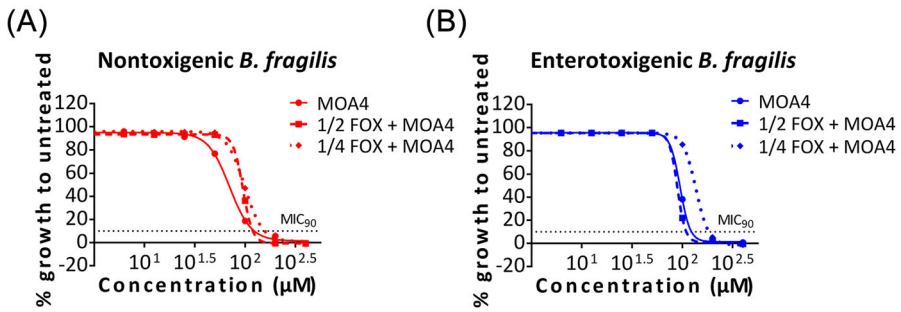

FIGURE 5 | Analysis of synergistic effects between FOX (at fixed concentration calculated from its MIC_{90} [1×]) and MOA4 compound (at different concentrations by serial dilutions) in both (A) nontoxigenic and (B) enterotoxigenic *B. fragilis* cultures. As a control for the synergy assays, the effect of MOA4 alone was represented. Data depicted is the mean of two independent experiments, and the SD is < 10%.

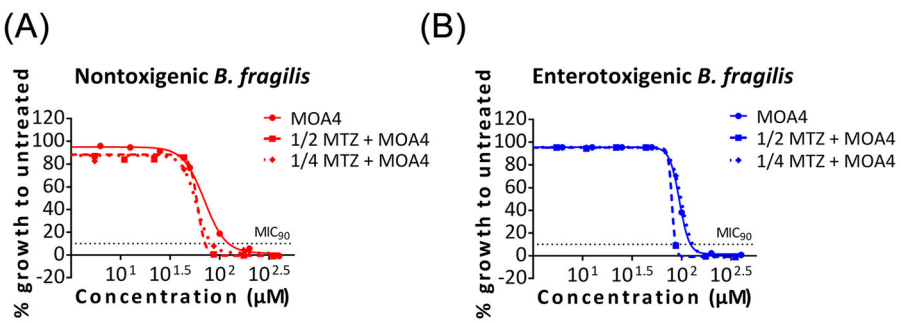

FIGURE 6 | Analysis of synergistic effects between MTZ (at fixed concentration calculated from its MIC_{90} [1×]) and MOA4 compound (at different concentrations by serial dilutions) in both (A) nontoxigenic and (B) enterotoxigenic *B. fragilis* cultures. As a control for the synergy assays, the effect of MOA4 was represented. Data depicted is the mean of two independent experiments, and the SD is < 10%.

evaluation of potential synergy between MOA4 and MTZ (Table 3) were determined. Notably, in the case of 1/4× FOX + MOA4, the MIC_{90} values were higher than those of the control (MOA4 alone), suggesting certain antagonism.

These results indicate that the combination of MTZ with MOA4 showed a higher antibacterial effect than either compound alone. As a first criterion for synergy, we determined whether the MIC_{90} of MOA4 in the presence of a subinhibitory concentration of MTZ was reduced ≥ four-fold compared to the addition of MIC_{90} of MOA4 and MIC_{90} of MTZ; since this was not the case (Table 3), a clear synergistic effect was not observed. As a second criterion for synergy, we determined the FIC of MOA4 compound (Table 3); for a synergistic effect, the FICI (addition of FIC of MOA4 plus FIC of MTZ) must be below 0.5, and since this was not the case (the FIC of MOA4 was above 0.5 in all cases), again, a clear synergistic effect could not be determined. In conclusion, none of the combinations that were analyzed complied with the above criteria for synergy, so the interaction observed between MOA4 and MTZ could not be defined as synergistic *sensu stricto*. However, a boosting effect was observed when MOA4 and MTZ were used in combination.

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TABLE 2 | MIC_{90} determination of MOA4 in combination with subinhibitory concentrations of MTZ and FOX in both *B. fragilis* strains.

	MIC_{90} of MOA4	
	Nontoxigenic strain	**Enterotoxigenic strain**
Alone	29–30 μg/mL (110–115 μM)	30–31 μg/mL (117–120 μM)
1/2× FOX	30–31 μg/mL (117–120 μM)	29–30 μg/mL (110–115 μM)
1/4× FOX	37–39 μg/mL (143–150 μM)	42–47 μg/mL (160–180 μM)
1/2× MTZ	18–19 μg/mL (71–73 μM)	22–23 μg/mL (85–87 μM)
1/4× MTZ	21–22 μg/mL (80–82 μM)	31–33 μg/mL (120–127 μM)

Note: MIC_{90} is represented as a range from the analysis of three assays.

3.2 | Compound Activity Against *B. fragilis* in an In Vivo Infection Model

3.2.1 | Intrinsic Compound Toxicity Determination on *G. mellonella* Larvae

To observe the intrinsic cytotoxic effect of the selected potential BFT-3 inhibitors on noninfected *G. mellonella* larvae, 10 μL injections of 1× PBS to simulate a bacterial infection and 10 μL injections of different concentrations of MOA4, MOA9, and MOA10 (or PBS/DMSO as a control) were administered to the worms. Figures S1–S4 summarize the 24 h monitoring of the different conditions tested (Supplementary Material).

In the case of MOA4 (Figure 7A and Table 4), at the highest concentration tested (1200 μM; i.e., 314 μg/mL; 3.14 μg/larva), a clear toxic effect on the worms was observed after approximately 24 h. At lower concentrations (≤ 400 μM; i.e., ≤ 105 μg/mL; ≤ 1.05 μg/larva), the worms maintained a survival rate of 60%–80%, similar to that observed in the nontreated controls. Then, MOA4 seemed to be nontoxic at concentrations up to 400 μM (i.e., 105 μg/mL; 1.05 μg/larva).

In the case of MOA9 (Figure 7B and Table 4), at the highest concentration of 1200 μM (i.e., 369 μg/mL; 3.69 μg/larva) and 400 μM (i.e., 123 μg/mL; 1.23 μg/larva), a survival rate of 30% and 40%, respectively, was observed after 48 h approximately. The percentage of survival continued to decrease to 10% and 40% at 96 h after administering 1200 and 400 μM, respectively. Administration of MOA9 at 133.33 μM (i.e., 41 μg/mL; 0.41 μg/larva) resulted in a survival rate of 70% after 24 h and beyond, similar to that observed in the untreated controls.

Finally, in the case of MOA10 (Figure 7C and Table 4), higher toxicity was observed at the concentrations tested compared to MOA4 and MOA9 compounds.

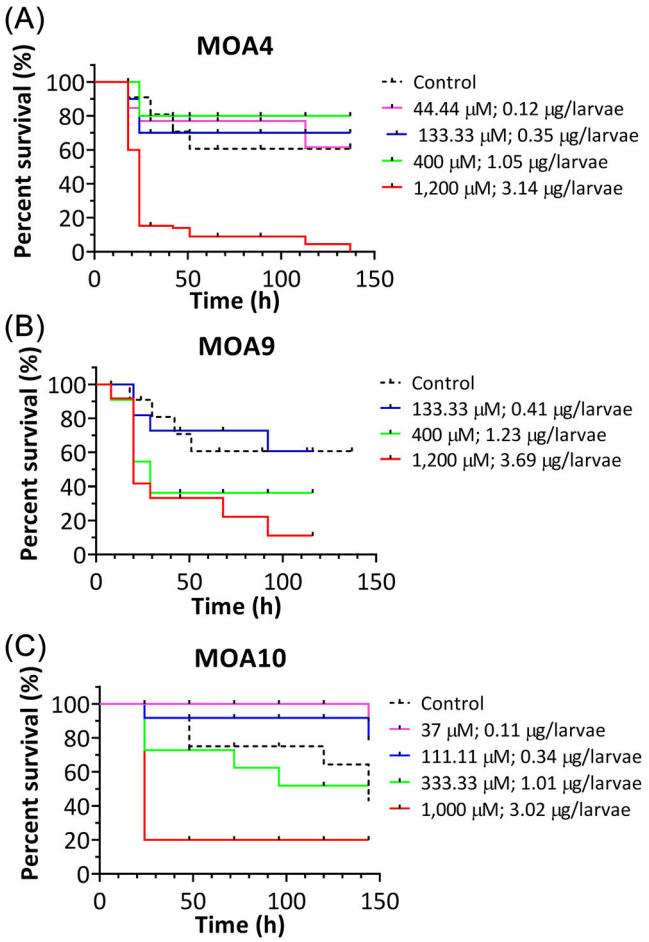

FIGURE 7 | Survival rates of *G. mellonella* larvae treated with different concentrations of (A) MOA4, (B) MOA9, and (C) MOA10 compounds. Survival analysis rates were determined using the Kaplan–Meier estimator, and the data came from the means of two independent experiments, and the SD is < 10%.

3.2.2 | Development of a *B. fragilis* Infection Model in *G. mellonella* Larvae

Since the use of *G. mellonella* to evaluate the infection produced by the gut pathogen *B. fragilis* or to search for new drugs against *B. fragilis* has not been described yet, it was necessary to establish this infection completely in a pharmacological model for *B. fragilis* based on *G. mellonella*.

The infection process, defined as the entry of bacteria causing diseases into the body resulting in detectable pathological effects, can be easily observed by visual inspection of these worms through the process of melanization, as has been observed for other bacterial pathogens [57, 61, 62]. In this process, worms change from a yellowish/tan coloration to a black spot pattern along the body and, after a while, a completely blackish color once the infection has progressed severely, resulting in death.

First, we determined the minimum CFU/mL of *B. fragilis* necessary to produce a clear and rapid infection of *G. mellonella*

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TABLE 3 | Evaluation of potential synergy between MOA4 and MTZ.

Condition	Drug synergy studies on *B. fragilis* cultures: MIC_{90} (μM)			
	MIC_{90} of MTZ	MIC_{90} of MOA4	MIC_{90} of MOA4 in the presence of subinhibitory concentration of MTZ	$FIC_{MOA4} = \frac{MIC_{90}\ (MOA4+MTZ)}{MIC_{90}\ (MOA4)}$
1/2× MTZ + MOA4 Nontoxigenic strain	1.2	115	71	0.62
1/2× MTZ + MOA4 Enterotoxigenic strain	4.1	120	85	0.71
1/4× MTZ + MOA4 Nontoxigenic strain	1.2	115	80	0.7

Note: Of note, only data where possible synergy could exist were analyzed. The remaining conditions, where it was clear that there was no synergy, are not represented.

TABLE 4 | Comparison of the percentage of survival of *G. mellonella* after injection of different compound concentrations at two time points.

	Compound concentration	Percentage of survival (%) at 24 h	Percentage of survival (%) at 48 h
MOA4	Control: 0 μg/mL	90	70
	12 μg/mL; 0.12 μg/larva(44.44 μM)	78	78
	35 μg/mL; 0.35 μg/larva(133.33 μM)	70	70
	105 μg/mL; 1.05 μg/larva(400 μM)	80	80
	314 μg/mL; 3.14 μg/larva(1200 μM)	17	15
MOA9	Control: 0 μg/mL	90	70
	41 μg/mL; 0.41 μg/larva(133.33 μM)	83	74
	123 μg/mL; 1.23 μg/larva(400 μM)	55	37
	369 μg/mL; 3.69 μg/larva(1200 μM)	40	36
MOA10	Control: 0 μg/mL	90	70
	11 μg/mL; 0.11 μg/larva(37 μM)	100	100
	34 μg/mL; 0.34 μg/larva(111.11 μM)	92	92
	101 μg/mL; 1.01 μg/larva(333.33 μM)	73	73
	302 μg/mL; 3.02 μg/larva(1000 μM)	20	20

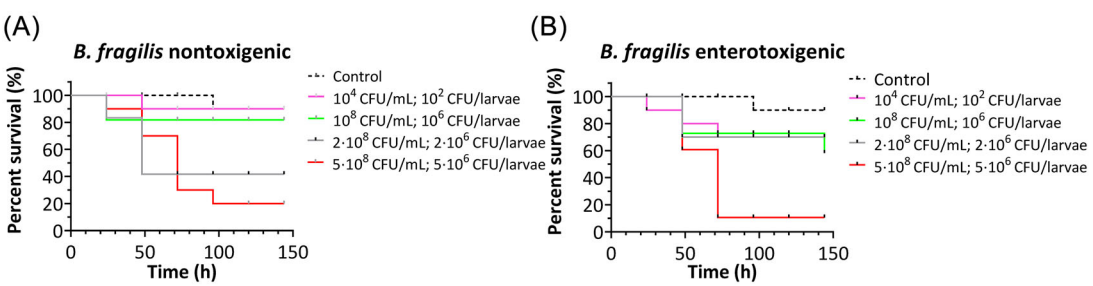

FIGURE 8 | Survival rates of *G. mellonella* larvae infected with different doses of (A) nontoxigenic and (B) enterotoxigenic *B. fragilis* injections. Analysis of survival rate was determined using the Kaplan–Meier estimator, and the data came from the means of three independent experiments, and the SD is < 10%.

larvae (Figure 8 and Table 5). For this purpose, an injection of 10 μL of a series of bacterial suspensions containing $5{\cdot}10^8$, $2{\cdot}10^8$, 10^8, 10^4, and 0 CFU/mL (control) (i.e., $5{\cdot}10^6$, $2{\cdot}10^6$, 10^6, 10^2, and 0 CFU/larva), followed by an additional injection of 10 μL 1× PBS injection to simulate a treatment, was carried out in each worm. Figures S5–S8 summarize the 24 h monitoring of the different conditions tested in the worms (Supplementary Material).

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TABLE 5 | Comparison of the percentage of survival at different bacterial concentrations and time points.

	Bacterial concentration	Percentage of survival (%) at 24 h	Percentage of survival (%) at 48 h
***B. fragilis* nontoxigenic**	Control: 0 CFU/larva	100	100
	10^4 CFU/mL; 10^2 CFU/larva	100	90
	10^8 CFU/mL; 10^6 CFU/larva	83	83
	$2{\cdot}10^8$ CFU/mL; $2{\cdot}10^6$ CFU/larva	83	42
	$5{\cdot}10^8$ CFU/mL; $5{\cdot}10^6$ CFU/larva	89	70
***B. fragilis* enterotoxigenic**	Control: 0 CFU/larva	100	100
	10^4 CFU/mL; 10^2 CFU/larva	90	79
	10^8 CFU/mL; 10^6 CFU/larva	100	72
	$2{\cdot}10^8$ CFU/mL; $2{\cdot}10^6$ CFU/larva	100	70
	$5{\cdot}10^8$ CFU/mL; $5{\cdot}10^6$ CFU/larva	100	61

At the maximum concentration ($5{\cdot}10^8$ CFU/mL; i.e., $5{\cdot}10^6$ CFU/larva) of any of the nontoxigenic or enterotoxigenic strains of *B. fragilis*, a reduction in the survival rate of 40% of the worms at 48 h was observed, after which the survival rate dropped to 20%–10% over the next 48 h; in fact, 80%–90% of the infected worms were dead within 72 h of infection. Lower concentrations of bacteria ($2{\cdot}10^8$, 10^8, 10^4 CFU/mL; i.e., $2{\cdot}10^6$, 10^6, 10^2 CFU/larva) resulted in higher survival rates after 48 h, independent of the strain used. In the case of uninfected controls (injected with 10 μL of 1× PBS, i.e., 0 CFU/mL; 0 CFU/larva), no significant reduction in survival rate was observed over the time period analyzed (with survival rates of approximately 90% at the end of the assay).

Altogether, we observed a dose-dependent effect, thus confirming that *G. mellonella* can be successfully used as an animal model to study infections caused by the gut pathogen *B. fragilis*.

3.2.3 | Treatment of Infected *G. mellonella* Larvae with MOA Compounds

Finally, the antibacterial effects of MOA4, MOA9, and MOA10 on *G. mellonella* worms infected with *B. fragilis* strain were tested. The goal was to assess whether the compounds were able to reduce the toxicity associated with *B. fragilis* infection in infected worms, compared to untreated infected control worms.

To this end, an initial 10 μL injection of 10^9 CFU/mL (10^7 CFU/larva) of either ETBF or NTBF *B. fragilis* was performed on worms, followed by a second 10 μL injection of the MOA4, MOA9, and MOA10 compounds at a fixed concentration (200 μM). Figures S9–S12 summarize the 24 h monitoring of the different conditions tested in the worms (Supplementary Material).

As it can be observed (Figure 9 and Table 6), in the case of ETBF, survival rate of infected worms treated with 200 μM MOA4 (i.e., 52 μg/mL; 0.52 μg/larva) was around 80% after 122 h, while that for untreated infected worms (controls) was only 20% after 24–48 h, with a rapid onset of melanization. Therefore, administration of MOA4 increased four-fold survival of infected larvae (from 20% to 80%), increasing the average survival time by five-fold. Similar results were obtained when worms were infected with the nontoxigenic strain and treated with MOA4; however, the survival rate was slightly higher. In the case of larvae treated with 200 μM MOA9 (i.e., 61 μg/mL; 0.61 μg/larva), in those infected with the nontoxigenic strain, there was a moderate survival increase compared to the control group (MOA9 treated worms had a 30% survival increase after 24 h and 10% survival increase after 96 h), but in those infected with the enterotoxigenic strain, a higher survival rate of the MOA9 treated worms was observed (30% from 24 to 144 h). Therefore, MOA9 also increased the average survival time four-fold, and the activity of this compound seems to be strain-dependent. For larvae infected with NTBF and treated with 200 μM MOA10 (i.e., 60 μg/mL; 0.6 μg/larva), there was an increase in survival (30%–40%) at 48 h in comparison with untreated controls, but it reached similar values to those of the control group at 144 h.

Because MOA4 was less toxic in worms and caused greater inhibition of bacterial growth, it was tested at a lower concentration in infected worms (Figure 10 and Table 7). When larvae were infected with 10^7 CFU/mL (10^5 CFU/larva) *B. fragilis* and treated with 100 μM of MOA4 (i.e., 26 μg/mL; 0.26 μg/larva), the survival rate was 80% in the case of the nontoxigenic strain, and 70% in the case of the enterotoxigenic strain.

Summarizing, MOA4 was the most effective compound, even at concentrations as low as 100 μM (i.e., 26 μg/mL; 0.26 μg/larva), in vivo against *B. fragilis*-infected *G. mellonella* worms, increasing their survival rates compared to untreated infected controls.

4 | Discussion

Antimicrobial resistance (AMR) is a growing global health emergency, mainly due to the continuous increase in demand for antibiotics in many sectors, natural selection of resistant bacteria, irresponsible overuse of drugs, and scarcity of novel antimicrobials with novel mechanisms of action [63]. In fact, the WHO has long warned of the enormous problem of multidrug-resistant bacteria, which are causing (and will cause) more deaths worldwide. Specifically, it is predicted that the world could

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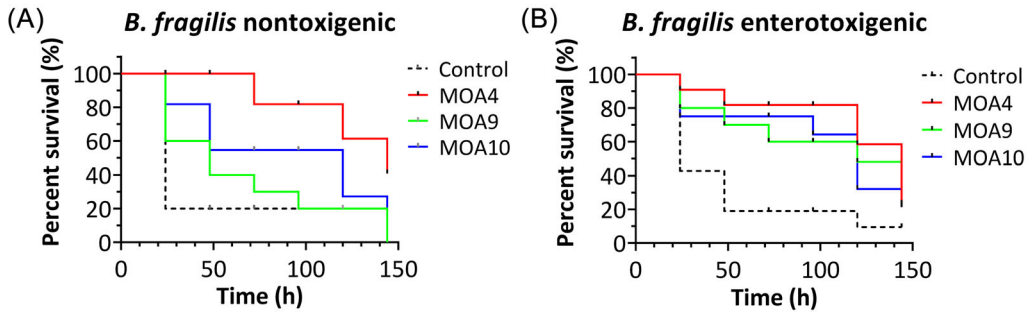

FIGURE 9 | Survival rates of *G. mellonella* larvae infected with 10^9 CFU/mL (10^7 CFU/larva) of (A) nontoxigenic or (B) enterotoxigenic *B. fragilis* strains, and treated with 200 μM of MOA4 (i.e., 52 μg/mL; 0.52 μg/larva), 200 μM of MOA9 (i.e., 61 μg/mL; 0.61 μg/larva), or 200 μM of MOA10 (i.e., 60 μg/mL; 0.6 μg/larva). Analysis of survival rate was determined using the Kaplan–Meier estimator, and the data came from the means of two independent experiments, and the SD is < 10%.

TABLE 6 | Comparison of the percentage of survival at 200 μM of compound, 10^9 CFU/mL (10^7 CFU/larva) of *B. fragilis*, and different time points.

	Compound	Compound concentration	Percentage of survival (%) at 24 h	Percentage of survival (%) at 48 h
***B. fragilis* nontoxigenic (10^9 CFU/mL; 10^7 CFU/larva)**	Control	0 μg/mL	20	20
	MOA4	52 μg/mL; 0.52 μg/larva (200 μM)	100	100
	MOA9	61 μg/mL; 0.61 μg/larva (200 μM)	60	40
	MOA10	60 μg/mL; 0.6 μg/larva (200 μM)	82	55
***B. fragilis* enterotoxigenic (10^9 CFU/mL; 10^7 CFU/larva)**	Control	0 μg/mL	44	20
	MOA4	52 μg/mL; 0.52 μg/larva (200 μM)	92	83
	MOA9	61 μg/mL; 0.61 μg/larva (200 μM)	90	72
	MOA10	60 μg/mL; 0.6 μg/larva (200 μM)	75	75

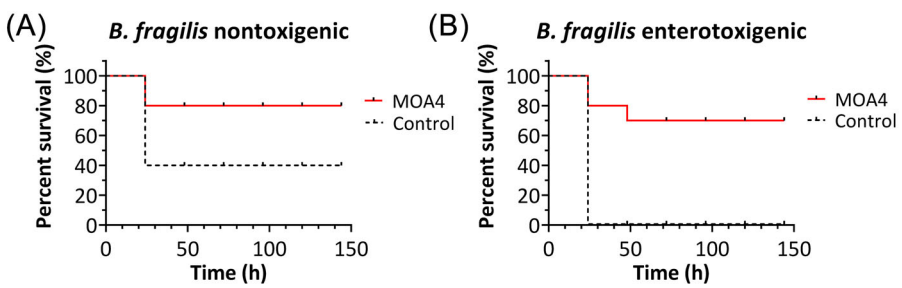

FIGURE 10 | Survival rates of *G. mellonella* larvae after the infection with 10^7 CFU/mL (10^5 CFU/larva) of (A) nontoxigenic and (B) enterotoxigenic *B. fragilis* strains and treated with 100 μM MOA4 (i.e., 26 μg/mL; 0.26 μg/larva). Analysis of survival rate was determined using the Kaplan–Meier estimator, and the data came from the means of two independent experiments, and the SD is < 10%.

enter a post-antibiotic era where infections might become more frequent and any minor injury could lead to death or severe disability if antibiotic resistance is not handled in a timely manner [64]. Moreover, the situation is expected to worsen in the long run if we do not act properly: up to 8.2 million people could die annually from AMR by 2050 [65]. Therefore, reducing the burden of AMR by making informed and location-specific policy decisions regarding control programs to prevent infections, access to essential antibiotics, and research on the development of new antibiotics, as well as vaccines, are crucial.

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TABLE 7 | Comparison of the percentage of survival at 100 μM of MOA4, 10^7 CFU/mL (10^5 CFU/larva) of *B. fragilis*, and different time points.

	Compound	Compound concentration	Percentage of survival (%) at 24 h	Percentage of survival (%) at 48 h
***B. fragilis* nontoxigenic (10^7 CFU/mL; 10^5 CFU/larva)**	Control	0 μg/mL	40	40
	MOA4	26 μg/mL; 0.26 μg/larva (100 μM)	80	80
***B. fragilis* enterotoxigenic (10^7 CFU/mL; 10^5 CFU/larva)**	Control	0 μg/mL	0	0
	MOA4	26 μg/mL; 0.26 μg/larva (100 μM)	80	70

In the context of AMR, *Bacteroides* spp. "have the most antibiotic-resistant mechanism and the highest resistance rates of all anaerobic pathogens" [1]. Appropriate use of effective treatments is essential for favorable clinical outcomes in infections caused by *B. fragilis*, a principal component of the human gut microbiota. Bacteria of this species are commonly exposed to different antimicrobials and antibiotics and have evolved from being susceptible to become resistant to a broad spectrum of antimicrobial agents [8, 66].

Importantly, there is a central consideration to be made: administering a broad-spectrum antibiotic may tackle the *B. fragilis* infection, but it may also affect the beneficial microbiota as well, facilitating the colonization of the gut by opportunistic microorganisms causing severe gastrointestinal diseases. Therefore, there is an urgent need to develop species-specific antimicrobial treatments, or to rethink or redesign the existing ones; along with that, the development of antivirulence compounds would be helpful in order to achieve specific inhibition of, for example, BFT to combat ETBF-mediated pathogenicity without disturbing the beneficial commensal microbiota [24–33]. Therefore, there is an indisputable need to better understand ETBF virulence mechanisms and factors, such as BFT-3, to design highly specific antimicrobials and antivirulence compounds to tackle them [8, 66, 67] and to develop biotechnological tools to drive and boost drug discovery efforts.

In our previous study, using large-scale compound screening, biophysical assays, in vitro and cell-based activity assays, and X-ray crystallography, we identified three FDA-approved small molecule drugs (flumequine—MOA4, foliosidine—MOA9, and hesperetin—MOA10) that target and inhibit BFT-3 in a dose-dependent manner through a novel allosteric mechanism of action based on the zinc-dependent conformation of BFT-3 described in our group for the first time [40]. Thus, these inhibitors do not bind to the active site but to an exosite far from the active site, stabilizing the inactive conformation. Considering that they could be repositioned as drugs for new therapeutic indications, preclinical studies are needed to evaluate their antimicrobial efficacy against *B. fragilis* in more complex scenarios. Therefore, we characterized the effect of these compounds in *B. fragilis* cultures and developed a new infection animal model to test these compounds in vivo.

Analysis of MIC_{90} showed that MOA4, MOA9, and MOA10 have antimicrobial effects on both strains (enterotoxigenic and nonenterotoxigenic) of *B. fragilis*. MOA4 was the compound that most reduced the bacterial growth, as estimated from both MIC_{90} and TKA assays, and, therefore, being the most effective compound (out of the three compounds selected in our screening) against both strains of *B. fragilis*. This is in agreement with the fact that MOA4 is an approved FDA antimicrobial for the treatment of bacterial infections (but not previously related to the gut pathogen *B. fragilis*).

Currently, due to the steadily increasing antibiotic resistance and shortage of antimicrobial development, there are limited therapeutic options for treating multidrug-resistant microorganisms. In this context, in addition to repurposing already approved drugs, synergistic studies and combination therapies are commonly employed to manage bacterial infections. We proposed to analyze if the most effective compound, MOA4, presented synergy when combined with the antibiotics cefoxitin (FOX) and metronidazole (MTZ), which are currently used in clinics for treating *B. fragilis* gut infections [59, 60]. The MIC_{90} determination allowed us to compare our *B. fragilis* strains with those of the same species reported in the literature (Figure S13). Based on this comparison, we concluded that our two strains of *B. fragilis* had a susceptibility pattern consistent with those reported in the literature for the antimicrobials MTZ and FOX (Table S2). Analysis of synergy revealed that the combination of MTZ with MOA4 showed a boosting effect at high concentrations of MTZ, although it could not qualify as a synergistic effect according to standard criteria. Further evaluation is needed, but this potentiating effect of MTZ on MOA4 underscores a new potential therapeutic scenario for combating multidrug-resistant strains.

To confirm whether the effects of these compounds in *B. fragilis* cultures were translated into in vivo efficacy, further studies are needed. Over the past decades, murine models have been the gold standard for studying microbial infections. In recent years, murine models have been the focus of ethical concerns in the entire scientific community, which advocates reducing the use of animals for research [43–45]. Furthermore, the use of these animals requires expensive infrastructure as well as lengthy and laborious protocols. As a bridge between in vitro studies and studies in mammals is necessary, alternative nonmammalian host models, such as *Acanthamoeba castellanii* (amoebae), *Artemia salina* (brine shrimp), *Caenorhabditis elegans* (roundworm), *Danio rerio* larvae (zebra fish), *Drosophila melanogaster* (fruit fly), and *Galleria mellonella* (greater wax moth), have been employed [46, 47].

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Since 2016, there has been a 42% increase in the use of the *G. mellonella* waxworm as an infection model for studying many pathogens and searching for new antimicrobial drugs [47]. A key point is the fact that the larval immune system of *G. mellonella* has remarkable functional and structural similarities with the innate immune response in mammals, including phagocytic cells and the production of antimicrobial peptides and both reactive oxygen and nitrogen species [48, 49]. These waxworms possess hemolymph, which is analogous to mammalian blood and contains immune cells called hemocytes. These cells can be compared to neutrophils in mammals in terms of their ability to phagocytose and kill pathogens by producing superoxide [49]. In addition, *G. mellonella* hemolymph cells produce soluble effector molecules, such as complement-like proteins (opsonins), melanin, and antimicrobial peptides [50], which are implicated in humoral responses. Hence, given that both *G. mellonella* and mammals are able to activate cellular and humoral mechanisms of defense against microbial pathogens, it is not surprising that there is a significant correlation between them, making *G. mellonella* an effective and appropriate bridge between in vitro and in vivo (mammals) infection models [45, 46, 49, 68, 69 70] (more information about *G. mellonella* as animal model is detailed in Annex I of Supplementary Material).

As already mentioned, the use of the *G. mellonella* waxworm as an infection model organism for studying many pathogens and searching for new drugs has greatly increased in recent years [47]. Currently, there are no published studies involving *G. mellonella* as an animal model of infection with the gut pathogen *B. fragilis*. Therefore, in this study, we developed a new infection animal model of *B. fragilis* infection using these worms and validated the antimicrobial activity of previously identified compounds.

For this purpose, we first evaluated the intrinsic toxicity of MOA4, MOA9, and MOA10 in *G. mellonella* larvae. Analysis of survival rates of *G. mellonella* larvae after administration of the compounds at different concentrations showed that MOA4 was the least toxic compound out of the three, with worms tolerating doses up to 400 μM (≤ 105 μg/mL for MOA4, ≤ 123 μg/mL for MOA9, and ≤ 121 μg/mL for MOA10). *G. mellonella* larvae were more susceptible to MOA9 and MOA10, with the highest tolerated doses of approximately 100–200 μM (31–61 μg/mL for MOA9, and 30–60 μg/mL for MOA10). We confirmed that the toxicity was dose-dependent.

After establishing the appropriate doses for the compounds, it was necessary to determine the appropriate number of CFU/mL (i.e., minimal dosage in CFU/larva) needed to induce a clear and rapid infection in *G. mellonella* worms. Analysis of the survival rates confirmed that at higher CFU/larva, lower survival rates were achieved compared with the uninfected controls. In particular, in the case of the nontoxigenic *B. fragilis* strain, a reduction in survival rates was observed for doses between 1 and $2{\cdot}10^6$ CFU/larva at 24–48 h; and in the case of the enterotoxigenic *B. fragilis* strain, a decrease in survival rates was observed for 10^6 CFU/larva. Therefore, a dose-dependent correlation was observed in both strains. Altogether, we implemented a new animal model for infection of the gut pathogen *B. fragilis* using *G. mellonella* larvae.

Next, we analyzed whether, after infecting *G. mellonella* larvae with *B. fragilis* cultures, treatment with the selected compounds MOA4, MOA9, or MOA10 could result in a reduction in the gut infection process. This assay was performed at fixed concentrations of MOA4, MOA9, and MOA10, and both strains of *B. fragilis*. Ideally, infected and untreated control larvae should have the lowest survival rates. In the case of MOA4, a much higher survival rate than that of the control was observed upon infection with any of the bacterial strains, indicating that the effects of infection were greatly reduced when the worms were treated with MOA4. In the case of infected worms treated with MOA9, differences were observed between strains because although small differences were observed in the nontoxigenic strain with respect to the control, a higher survival rate was observed in the larvae infected with the enterotoxigenic strain, indicating a strain-dependent effect for MOA9. In the case of treatment with MOA10, there was a slight increase in survival rates in larvae infected with the nontoxigenic strain, but much less than that of larvae treated with MOA4, and the increase in survival rate was more evident in those infected with the enterotoxigenic strain.

Because MOA4 was less toxic for *G. mellonella* worms, and it also produced a higher growth inhibition in bacterial cultures, MOA4 was tested at a lower concentration in worms to evaluate whether similar survival rates could be observed at a lower dose. And, as expected, a 100 μM MOA4 treatment, that is, 26 μg/mL; 0.26 μg/larva, also increased survival rates in larvae infected with any of both strains.

An important aspect to highlight is that in all experiments involving *G. mellonella*, two injections were required to compare results between conditions and assays (i.e., compound toxicity, effectiveness of infection, and compound activity), one injection corresponding to the infection, and the other injection corresponding to the treatment. In the case of the assay to determine the minimal dose of bacteria to trigger infection, a mock treatment injection with 1× PBS was made, and in the case of the assay to determine the maximal tolerated compound concentration, a mock bacterial infection with 1× PBS was made. Therefore, there were always two punctures, which allowed a good comparison between assays and always produced the same level of potential physiological damage or response in worms. Oral bacterial inoculation and compound administration through food or water is a possibility that we considered at the beginning. However, this would result in high variability among specimens, which must be avoided when assessing the performance of the animal infection model. That is why we relied on direct inoculation and administration by injection to guarantee reproducibility.

Focusing on MOA4, we have evidence of BFT-3 inhibition at concentrations that do not significantly affect the growth of *B. fragilis*. According to our previous data, the dissociation constant for the direct interaction of BFT-3 and MOA4 is around 10 μM, which is close to the IC_{50} observed in in vitro enzyme assays [40]. Furthermore, in cell assays, we observed that MOA4 completely abolished E-cadherin processing [40]. On the other hand, we observed that the MIC_{90} for MOA4 is a bit higher than 100 μM, and this concentration did not significantly affect bacterial growth according to time-kill assays. Therefore, no bacteriostatic

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or bactericidal effects were observed at concentrations that completely suppressed BFT-3 activity. These data indicate that MOA4 suppresses BFT-3 activity independently of bacterial viability, that is, toxin inhibition is well separated from antibacterial effects. Then, MOA4 could be a promising candidate as a therapeutic agent for neutralizing the harmful effects of the *B. fragilis* toxin.

We have conducted experiments in which cells were treated with BFT-3 and showed quick morphological changes (round shape and loss of intercellular adhesion), which could be explained by the cleavage of E-cadherin on the surface of the cells. This is something previously reported [14]. In our case, when the compounds were administered, the effect induced by BFT-3 was absent or delayed, confirming their inhibitory effect on BFT-3. Therefore, secretion of BFT-3 may induce some changes resulting in loss of intracellular adherence and generation of an environment facilitating the colonization by *B. fragilis* and even the possible migration of cells (as it would occur in metastatic tumors) [22].

From the presented results, we can conclude that (1) a new animal model for *B. fragilis* infection using *G. mellonella* has been established, which could be useful for identifying and testing potential antimicrobial candidates against this gut pathogen; (2) MOA4, MOA9, and MOA10 are reasonably well tolerated by *G. mellonella*; and (3) MOA4, MOA9, and MOA10 possess antibacterial activity in vitro in bacterial cultures and in vivo in the *G. mellonella* model. These compounds represent promising candidates for either direct repurposing for therapeutic treatment of *B. fragilis* chronic infection, diminishing the risk of inflammation and colorectal cancer development, or, given their small molecular mass, further optimization to improve their affinity for BFT-3, increase their antimicrobial activity, and decrease their toxicity.

In summary, the identified compounds, particularly MOA4, hold promise for integration into clinical strategies. Their ability to inhibit BFT-3 suggests their potential as adjuvants with standard antibiotics, enhancing efficacy while minimizing AMR by targeting virulence rather than bacterial growth. This dual approach could reduce infection severity and preserve the gut microbiome balance, warranting further preclinical evaluation.

Author Contributions

A.J.-A. performed the experiments. M.G.-L. and J.A.A. supervised the work with the *B. fragilis* cultures. H.J. and S.V. contributed to experiments. A.J.-A., A.V.-C, and O.A. wrote the manuscript with input from all other authors. A.J.-A., M.G.-L., J.A.A., A.V.-C., and O.A. revised the manuscript. All the authors approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no competing financial interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors (A.V.-C. or O.A.), upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Material: nyas70255-sup-0001-SuppMat.pdf

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