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Evaluation of Changes Induced in the Probiotic *Escherichia coli* M17 Following Recurrent Exposure to Antimicrobials

M. J. A. Mbarga[1*], I. V. Podoprigora[1], E. G. Volina[1], A. V. Ermolaev[1] and L. A. Smolyakova[1]

[1]Department of Microbiology and Virology, Institute of Medicine, RUDN University, Moscow, Russia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: It is already well known that the exposure of certain bacteria, pathogenic or not, to antimicrobials is likely to increase their virulence and induce the development of direct or cross resistance to antimicrobials, but there is almost no information available regarding probiotics.
Aim: To assess the changes induced in susceptibility to antibiotics, biofilm formation, growth rate and relative pathogenicity in the probiotic *Escherichia coli* M17 (EC-M17) after long exposure to antimicrobials namely ampicillin, kanamycin, cefazolin and silver nanoparticles (AgNPs).
Methods: After determining the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the 4 antimicrobials above-mentioned by the microdilution method, EC-M17 was exposed to increasing subinhibitory doses ranging from MIC/8 to MIC for 8 days. The susceptibility to antibiotics of the mutants obtained was assessed by the Kirby Bauer disc diffusion method, biofilm formation by the Congo red agar method and with crystal violet bacterial attachment assay, and relative pathogenicity was assessed using a *Galleria melonella* waxworm model.
Results: Exposure to antimicrobials induces noticeable changes in EC-M17. The highest adaptation to antimicrobials was observed on AgNPs with 8-fold increase in MIC and 16-fold

**Corresponding author: E-mail: josepharsenembarga@yahoo.fr;*

increase in MBC of AgNPs. EC-M17 exposed to ampicillin, kanamycin and silver nanoparticles became resistant to ampicillin, ceftazidime, ceftazidime/clavulanate and tetracycline while exposure to cefazolin induced a significant decrease in sensitivity to tetracycline and ampicillin and resistance to ceftazidime/clavulanate and ceftazidime. The strain exposed to ampicillin was the only one to produce more biofilm than the control strain and except the EC-M17 exposed to cefazolin, all other EC-M17 strains were more pathogenic on *G. melonella* model than the control.

Conclusion: Data in this investigation suggest that repeated exposure of the probiotic EC-M17 to antimicrobials may induce changes in antimicrobials susceptibility, biofilm formation, growth rate, and relative pathogenicity. Therefore, as far as possible, the probiotic *E. coli* M17 should not be used in combination with antibiotics and further investigations are required to expand similar work on more probiotics in order to avoid resistance build-up which might be transmitted by horizontal transfer.

Keywords: Escherichia coli M17; susceptibility to antibiotics; biofilm formation; growth rate; motility and pathogenicity.

1. INTRODUCTION

The probiotic *Escherichia coli* M17 (EC-M17) was identified in Russia more than 90 years ago. EC-M17 is a non-pathogenic bacterium [1,2] of the family Enterobacteriaceae and a non-spore-forming gram-negative rod of the serotype O2 with flagellar antigen H type 41 [2]. EC-M17 should not be confused with *E. coli* Nissle 1917. Although no direct comparison has yet been made to establish the differences between these two strains, Kokesova et al. [3] and Fitzpatrick et al. [2] reported that Nissle strain differs from M17 in both serotype (O6 v. O2 for M17) and flagellar H antigen (H1 v. H41). The parent strain EC-M17 was deposited under the designation ATCC 202226 in the American Type Culture Collection [2].

EC-M17 is used in both humans and animals for its beneficial effects on the gastrointestinal tract [4]. This strain is particularly known for its effectiveness against a variety of gastrointestinal problems, including inflammatory bowel disease (ulcerative colitis or Crohn's disease) and diarrhea in infants and children [2,5]. EC-M17 has been reported to have had a positive result in the treatment of dysentery in thousands of individuals [6,7]. Moreover, the toxicity, immunogenic properties and protective activity of the live culture of EC-M17 and antigenic preparations obtained from cell suspensions of this strain have been studied by Levina et al. [3], and the results showed the capacity of EC-M17 to induce the production of 02 and H6 antibodies and to play a significant immunomodulatory role in rabbits. In addition, a study conducted by Fitzpatrick et al. [2] demonstrated the beneficial effect of EC-M17 combined with an antibiotic (metronidazole) on the immunomodulation and attenuation of murine colitis, probably due to an inhibitory effect on NF-kB signaling. However, this study did not attempt to assess the consequences that such a combination could have on EC-M17 itself. Indeed, several recent studies have reported that microorganisms, including *E. coli,* whether pathogenic or not, are likely to develop resistance to antibiotics and increase the expression of their virulence factors following exposure to biocides and antimicrobials more globally [8-13].

Since there is almost no information on the potential impacts of antimicrobials on probiotics, this investigation aimed to assess the changes induced in susceptibility to antibiotics, biofilm formation, growth rate and relative pathogenicity in the probiotic *Escherichia coli* M17 (EC-M17) after long exposure to antimicrobials namely ampicillin, kanamycin, cefazolin and silver nanoparticles (AgNPs).

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

In this investigation, we used the standard strain *Escherichia coli* M17 provided by the laboratory of microbiology and virology of the Peoples' Friendship University of Russian. All the cultures were made on BHIB (Brain Heart Infusion Broth) (HiMedia™ Laboratories Pvt. Ltd., India) and Muller Hinton Agar (MHA HiMedia™ Laboratories Pvt. Ltd., India) and incubated aerobically at 37°C for 18-24h.

2.2 Stocks Solution of Antibiotics and AgNPs

Stock solutions of each antimicrobial were prepared at a concentration of 1024 µg/ml and dilutions were made as needed. Ampicillin, cefazolin and kanamycin were prepared in physiological water (NaCl 0,9%) and 2 nm silver nanoparticles (Nanoserebro Argitos, OOO NPP Sintek Nano, Russia) were prepared in distilled water. All the solutions were sterilized by microfiltration (0.45 µm) prior to use.

2.3 Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC and MBC were determined as previously reported [14]. In brief, two 5-ml overnight cultures of test bacteria were prepared in BHIB prior to overnight incubation (18 to 24h) at 37°C. Cultures were diluted to a turbidity equivalent to 0.5 McFarland, then the stock solutions of antimicrobials (100µL) above-mentioned were submitted to serial twofold dilutions in sterile BHIB (100µL) on U-bottom 96-well microplates prior to addition of the bacterial inoculum (10 µL). The plates were incubated overnight (18 to 24 h) at 37°C. The MIC was defined as the lowest concentration of antimicrobial at which growth was completely inhibited (viewed as turbidity relative to that for a sterile negative control). Further, MBCs were determined by subculturing the wells without visible growth (with concentrations ≥ MIC) on MHA plates. Inoculated agar plates were incubated at 37°C for 24 h. MBC was considered the lowest concentration that did not give any bacterial growth on agar.

2.4 Long-term Exposure of Bacteria to Antibiotics and Silver Nanoparticles

Bacteria were exposed to increasing concentrations of ampicillin, cefazolin, kanamycin and AgNPs using U-bottom 96-well microplates. 8 concentrations of each antimicrobial were prepared in sterile BHIB then the mixture was sterilized by microfiltration prior to use. The concentrations varied from MIC/2 to MIC with an increment of MIC/16. For each antimicrobial, 200 µL of the preparation of MIC/2 concentration was introduced into line 1 of the microplate, then MIC/2 + 2MIC/16 in line 2, MIC/2 + 3MIC/16 in line 3, etc. 15 µL of overnight culture of *Escherichia coli* M17 prepared at a concentration equivalent to 0.5 of McFarland was inoculated in the first line and after 24h of incubation at 37°C, the wells of line 1 were homogenized and 15 µL was transferred to line 2 of the corresponding columns and the same operation was repeated for the following lines until the 8th day. Bacteria passaged 8 times on antimicrobial-free medium were also included and were considered as the controls. During incubation, the microplates were placed in a container containing distilled water to limit water loss by evaporation. After successive passages, the bacteria were kept at −80°C in cryovials (Cryoinstant; Deltalab, Spain) for subsequent testing.

2.5 Sensitivity of Bacteria to Antibiotics

The modified Kirby-Bauer's disc method described by Mbarga et al. [15] was used to assess the sensitivity to antibiotics of the original *Escherichia coli* M17 and the mutant strains obtained. Briefly, after bringing the bacteria to room temperature, they were cultured at 37°C for 24 hours in sterile BHIB. 1.5 ml of each overnight culture was centrifuged (Eppendorf Centrifuge 5415 R) for 10 minutes at 3000 RCF and the pellet was collected, washed 3 times with Phosphate buffer saline (PBS) and resuspended in 5 ml of physiological water to obtain a concentration equivalent to 0.5 McFarland. 100 µL of the culture was plated on Muller Hinton Agar (MHA) (HIMEDIA®, Ref 173-500G) and the antibiotic discs were placed aseptically using a dispenser. After 18-24 hours of incubation, the inhibition diameters were measured and interpreted referred to the Clinical & Laboratory Standards Institute [16]. The petri dishes were again incubated for 48 hours at 37°C and the bacteria of the second growth in the inhibition zones were isolated and subjected to a second antibiogram as described above. The 11 antibiotics used were: tetracyclin (TE), 30 µg/disc, ceftazidime/clavulanic acid (CAC), 30/10 per disc; ceftazidime (CAZ), 30 µg/disc; ceftriaxone (CTR), 30 µg/disc; ciprofloxacin (CIP), 30 µg/disc; imipenem (IMP), 10 µg/disc; nitrofurantoin (NIT), 200 µg/disc; trimethoprim (TR), 30 µg/disc; Fosfomycin (Fo), 200 µg/disc; Amoxicillin (AMC), 30 µg/disc and ampicillin (AMP), 25 µg/disc.

2.6 Evaluation of Biofilm Formation by Crystal Violet Bacterial Attachment Assay

The biofilm formation of original strains and exposed strains was assessed in sterile 96-well microtiter plate. 200µL of sterile BHIB was

introduced in each well and was inoculated by the corresponding overnight culture (18 to 24 h at 37°C and 100 rpm) centrifugated and resuspended in physiological water to obtain a turbidity equivalent to 0.5 of McFarland as described above. Sterile controls were also included. The plates were incubated statically for 48 h at 37°C. 100µL of the medium was transferred in the corresponding well in another microtiter plate for planktonic measurement. The remaining medium was removed from the wells and replaced with 200µl of 1% (w/v) crystal violet solution during 90s. The wells were rinsed three times with distilled water prior to drying at 37°C. The biofilm-bound crystal violet was solubilized in 200 µl of 100% ethanol and the A450 was determined and compared with the negative controls. Each test was repeated 6 times and each repeat was read 3 times.

2.7 Planktonic Growth Rate Measurement

Overnight suspensions of original and each adapted strain were diluted 1:10 in BHIB (200ml) and incubated at 37°C with shaking at 150 rpm (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany). Samples were regularly removed and diluted as appropriate. Optical density was measured at 490nm.

2.8 *Galleria melonella* Pathogenesis Assay

Relative pathogenicity was assessed as described by Henly et al. [8] with slight modifications. Briefly, final larval-stage *G. melonella* (ECO BAITS, Moscow, Russia) was stored in the dark at 4°C for less than 7 days, before randomly assigning 20 to each treatment group and incubating at 37°C for 30 min. After this acclimatation phase, aliquots (15µL) of each suspension (eq 0.5 of McFarland) of overnight *Escherichia coli* M17 strains were injected into the hemocele of each larva via the last left proleg. Larvae were incubated at 37°C in sterile petri dishes and the number of surviving individuals was recorded after 12, 24, 48 72 and 96 hours. The group injected with sterile PBS were used as negative controls. The larvae were considered dead when were unresponsive to touch and appeared black

2.9 Statistical Analysis

All experiments were carried out at least in triplicate. The statistical significance was set at $p \leq 0,05$ and all the statistical analysis were carried out using the statistical software XLSTAT 2020 (Addinsof Inc., New York, USA). All the graphs were plotted by Excel software or SigmaPlot 12.5 (Systat Software, San Jose, CA, USA).

3. RESULTS

3.1 Changes in MIC and MBC after Long Exposure to Ampicillin, Cefazolin, Kanamycin and Silver Nanoparticles

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were determined for Escherichia coli M17 before and after exposure to kanamycin, cefazolin, ampicillin and Silver nanoparticles (AgNPs) (Table 1). The results were interpreted as the fold change relative to the original strain. No variation was observed in the MICs and MBCs between the initial *E. coli* M17 and it analogue which underwent 8 repeated passages in the BHIB antimicrobials-free. In contrast, all *E. coli* M17 exposed to antimicrobials developed direct and cross resistance. With regard to the MIC, we observed ≥2-fold increase in 2/4 strains for ampicillin and cefazolin in 3/4 strains for kanamycin and AgNPs. Regarding the MBCs, for ampicillin there was 2-fold increase in bacteria exposed to kanamycin and ampicillin while for Cefazolin there was 4-fold increase for bacteria exposed to cefazolin and 2-fold increase for bacteria exposed to ampicillin and kanamycin. Interestingly, direct adaptation appears to be more significant on *E. coli* M17 exposed to AgNPs with 8-fold increase in MIC and 16-fold increase in MBC of AgNPs.

3.2 Changes in Susceptibility to Other Antibiotics

The original strain *E. coli* M17 and the control (passed in BHIB-antimicrobial free) were both sensitive to all antibiotics tested. However, exposure to antimicrobials has led to significant changes in susceptibility to some antibiotics (Fig. 1). Indeed, *E. coli* M17 strains exposed to ampicillin, kanamycin and silver nanoparticles became resistant to ampicillin, ceftazidime, ceftazidime/clavulanate and tetracycline. In addition, exposure to cefazolin induced a significant decrease in sensitivity to tetracycline and ampicillin and resistance to ceftazidime and ceftazidime /clavulanate. No significant change in susceptibility was observed on Fosfomycin and

trimethoprim while changes occurred in susceptibility to ciprofloxacin, Ceftriaxone, nitrofurantoin and imipenem without causing resistance.

3.3 Biofilm Formation, Growth Rate and Relative Pathogenicity on *G. melonella* Model

As shown in Figs. 2 and 3, the original bacteria and the control were both biofilm producers. However, exposure to kanamycin, cefazolin and AgNPs resulted in decreased biofilm formation while the strain exposed to ampicillin became more biofilm-producing than the original bacteria. Interestingly, as reported by others [17,18] there was a good correlation between the evaluation of biofilm formation on Congo red agar (Fig. 2) and the assessment by crystal violet attachment assay (Fig. 3). In addition, compared to the original strain, *E. coli* M17 exposed to ampicillin grew faster, followed by the strain exposed to kanamycin while all others grew relatively similar to the control (Fig. 4). Finally, the pathogenicity assay performed with the *Galleria melonella* waxworm model showed that exposure to Cefazolin significantly attenuates the potential pathogenicity of *E. coli* M17 while exposure to kanamycin, ampicillin and AgNPs tend to accentuate this pathogenicity (Fig. 5).

Table 1. MICs and MBCs of *Escherichia coli* M17 before and after long exposure to antimicrobials

Exposure	MIC (µg/mL)				MBC (µg/mL)			
	AMP	CZ	Ka	AgNPs	AMP	CZ	Ka	AgNPs
EC M17-Initial	256	4	32	16	256	8	32	16
EC M17-Unexposed	256	4	32	16	256	8	32	16
EC M17-AMP	512	8	64	64	512	16	128	64
EC M17-CZ	256	16	64	16	256	32	128	32
EC M17-Ka	512	8	64	32	512	16	64	32
EC M17-AgNPs	256	4	32	128	256	8	64	256

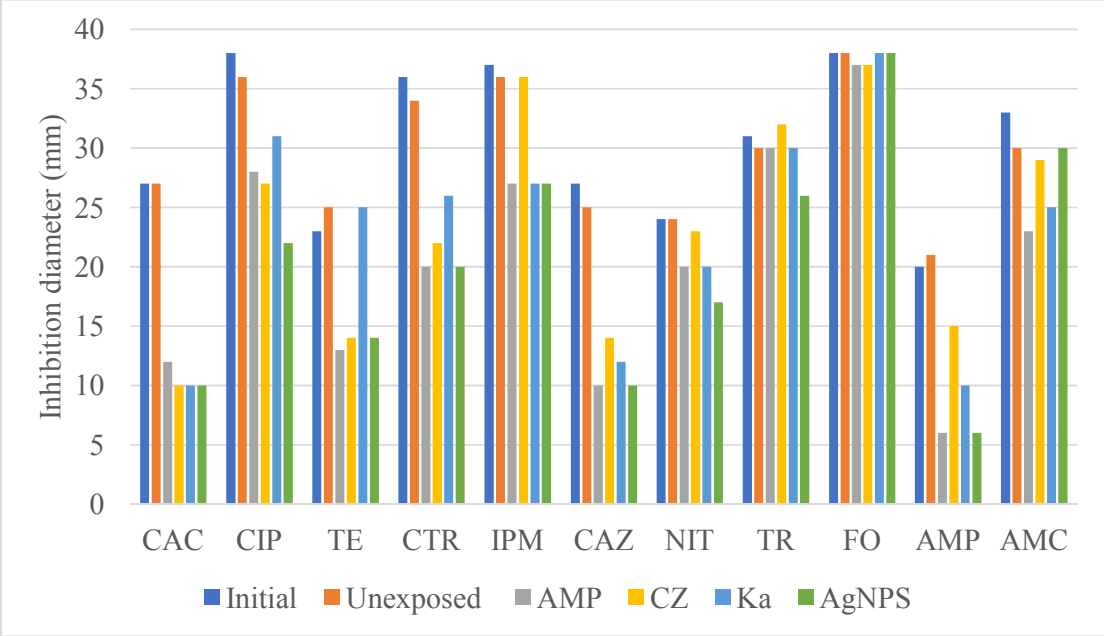

Fig. 1. Susceptibility to antibiotics before and after long exposure to antimicrobials

Data show the mean (rounded the unit) of antibiotic inhibition zones for E. coli M17 and their analogues before and after antimicrobial exposure and represent the results for samples taken from two separate experiments each with three technical replicates. Unexposed =strain passed 8 times in BHIB antimicrobials-free, Ka= exposed to Kanamycin, CZ=cefazolin, AMP= to ampicillin and AgNPs= Silver nanoparticles (AgNPs). Referred to 2-6 for antibiotic names.

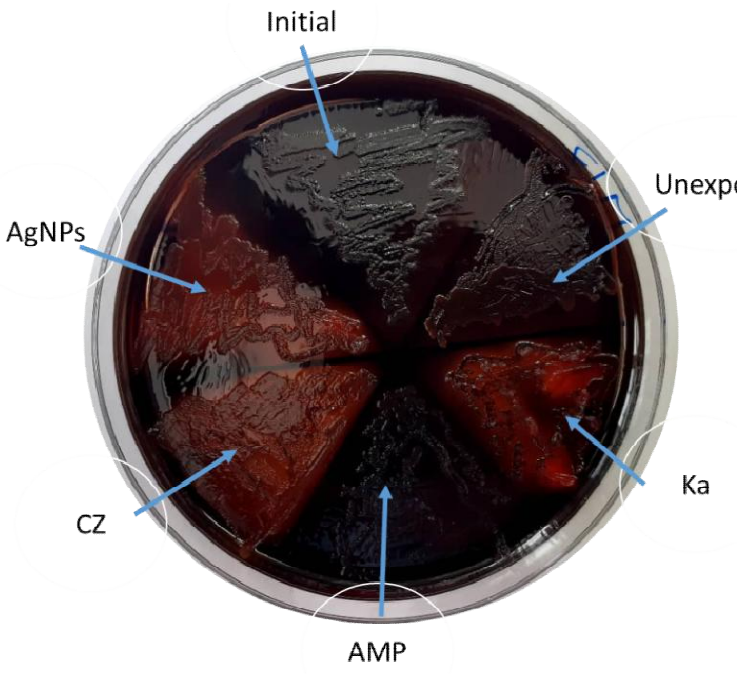

Fig. 2. Biofilm formation on Congo Red Agar of *E. coli* M17 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexp), strains exposed to kanamycin (Ka), cefazolin (CZ), ampicillin (AMP) and Silver nanoparticles (AgNPs)

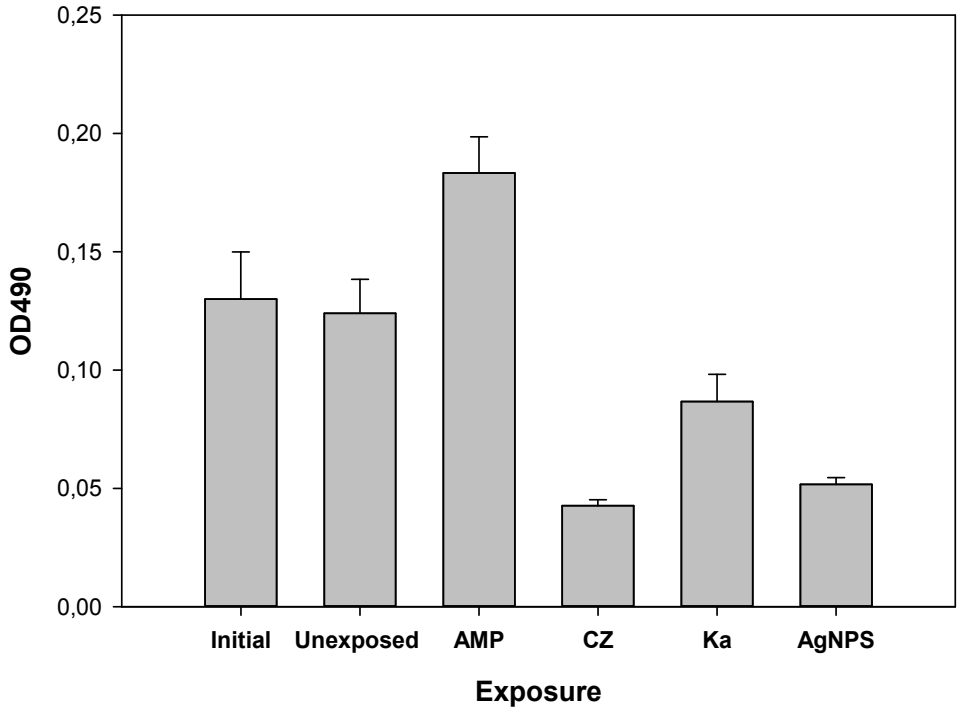

Fig. 3. Biofilm formation of *E. coli M17* parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexposed), strains exposed to kanamycin (Ka), cefazolin (CZ), ampicillin (AMP) and Silver nanoparticles (AgNPs)

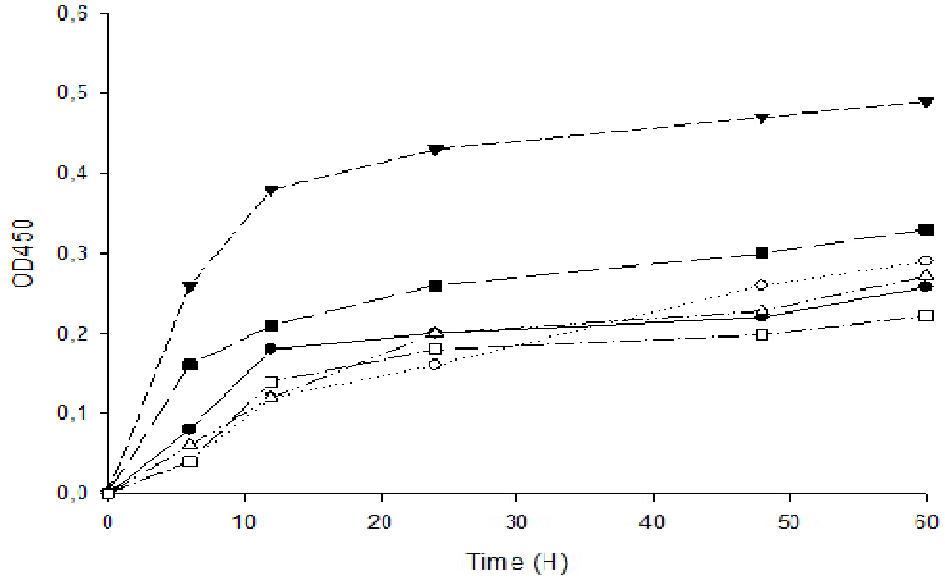

Fig. 4. Planktonic growth rate of *E. coli* M17 parent strain (white circles), strain passed 8 times in BHIB antimicrobials-free (white triangles), strains exposed to kanamycin (black square), cefazolin (white square), ampicillin (black triangles) and silver nanoparticles (black circles)

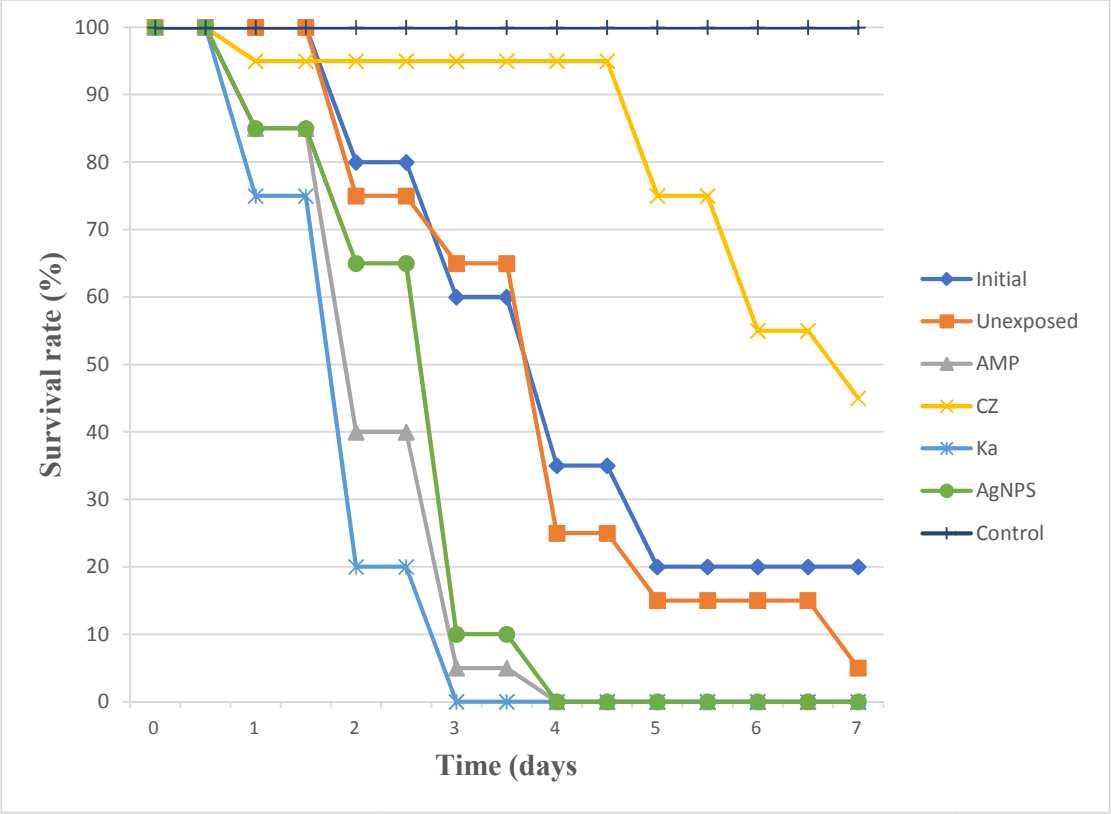

Fig. 5. Relative pathogenicity of *E. coli* M17 parent strain (Initial), strain passed 8 times in BHIB antimicrobials-free (Unexposed), strains exposed to kanamycin (Ka), cefazolin (CZ), ampicillin (AMP) and silver nanoparticles (AgNPs)

4. DISCUSSION

It is very common to determine the susceptibility to antibiotics of probiotics when studying their probiotic suitability [19,20] and for their potential combination with antibiotics [20,21]. However, to date no study in the literature has been focused on the consequences that exposure to antimicrobials could have on probiotics. In this study we evaluated the impact of antimicrobial exposure in the probiotic *Escherichia coli* M17. Data of this investigation suggest that long-exposure of the probiotic *E. coli* M17 to antimicrobials may influence it antimicrobials susceptibility, biofilm formation, growth rate, and even relative pathogenicity. These results are similar to those reported on common bacteria [8-13].

The potential causes of the changes in susceptibility observed in this study may be attributed to the stress response [8], structural changes on the membrane which changes the interaction with antimicrobial agents [8], the activation of efflux pumps that make bacteria able to expel antimicrobials from the cell [8,11].

Adaptation to AgNPs was particularly high with 8-fold increase in MIC and 16-fold increase in MBC of AgNPs. Previous investigations have also documented the selection of silver resistance in Gram-negative pathogens [22], including *E. coli* and other Enterobacteriaceae [23]. This resistance has been correlated with increased efflux activity [24] or a loss of outer membrane porins [25], thereby decreasing cell permeability which may explain the decrease induced in AgNPs susceptibility observed in the *E. coli* M17 exposed to AgNPs.

Regarding biofilm formation, only the *E. coli* M17 exposed to ampicillin produced more biofilm than the original strain. Machado et al. [26] and Henly et al. [8] reported that adaptation to antimicrobials is correlated with an increase in biofilm biomass in *E. coli,* which is believed to be due to an increase in protein and polysaccharide content within the extracellular polymeric substance (EPS). This change in EPS composition may lead to reduced Ampicillin susceptibility, as observed in our Ampicillin-adapted isolates. Moreover, Microarray analysis performed by Niba et al. [27] revealed that antimicrobial exposure resulted in an increase in the expression of *fimDFHI,* which encodes proteins involved in fimbrial biosynthesis, which has been shown to be positively associated with an increase in biofilm formation. This may provide a potential link between the increase in biofilm formation and the resistance caused by ampicillin exposure in *E. coli* M17.

In general, the probiotic *E. coli* M17 has low virulence and moderate toxicity [1]. However, the attenuation of the relative pathogenicity observed in the *G. melonella* model following exposure to cefazolin is one of the most interesting findings of this study. This presents prospects for attenuating virulence, not only for probiotics but also for pathogenic bacteria, and should be further investigated and extended to more microorganisms. Furthermore, further studies should be conducted to elucidate the mechanism of action involved in this attenuation of pathogenicity.

5. CONCLUSION

In this investigation we evaluated the changes induced in the probiotic *E. coli* M17 following exposure to ampicillin, kanamycin, Cefazolin and silver nanoparticles. The results revealed that these antimicrobials may induce significant changes on this probiotic, including an increase in the relative pathogenicity (assessed here on *Galleria melonella* waxworm model), except the strain exposed to cefazolin. Therefore, as far as possible, the probiotic *E. coli* M17 should not be used in combination with antibiotics and further investigations are required to expand similar work on more probiotics in order to avoid resistance build-up which might be transmitted by horizontal transfer.

CONSENT

Not applicable

ETHICAL APPROVAL

Not applicable

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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