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## EDITED BY

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## REVIEWED BY

Sebastian Strauch,  
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Erlangen-Nürnberg, Germany  
Madhan Tirumalai,  
University of Houston, United States

## \*CORRESPONDENCE

Hugo Castillo,  
✉ castilh2@erau.edu

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# Phenotypical changes in *Escherichia coli* K12 after prolonged exposure to simulated microgravity

Alba Chavez<sup>1</sup>, Collin Topolski<sup>2</sup>, Janelle Hicks<sup>2</sup>, Mitchell Villafania<sup>1</sup>,  
Natalie Baez<sup>3</sup>, Marissa Burke<sup>1</sup> and Hugo Castillo<sup>1\*</sup>

<sup>1</sup>Human Factors and Behavioral Neurobiology Department, Embry-Riddle Aeronautical University, Daytona Beach, FL, United States, <sup>2</sup>Mechanical Engineering Department, Embry-Riddle Aeronautical University, Daytona Beach, FL, United States, <sup>3</sup>Division of Sciences and Technology, Universidad Ana G. Mendez Cupey Campus, San Juan, PR, United States

Over the past few decades there has been a steady increase in interest in the study of the role of space environment in the genetic and phenotypical changes of microorganisms. More specifically, there are concerns with astronaut health being compromised during missions to the Moon and beyond from changes in many conditions. These include changes in the physiology of bacteria leading to alterations directly related to human health such as virulence and antibiotic resistance or to the functioning of life support systems such as the increase in biofilm formation in the water supply or treatment components. The effects of space conditions on microorganisms have been studied for more than a decade; however, there is still a need to determine the impact of the physiological effect of microgravity not only of bacterial growth, but also on the different virulence-related phenotypes that might contribute to phenotypic plasticity and microbial adaptation. This study focuses on deciphering the phenotypical changes of the commensal bacterium *E. coli* K12 after growth under simulated microgravity conditions using a 2D microgravity analog. Using a 2D clinostat, *Escherichia coli* was grown up to 22 days and used to measure changes in phenotypes commonly related to virulence. The phenotypes measured included cell population growth, biofilm development and the response to acidic pH and oxidative stress. Results from our studies showed the tendency to enhanced biofilm formation and a decreased resistance to oxidative stress and to grow under acidic conditions. These results suggest that microgravity regulates the adaptation and phenotypic plasticity of *E. coli* that could lead to changes in virulence.

## KEYWORDS

*Escherichia coli*, microgravity, biofilm, oxidative stress, antibiotic resistance

## Introduction

Environmental conditions in space, such as microgravity, radiation, and extreme temperature fluctuations, present challenges to the survival and growth of organisms, including bacteria (Milojevic and Weckwerth, 2020). Microgravity, in particular, affects bacterial behavior and physiology such as the regulation of growth rates, gene expression, and resistance to antibiotics, among others (Nickerson, et al., 2000; Klaus and Howard, 2006; Castro et al., 2011). Given the constraints to experiment under real microgravity conditions, the use of analogs allows us to simulate some of the effects of microgravity on cells such as reduced sedimentation due to a constant state of free falling and the reduction of shear forces

(Klaus, 2001). These analogs significantly increase our capacity to formulate better hypotheses and to predict biological changes at different levels. For example, experiments using *E. coli* have reported changes in growth rate and biofilm development, reduction in the resistance to acidic pH, alterations in the transport and utilization of metal ions, increase in antibiotic resistance, increase in membrane fluidity and the regulation of gene expression (Lynch et al., 2006; Kim and Rhee, 2016; Yim et al., 2020; Allen et al., 2022; Kim et al., 2022). Another important observation has been the expression of virulence factors such as the LT-1 toxin in entero-toxigenic *Escherichia coli* (Chopra et al., 2006) and the increase in adhesiveness of adherent-invasive *E. coli* to mammalian gastrointestinal cells (Allen et al., 2008). These responses were observed in cultures under aerated conditions typical for *E. coli* and after the exposure to simulated microgravity that lasted between 12 and 48 h, suggesting *E. coli* cells sensed and responded to a change in their physical environment within only a small number of generations. Although there are multiple studies that emphasize the effect of prolonged exposure to microgravity in bacterial growth dynamics and other phenotypes. An important consideration is that in a real-life scenario *E. coli* cells in the human microbiome are subjected to microgravity conditions that last from several days to weeks during stays at the International Space Station, creating the possibility of responses or adaptations not previously observed in short-term experiments. To address this issue, two studies that grew *E. coli* for 1,000 generations under simulated microgravity reported mutations in genes coding for pili biogenesis (*surA*), adhesion (*fimH*) and osmotic stress (*betA*) (Tirumalai et al., 2017), as well as the increase in resistance to the antibiotics chloramphenicol and cefalotin that persisted for over 100 generations after the exposure to the treatment (Tirumalai et al., 2019), suggesting that prolonged exposure to microgravity selects for phenotypes better adapted to this unusual condition. In this study, we exposed *E. coli* to simulated microgravity conditions for 22 days, equivalent to approximately 128 generations, in rich media and under non-aerated conditions to induce the microaerophilic growth common to the human gut. *Escherichia coli* is one of the most well studied bacterial species and it is widely reported to be an important member of the healthy human gut microbiome (Martinson and Walk, 2020); it is also a commonly used model system to study alterations in both its genotype and phenotype when exposed to environmental stressors (Jang et al., 2017). Furthermore, the wide variety and consistency of the altered stress responses reported in this model system have been useful for understanding the overall adaptive mechanisms that occur under the effects of short exposure to microgravity (Aunins et al., 2018). Our objective was to measure the regulation of three phenotypes (biofilm development, resistance to oxidative stress and acid tolerance stress) commonly associated with virulence in our model species, when *E. coli* strain K12 was exposed to 22 days under clinorotation using the recently developed microgravity analog called the EagleStat (Topolski et al., 2022). This system is a device that is specifically designed to be ergonomically efficient and to allow manipulation of multiple microbiological samples under fractional gravitational loads or microgravity. Prolonged exposure to simulated microgravity has been reported before in *Stenotrophomonas maltophilia* cultures using a high-aspect rotating vessel for a total of 14 days (Su et al., 2021). Our experiment reports a similar treatment

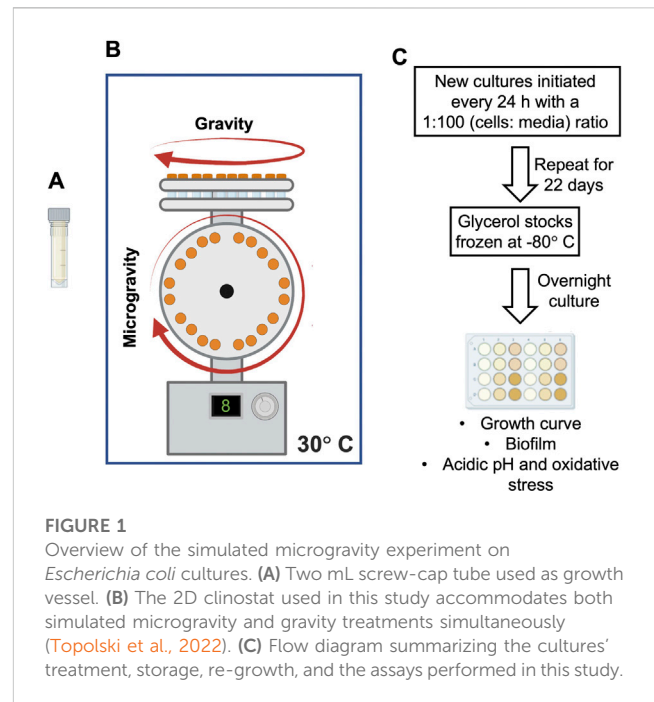


FIGURE 1

Overview of the simulated microgravity experiment on *Escherichia coli* cultures. (A) Two mL screw-cap tube used as growth vessel. (B) The 2D clinostat used in this study accommodates both simulated microgravity and gravity treatments simultaneously (Topolski et al., 2022). (C) Flow diagram summarizing the cultures' treatment, storage, re-growth, and the assays performed in this study.

using *E. coli* K12 exposed 22 days of simulated microgravity that describes phenotypical changes known to contribute to virulence.

## Materials and methods

### Prolonged exposure of bacteria to simulated microgravity

Three overnight cultures of *E. coli* K12 MG1655 were grown in nutrient broth (0.3% beef extract, 0.5% peptone, 1% sodium chloride) at 30°C in a microbiological incubator under constant agitation (200 rpm) for 15 h. The prolonged exposure to simulated microgravity was performed using a 2D clinostat designed for microbial studies as previously described in Topolski et al. (2022). In brief, 2 mL aliquots of overnight cultures diluted 1:100 in nutrient broth (NB) were transferred to 2 mL screwcap microtubes while avoiding the presence of visible air bubbles to ensure low-shear force conditions required for microgravity simulation. The tubes were mounted on the clinostat on their respective simulated microgravity (SM) and gravity (G) positions (Figure 1) inside a microbiological incubator at 30°C and constant rotation at 8 rpm. For the prolonged exposure to SM and G conditions, 20 µL of each tube were transferred to new tubes containing 1980 µL of nutrient broth for a 1:100 dilution, daily, for 22 days. On each transfer, aliquots of 100 µL of culture were mixed with an equal volume of 50% glycerol and frozen at -80°C.

### Bacterial growth dynamics

In preparation for all the assays here reported, overnight cultures (OC) from days 4 and 22 samples were prepared by inoculating 2 mL of nutrient broth with 10 µL of each glycerol stock and grown

at 30°C under constant agitation at 200 rpm for 15 h. The growth of the cultures was measured from 100 µL aliquots transferred to a 96 well plate to read optical density at 630 nm using a microplate reader (Biotek, Winoosky, VT) and their density adjusted to 0.5 units of absorbance to proceed with the experiment. Normalized OC were diluted 1:100 in nutrient broth and 1 mL aliquots were transferred into multiple wells of a 24-well microtiter plate, using separate plates for days 4 and 22. The plates were incubated at 30°C and their absorbance was measured every hour for 15 h as previously described. Cell count was estimated from serial 10-fold dilutions and plating 10 µL drops, in triplicate, on NB agar plates.

## Biofilm formation

Biofilm biomass was measured using the tetrazolium XTT and crystal violet (CV) assays (Kuhn et al., 2003; O'Toole, 2011). For this purpose, 1 mL aliquots of 1:100 dilutions of overnight cultures were prepared in nutrient broth and transferred to individual wells on a flat-bottom, polystyrene 24-well microtiter plate (Corning, Sigma-Aldrich CLS3628, St. Louis, MO) and incubated for 24 h under static conditions at 30°C. After incubation, planktonic cells (those not forming biofilms) were removed by shaking the plate and attached cells (biofilm formers) were washed three times with sterile saline solution (0.85% sodium chloride). Three wells on the plate were reserved for sterile negative controls (only sterile media added). For the CV assay, 1 mL of crystal violet (1% aqueous solution) was added to each well and incubated at room temperature for 20 min. After incubation, the CV was transferred to a waste container and the plate was rinsed five times with sterile saline solution to remove the unbound dye. After the rinse, the wells were treated with 1 mL of 95% isopropanol to solubilize the CV and optical density at 550 nm was measured from three 100 µL aliquots on a 96-well microplate.

For the XTT assay, plates rinsed as previously described for the CV assay were treated with 1 mL of a solution containing 0.010 mol/L menadione (Sigma-Aldrich, St. Louis, MO) stock solution (diluted in acetone) mixed with XTT/Saline solution (0.5 g of XTT -2,3-bis-2-methoxy-4-nitro-5-sulfophenyl-5-phenylaminocarbonyl-2H-tetrazolium hydroxide-from Sigma diluted in Saline Solution) at a final concentration of 1 µmol/L. The plates were then covered in aluminum foil and incubated for 2 h at 37°C degrees. The XTT was actively and effectively reduced by metabolically active cells (biofilm formers) and the original clear solution was transformed into an orange solution that can be measured at an absorbance of 490 nm. Three technical replicates (from each biological replicate) were transferred to a 96-well microplate and measured at the respective optical density. For both CV and XTT assays, negative controls were treated and measured for experiment quality.

## Oxidative stress resistance assay

To measure resistance to oxidative stress, planktonic cells were diluted 1:100 in nutrient broth containing 3 mM hydrogen peroxide (Millipore Sigma). Media without hydrogen peroxide was used as

control. One mL aliquots were transferred to a 24 well microplate (in triplicate with unamended and cell-free media as controls) and incubated for 1 h at 37°C and constant agitation at 150 rpm. After incubation, the cultures were centrifuged at 10,000 rpm for 5 min, the supernatant was removed, and the cell pellet was rinsed twice with saline solution before starting a 10-fold serial dilution for drop plating to evaluate colony forming units.

## Acidic pH tolerance assay

For growth in acidic media, cells were diluted 1:100 in nutrient broth buffered to pH 4.5 using HCl. One mL aliquots were transferred to a 24 well microplate (in triplicate with media pH 7.5 as control) and incubated for 1 h at 37°C with constant agitation at 150 rpm. After incubation, the cultures were serially diluted, 10-fold, in saline solution and plated using the drop plate technique to estimate CFU/mL.

## Differential gene expression

The differential expression of genes coding for enzymes involved in the antioxidant response (*oxyR*, *soxR*, *cysD*) and tolerance to acidic pH (*fabA*, *cpxR*, *cpxA*) was analyzed using RT-qPCR (Table 1). For this purpose, cDNA was synthesized from total RNA with the PRIMA qMax first strand cDNA synthesis flex kit (Midsci, Valley Park, MO), using a program of priming at 25°C for 5 min, reverse transcription at 46°C for 20 min and RT inactivation at 95°C for 1 min. Quantitative PCR reactions, in 10 µL volumes, were ran in triplicate using the Applied Biosystems PowerUp SYBR green master mix (Applied Biosystems), 0.5 µM of each primer and 1 ng of cDNA as template. The relative gene expression of the target genes (Table 1) was normalized against the expression of the *gapA* gene using the efficiency-corrected model by Pfaffl et al., 2002. Each comparison was calculated from 7 to 9 Ct values from three biological replicates.

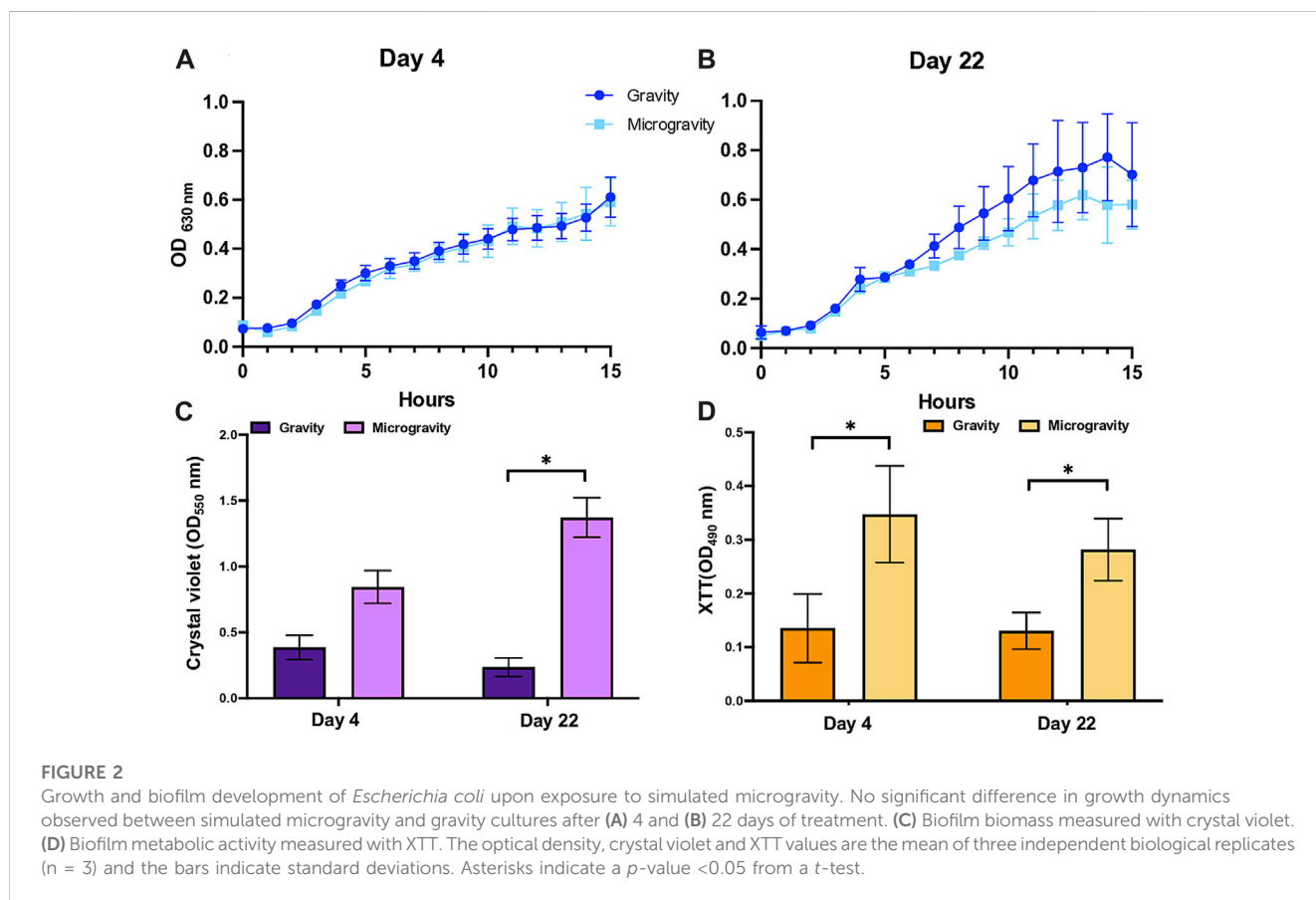
## Results

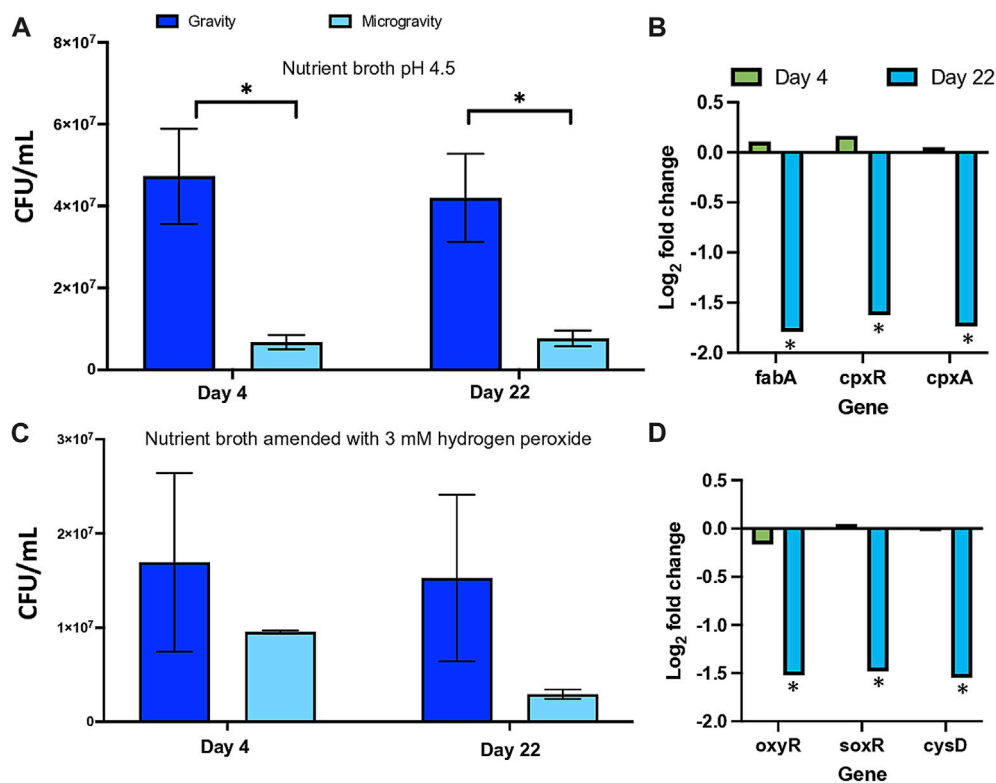
### Growth and biofilm formation, after exposure to simulated microgravity

We present the results of *E. coli* cultures grown under SM for 4 and 22 days, corresponding to approximately 53 and 128 generations, respectively. The long generation times observed (1.8–4 h) were most likely due to the microaerophilic conditions expected to develop on the micro tubes used as growth vessels and the absence of headspace required to attain the low shear conditions needed for microgravity simulation. Our experiments show that SM did not regulate the number of cells on cultures from either exposure time (Figures 2A, B). However, the biofilm biomass (CV) and metabolic activity (XTT) assays showed a significant increase in cultures grown under simulated microgravity, compared to those from the gravity control (Figures 2C, D), similar to a previous observation in 24 h cultures (Topolski et al., 2022).

TABLE 1 Primers used in this study.

Gene	Name	Forward (5'-3')	Reverse (5'-3')	Amplicon length (bp)
<b>Antioxidant response genes</b>				
<i>oxyR</i>	Hydrogen peroxide-inducible genes activator	AGCGGGATCACTTTA CTGCC	CGGTGAGCCAGGACG ATAAA	129
<i>cysD</i>	Sulfate adenylyltransferase subunit 2	CAACGGGCAAACAAT GTCG	GATATAGCGGAACAA TGTCG	131
<i>soxR</i>	Redox-sensitive transcriptional activator of soxS autorepressor	CCATTGGTGAAGCGT TTGGC	ACATCCGTCCAGTTC GTCAC	143
<b>Tolerance to acidic pH response genes</b>				
<i>fabA</i>	3- hydroxydecanoyl-[acyl-carrier-protein] dehydratase	CAATTGCCAGCACCG AACAT	GGCATCCGAAGAACC ACAGA	130
<i>cpxA</i>	Sensor histidine kinase CpxA	CGCAGGTGCCAGTTT TAACC	AGTAACGCCGTACCC AGTTG	132
<i>cpxR</i>	Transcriptional regulatory protein CpxR	GCAAACGCTGGAGTT AACCG	GCATATCAATAGCGC GGTCG	143
<b>Housekeeping, References gene</b>				
<i>gapA</i>	Glyceraldehyde-3-phospahte dehydrogenase A	ATATGCTGGCCAGGA CATCG	TCTTTGTGAGACGGG CCATC	165





**FIGURE 3**

Regulation of the acid tolerance (A) and oxidative stress (C) in *Escherichia coli* by simulated microgravity and the differential expression of genes coding for proteins related to growth under acidic pH (B) and to the response to oxidative stress (D). Bar graphs represent the mean of triplicated experiments ( $n = 3$ ) with standard deviation as error bars. The asterisks represent significant differences between simulated microgravity and gravity cultures ( $* < 0.05$ ).

## Acidic pH and oxidative stress tolerance assay

We tested the effect on microgravity on the regulation of tolerance to media acidified to pH 4.5 and amended with 3 mM hydrogen peroxide, using media pH 7.5 and unamended media as controls, respectively. Our data shows a marked increase in the culture's sensitivity to acidic pH when cultures were grown under simulated microgravity conditions that did not change, significantly, with the number of generations (Figure 3A). Similarly, the effect of oxidative stress induced by the addition of hydrogen peroxide caused a decrease in cell viability in cultures that experienced reduced gravity, but only after cells grew under this condition for 22 days (Figure 3B).

## Differential expression of genes involved in the response to oxidative stress and tolerance to acid pH

We measured the differential expression of genes involved in the tolerance of *E. coli* to acidic pH (*fabA*, *cpxR* and *cpxA*) and the response to oxidative stress (*oxyR*, *soxR*, *cysD*). Our results show that expression of these genes initially not regulated in cells growing under SM conditions for 4 days, decreased upon the prolonged exposure to the treatment for 22 days (Figures 3B, D).

## Discussion

Human presence in space becomes more frequent, longer and it will expand beyond the limits of the International Space Station. In their quest to reach other planets, humans will rely not only on traditional life support systems to provide sustenance and protection, but also on their relationship with other organisms such as bacteria that significantly influence their interaction with the immediate physical environment. For this reason, it is imperative that we understand better the potential for the metabolic regulation of microbial activity in response to components of the space environment such as microgravity. The primary objective of this investigation was to examine the influence of the continuous exposure to simulated microgravity, from several days to weeks, under oxygen-limited conditions. The use of clinostats and other microgravity analogs present limitations in their results as they only partially mimic the effects of real microgravity conditions, a condition previously discussed (Ferranti et al., 2021; Allen et al., 2022). However, their use allows us to explore the biological effects of near weightlessness in bacterial cultures in a systematic way. Our results showed no significant difference in the growth kinetics between SM and G cultures on either exposure time (Figure 1), similar to the response in a previous experiment using a different microgravity analog with comparable growth conditions (Allen et al., 2022) and the device here discussed in 24 h old cultures (Topolski et al., 2022). This could

be explained as the result of the cellular homeostatic control present in *E. coli* in the event that the constant state of free-falling imposed by clinorotation was in fact sensed as a stress. However, when we analyze the results of our biofilm assays, we observed that SM conditions selected for cells capable of forming increasing amounts of biofilm biomass, as well as a higher metabolic activity within biofilms, suggesting that our treatment led to stable phenotype changes in our model. It is known that biofilm development provides bacteria with advantages such as a higher resistance to unfavorable environments and antimicrobial agents by enhancing their ability to colonize solid surfaces or to alter the expression of genes, which translates into the potential increase in virulence and/or antibiotic resistance (Katongole et al., 2020; Behzadi et al., 2022). In contrast, the reduction in the viability of cells under acidic (days 4 and 22) and oxidative stress (day 22), supported by the downregulation of several genes involved in these responses, suggest that either SM favors the growth of cells with an impaired ability to survive the passage through the stomach, colonize the gut surfaces and to express virulence genes or it regulates the expression of the genes coding for enzymes involved in these processes. Although perceived as desirable traits, it is important to consider the majority of *E. coli* strains are not pathogenic, but form commensalistic relationships with humans (Martinson and Walk, 2020), hence the changes in the growth and survival of this organism could alter its dynamics in the gut of an astronaut during long-term space residence. Interestingly, our results are in accordance with a previous report for *E. coli* in spaceflight for 12 days that found a comparable reduction in viability after exposure to an acidic environment (Liu et al., 2023). Two other experiments showed responses comparable to ours, one with the downregulation of the *soxR* gene during a 49-h experiment exploring the effect of antibiotics on *E. coli* at the ISS (Aunins et al., 2018) and a second with the decreased survival of *Salmonella enterica* that grew the cells in a microgravity analog for 10 h (Wilson et al., 2002). The interesting finding of decreased viability under oxidative stress, induced by hydrogen peroxide, in prolonged exposure to microgravity reaffirms a complicated network of regulation to stress that may be attributed to interfering gene expression profiles caused by microgravity; however, our study shows that the *soxR* global regulator pathway is affected by oxidative stress after 22 days of exposure to microgravity. One interesting finding also reported by Aunins et al., 2018 is that downregulation of *soxR* was parallel to an upregulation of genes responsible of antibiotic resistance (particularly those that target the 30 S ribosomal subunit). Future studies in our system will include a comprehensive profile of antibiotic resistance patterns under prolonged exposure to microgravity. Our findings include a higher susceptibility to acidic conditions when cells were grown under microgravity. This finding can be explained by observing the higher quantity of biofilms formed under microgravity. Biofilms are known to have high tolerance to low pH. However, the Extra Polymeric Substances (EPS) that are part of the biofilm matrix may create zones of variable pH that could expose individual cells (without the EPS matrix) to low pH and affect dramatically their viability. Future studies will include exposure of cells grown under microgravity to high

pH environments and the formation of alkaliphilic biofilms that have been reported to confer a greater level of control and survival in adverse environmental conditions (Charles et al., 2022). The composition of the EPS matrix will also be studied since it has been reported that alkaliphilic biofilms contain a higher amount of DNA when compared to protein, cellulose or lipid content (Charles et al., 2022). This data suggest that the direct effect of an acidic environment and oxidative stress conveyed by the condition of microgravity can increase biofilm formation along with resistance to antibiotics, a phenotype that will be addressed in the future. Overall, our experiment adds to the previously reported need to develop more realistic simulations of how microorganisms grow in space by exploring the interaction of the prolonged exposure to microgravity with microaerophilic conditions on the selection for phenotypes directly related to cell survival and the expression of virulence in the human gut.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

AC: Conceptualization, Investigation, Methodology, Writing–review and editing. CT: Conceptualization, Data curation, Methodology, Writing–review and editing. JH: Methodology, Writing–review and editing. MV: Formal Analysis, Methodology, Visualization, Writing–review and editing. NB: Methodology, Visualization, Writing–review and editing. MB: Methodology, Writing–review and editing. HC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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## Conflict of interest

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