Received: 4 April 2023 | Accepted: 28 September 2023

DOI: 10.1111/1462-2920.16522

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Modelled microgravity impacts *Vibrio fischeri* population structure in a mutualistic association with an animal host

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Funding information
National Aeronautics and Space Administration, Grant/Award Numbers: 80NSSC18K1465, 80NSSC19K0138

Abstract

Perturbations to host–microbe interactions, such as environmental stress, can alter and disrupt homeostasis. In this study, we examined the effects of a stressor, simulated microgravity, on beneficial bacteria behaviours when colonising their host. We studied the bacterium *Vibrio fischeri,* which establishes a mutualistic association in a symbiosis-specific organ within the bobtail squid, *Euprymna scolopes*. To elucidate how animal–microbe interactions are affected by the stress of microgravity, squid were inoculated with different bacterial strains exhibiting either a dominant- or sharing-colonisation behaviour in High Aspect Ratio Vessels, which simulate the low-shear environment of microgravity. The colonisation behaviours of the sharing and dominant strains under modelled microgravity conditions were determined by counting light-organ homogenate of squids as well as confocal microscopy to assess the partitioning of different strains within the light organ. The results indicated that although the colonisation behaviours of the strains did not change, the population levels of the sharing strains were at lower relative abundance in single-colonised animals exposed to modelled microgravity compared to unit gravity; in addition, there were shifts in the relative abundance of strains in co-colonised squids. Together these results suggest that the initiation of beneficial interactions between microbes and animals can be altered by environmental stress, such as simulated microgravity.

INTRODUCTION

Symbioses between horizontally transmitted microbes and their animal hosts rely on the encounter and acquisition of the correct symbiont from the environment. Perturbations to the environment can impact the resulting population within the host, and therefore, the health of the holobiont (Gilarranz et al., 2017). Although the environment can shape host colonisation (Anderson et al., 2016; Devevey et al., 2015; Lee & Ruby, 1994; Polzin et al., 2019), the bacterial symbiont population can also affect the response of the host to the environment (Morrissey et al., 2021). Although numerous studies have targeted the impact of specific microbial species on the host, the importance of microbes at the strain level is becoming increasingly evident (Ellegaard & Engel, 2019; Van Rossum et al., 2020; Vatanen et al., 2018). Therefore, addressing the impact of the environment, perturbations to that environment, as well as strain dynamics on host colonisation is essential to understanding mutualistic associations.

An ideal model system to explore these effects on host colonisation is the symbiosis between *Vibrio fischeri* and its animal host, the bobtail squid *Euprymna scolopes* (Figure 1). In the squid–vibrio symbiosis, the horizontally transmitted bacterium *V. fischeri* receives host nutrients in exchange for providing bioluminescence that is used as counterillumination by the squid for nocturnal camouflage (Ruby & McFall-Ngai, 1992). The symbiosis is induced by a molecular dialogue between host and symbiont, where *V. fischeri* colonises and establishes itself within a specialised bilobed light organ within the squid with each lobe containing three epithelial lined crypt spaces where the bacteria reside

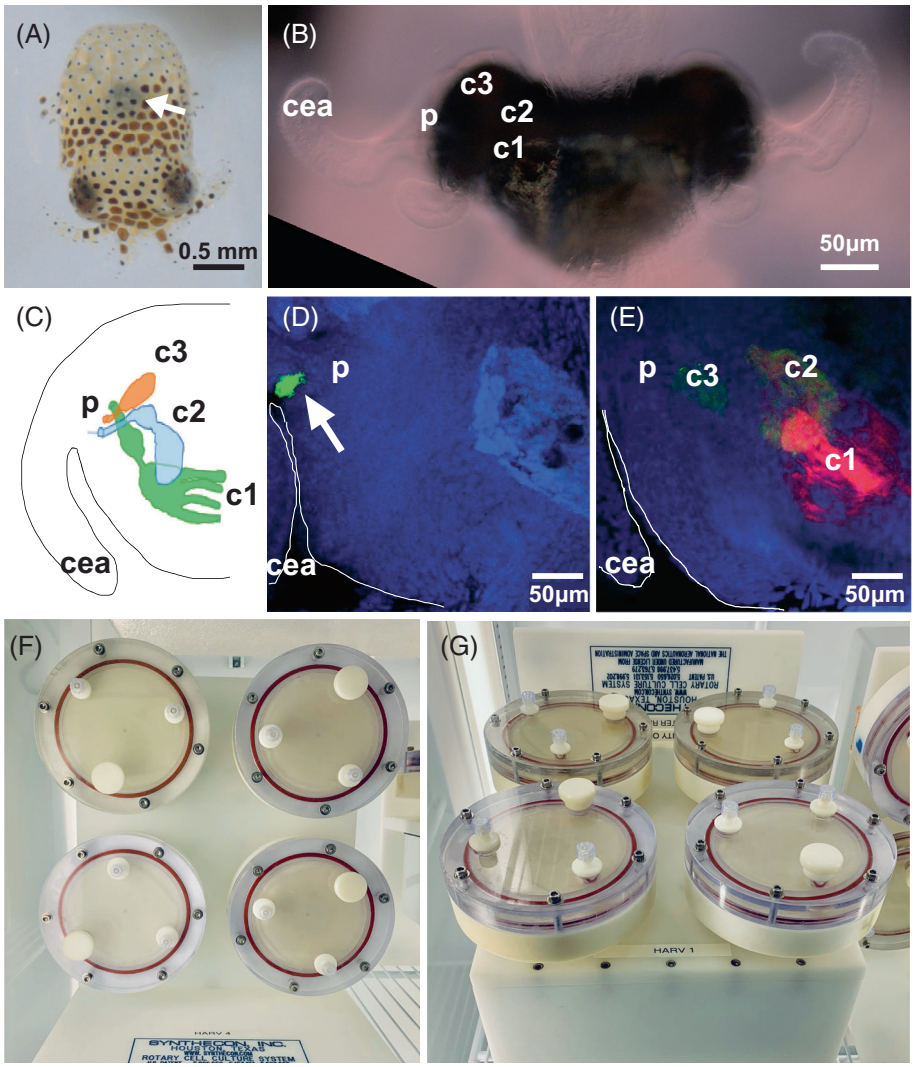

FIGURE 1 Overview of host symbiont tissues and modelled microgravity treatments. (A) Squid paralarvae after hatching. Arrow indicating the location of symbiotic light organ. (B) Micrograph of bilobed light organ morphology with ciliated epithelial appendages (cea) extending from either side of light organ. Pores (p) located on the surface of light organ in which symbionts begin the colonisation process to reside within one of three epithelial lined crypt spaces (c1, c2, and c3) on each side of the light organ. (C) Cartoon of one side of the light organ depicting internal structure of crypt spaces. The *Vibrio fischeri* are harvested from the environment by the ciliated epithelial appendages (cea), aggregate around the pores (p), then a limited number of cells cross different microenvironments to reach and colonise one of the three crypts (c1, c2, and c3) located within each side of the light organ. (D) Confocal micrograph depicting the early stages of colonisation where bacteria begin to aggregate (arrow) outside of the pore of the host light organ (blue). (E) Colonisation of the crypt spaces (c1, c2, and c3) by the different GFP- (green) or RFP- (red) labelled populations of *V. fischeri* can be visualised by 18 h post inoculation. (F and G) The stress of microgravity can be simulated using High Aspect Ratio Vessels in either the vertical low-shear modelled microgravity position (F) or in the horizontal position as a unit gravity control (G).

and grow (Figure 1A–E; McFall-Ngai & Ruby, 2021). The bacteria are harvested from the environment by ciliated epithelial appendages located on the surface of the light organ, which bring *V. fischeri* near pores that lead into the light organ (Figure 1B; Nawroth et al., 2017). Around the pores, the bacteria induce biofilm production factors and begin the process of aggregation (Figure 1C,D; Nyholm et al., 2000). The aggregation depends on the expression of the symbiosis polysaccharide locus (*syp*) and is regulated by multiple regulation factors (Visick, 2009; Visick et al., 2021), including but not limited to RscS, a histidine kinase involved in the colonisation competency of *V. fischeri* (Mandel et al., 2009). A few bacteria detach from the aggregate and migrate into the pores travelling through different microenvironments to reach the deep crypts of the light organ where the bacteria start growing (Figure 1C,E; Altura et al., 2013; McFall-Ngai et al., 2010).

The natural environment harbours an array of different *V. fischeri* strains, providing a wide diversity of potential symbionts (Bongrand et al., 2016; Wollenberg &

Ruby, 2009, 2012). These naturally occurring strains vary in several phenotypes, such as aggregation size (Koehler et al., 2019), the timing needed to reach the crypt (Bongrand & Ruby, 2019b) or the presence of a type VI secretion system (Speare et al., 2018), which together may contribute to shaping the population structure within the light organ (Bongrand & Ruby, 2019a). Of the many natural *V. fischeri* strains, two categories have emerged including sharing (S) and dominant (D) strains, which reflect bacterial populations within *E. scolopes* light organ crypt spaces (Bongrand et al., 2016; Bongrand et al., 2020). S-strains can co-colonise light organs, whereas D-strains typically occur as single populations within a crypt space. Although the complete mechanism resulting in these colonisation phenotypes remains unclear, D-strains are known to reach the crypt spaces earlier than S-strains (Bongrand & Ruby, 2019b). In addition, both strains have been isolated together in adult light organs (Wollenberg & Ruby, 2009) suggesting that the final microbial population structure is influenced by others factors, including the environment (Hodgson et al., 2002; Lee et al., 2014).

To explore whether perturbations to the environment can impact these bacterial colonisation strategies, the stress of simulated microgravity was used as it represents a global environmental stressor. Gravity has had a profound impact on the evolution of life on Earth and the 'free-fall' environment of simulated microgravity can alter gravity-dependent properties, such as sedimentation, convection and hydrodynamic stresses, thereby impacting numerous biological functions of both the host and its microbiota (Bizzarri et al., 2014; Garrett-Bakelman et al., 2019; Hariom et al., 2021; Todd, 1989). For example, microgravity, both natural and simulated, is known to affect bacterial motility, biofilm formation and growth (Gilbert et al., 2020; Kim et al., 2013; Su et al., 2021), which are critical phenotypes involved in colonisation success of animal–microbe associations (Raina et al., 2019; Schlomann et al., 2018).

Previous studies using simulated, or modelled, microgravity on the squid–vibrio symbiosis have suggested numerous changes to both the bacteria and host physiology resulting in alterations to the developmental timeline (Casaburi et al., 2017; Duscher et al., 2018; Foster et al., 2013; Vroom et al., 2021; Vroom et al., 2022). For example, a transcriptomic analysis of the squid under modelled microgravity showed a host stress response to the environment was attenuated when the symbiont is present (Casaburi et al., 2017). Additionally, *V. fischeri* also showed increased cell concentrations when grown in culture under modelled microgravity conditions compared to unit gravity, yet bioluminescence produced by the bacteria in colonised squid was lower in modelled microgravity (Grant et al., 2014). All these data suggest that both host and symbiont are altered in modelled microgravity and highlight the importance of deciphering the impact that environmental perturbations can have on the establishment of a host–microbe association. Here, we explored whether simulated microgravity influenced the establishment of the symbiosis and impacted the resulting symbiont population within the host light organ. Overall, this study demonstrated that an environmental stressor, such as simulated microgravity, can shape a symbiotic population, and therefore, a mutualistic association.

EXPERIMENTAL PROCEDURES

Bacterial strains

In this study, four *V. fischeri* strains isolated from the *E. scolopes* light organ were used including ES114, KB2B1, MB13B1 and MB13B2 (Boettcher & Ruby, 1990; Wollenberg & Ruby, 2009). Additionally, two RscS mutants of ES114 were used: one lacking the gene (KV6533; Tischler et al., 2018) and the other containing a mutation in the gene which results in a hyper aggregator phenotype (KV4366; Marsden et al., 2017). All the strains harboured a plasmid expressing either a Green Fluorescent Protein (GFP, pVSV102) or a Red Fluorescent Protein (RFP, pVSV208; Dunn et al., 2006). Each strain carried the same plasmid in either the single or co-colonisation experiments such that gravity was the only variable between conditions for the same inoculum. In addition, no significant loss of plasmid from the experimental populations was observed as these plasmids have been shown to be highly stable within *V. fischeri* (Dunn et al., 2006).

Squid rearing and welfare

All cephalopod procedures were approved by both the University of Florida (approval number 201910899) and Kennedy Space Center (approval number GDR-20-128) Institutional Animal Care and Use Committees (IACUC). Adult squid were collected from Maunalua Bay in O'ahu, Hawai'i, and transferred to the Space Life Sciences Lab where they were maintained and bred in a natural seawater aquarium system. Egg clutches from the female squid were transferred to aquaria containing natural seawater and incubated on a 12 h light–dark cycle at 23°C until hatching (~21 days). In all experiments, animals were anaesthetised and euthanised before processing using a 1:1 solution of 0.37 M $MgCl_2$ and FSW, which is the current practise for cephalopods (Abbo et al., 2021; Fiorito et al., 2015).

Squid colonisation under modelled microgravity stress

Overnight *V. fischeri* cultures were grown at 28°C with shaking in LBS media containing 20 g NaCl, 50 mL of 1 M Tris–HCl (pH 7.5), 10 g Bacto-Tryptone and 5 g yeast extract per litre. Strains containing chloramphenicol (pVSV208) or kanamycin (pVSV102) plasmids were supplemented with 2.5 or 100 μg ml per litre antibiotics, respectively. The overnight cultures were diluted 100× in seawater tryptone (SWT) media containing 5 g Bacto-Tryptone, 3 g yeast extract, 6 mL of 50% glycerol and 700 mL natural seawater per litre and grown until mid-exponential phase. For each condition, 100 mL of 0.22 μm-filter-sterilised seawater was inoculated with the bacterial culture for an aimed-final concentration of 5000 cells per mL and a 1:1 ratio when co-inoculated with another strain.

To simulate low-shear modelled microgravity conditions (LSMMG), 50-mL High Aspect Ratio Vessels (Figure 1F,G; HARVs; Synthecon, Houston, TX) were used with a rotary cell culture system as previously described (Foster et al., 2013; Schwarz et al., 1992; Vroom et al., 2022; Wolf & Schwarz, 1991). FSW containing the appropriate bacterial inoculum was loaded into four replicate HARV reactors and then hatchling paralarvae, up to 10 per reactor, were added to the HARV through one of the entry ports and sealed to prevent the formation of bubbles. All four of the replicate HARVs were incubated simultaneously at 23°C in a LED-illuminated Percival incubator (Percival Scientific, Inc., Perry IA) and rotated at 13 rpm for 18 h. The HARVs were either rotated around a horizontal axis to simulate the low-shear fluid forces of microgravity or rotated along a vertical axis to serve as a unit gravity control. Previous research has known no differences in dissolved oxygen levels of the seawater within the HARV reactors under LSMMG or gravity positions when incubated for up to 48 h (Vroom et al., 2022).

After incubation in the HARVs, the squid paralarvae were euthanised by over-anaesthetisation and either frozen at −80°C or fixed in 4% paraformaldehyde in mPBS (50 mM sodium phosphate buffer with 0.45 M NaCl, pH 7.4). The frozen paralarvae were homogenised in FSW and different dilutions were plated onto LBS agar plates, as previously described (Bongrand & Ruby, 2019b). The number of colony-forming units (CFUs) was determined and when necessary fluorescent CFUs were counted using a Nikon stereoscope located at the Kennedy Space Center. The animals preserved in fixative were rinsed in mPBS, counterstained with TOTO3 (Thermo Fisher Scientific, Inc, Waltham, MA) containing 1% Triton X-100, and mounted using Vectashield as previously described (Bongrand & Ruby, 2019b). Imaging was performed on a Nikon A1R confocal microscope, located in the Microgravity Simulation Support Facility at the Kennedy Space Center.

All experiments were performed within three replicates HARVs each containing ∼10 animals. No statistical differences between the technical replicate HARVs were observed and the results were combined unless otherwise noted. Normality was assessed using a D'Agostino and Pearson test. A *t*-test or a Mann–Whitney–Wilcoxon *U* test was performed to compare the LSMMG condition to the gravity condition when the data were normal or not normally distributed, respectively. Both parametric and non-parametric analyses were two-sided and used a significance cut-off of $p \leq 0.05$. Data were visualised and statistics were performed using GraphPad Prism version 9.4.0 for Mac OS X.

RESULTS

Modelled microgravity stress did not alter the strain behaviour of *V. fischeri*

To evaluate the impact of environmental perturbations on *V. fischeri* colonisation of host epithelial tissues, we examined whether the natural sharing or dominant strain behaviour in *V. fischeri* was maintained under the stress of modelled microgravity. We co-inoculated newly hatched squid with strains with well-characterised behaviours (Bongrand et al., 2016; Bongrand & Ruby, 2019b; Wollenberg & Ruby, 2009) including the S-strain ES114 and one of three strains: (i) D-strain KB2B1, (ii) D-strain MB13B2 or (iii) the S-strain MB13B1. These strains were selected as representatives of the natural environmental bacterial diversity since they harbour differences in their physiology (Wollenberg & Ruby, 2009) and in their colonisation efficiency (Bongrand et al., 2016). The animal and *V. fischeri* were co-incubated in the HARVs under LSMMG and unit gravity conditions for 24 h and then assayed by plating the light organs on LBS plates and determining which strains colonised the host tissues by counting the CFUs (Figure 2).

The results suggested there were no major behavioural changes to the *V. fischeri* strains under the stress of modelled microgravity compared with gravity controls. The vast majority of the squid co-inoculated with the S-strain ES114 and one of the tested D-strains were singly colonised by the D-strain, suggesting that the D-strains retained their dominant colonisation behaviour under the stress of modelled microgravity. Animals co-colonised with two sharing strains (i.e., ES114 and MB13B1), showed no statistical colonisation differences under LSMMG conditions or gravity controls (Figure 2).

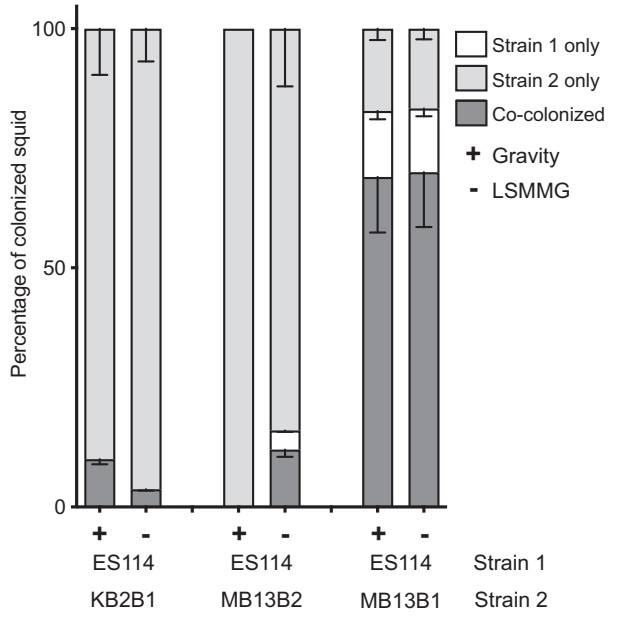

FIGURE 2 Colonisation state of juvenile squid co-inoculated with different *Vibrio fischeri* strains. Nascent squid were co-inoculated with sharing strain ES114 (Strain 1) and a second strain that exhibits either a dominant (KB2B1, MB13B2) or sharing (MB13B1) behaviour and incubated in High Aspect Ratio Vessels (HARVs) under low-shear modelled microgravity (−, LSMMG) or unit gravity (+, Gravity) conditions. After 24 h of incubation in the HARVs, colonisation state of squid light organs was determined by plating. Light organs exhibited different patterns of *V. fischeri* populations under the two gravity conditions including those only colonised by the sharing ES114 (white), colonised only by the second strain (light grey), or co-colonised by both strains (dark grey). Each bar represents the average colonisation percentage for each strain with three technical replicates performed for a total of 25–30 animals analysed per condition and error bars representing the 95% confidence intervals.

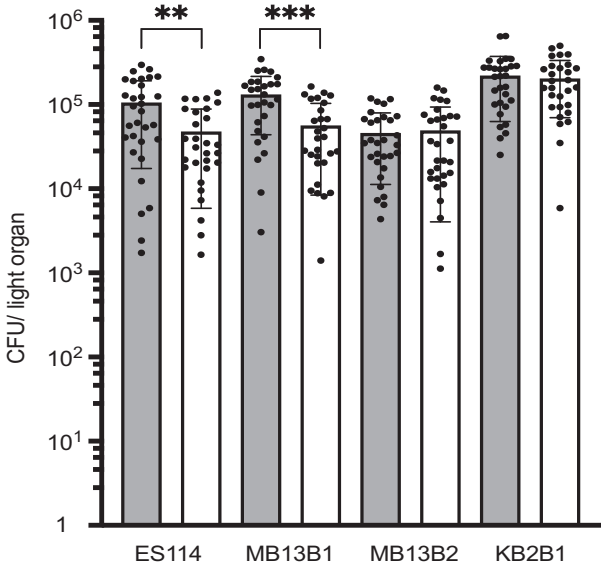

FIGURE 3 Colonisation of juvenile squid with single strains of *Vibrio fischeri* under modelled microgravity and unit gravity control conditions. Hatchling squid were colonised by either a sharing strain (ES114, MB13B1) or by a dominant strain (MB13B2 or KB2B1) and incubated for 24 h under unit gravity (Grey bars) or low-shear modelled microgravity (LSMMG, white bars) conditions. After 24 h, the average of colony-forming units (CFU) was assessed for each light organ (each dot represents a single squid light organ). Three technical replicates were performed for a total of 28 to 31 animals analysed per condition. The error bars indicate the standard deviation. A Mann–Whitney–Wilcoxon *U* test, which was used to compare unit gravity with LSMMG-treated animals, indicated that the CFU per light organ were significantly different for ES114 and MB13B1 (**$p \leq 0.01$; ***$p \leq 0.001$).

Sharing strains showed decreased population levels under modelled microgravity

Previous studies have shown that under spaceflight and modelled microgravity conditions, growth rates of some bacteria, including *V. fischeri*, increased compared to gravity controls (Grant et al., 2014; Su et al., 2021), however, it is not clear whether the ability to colonise tissues in vivo are affected. To assess whether modelled microgravity impacts levels of colonisation within the host light organ, hatchling squid were singly colonised by different strains of *V. fischeri* and incubated in the HARV reactors under LSMMG and gravity conditions. After 24 h, the number of CFU per light organ was determined and statistically compared (Figure 3). Interestingly, when colonised by S-strains the number of CFU per light organ was significantly decreased in modelled microgravity compared to unit gravity ($p = 0.0065$ and 0.0004 for ES114 and MB13B1, respectively). This phenotype was not observed when the squids were colonised with a D-strain ($p = 0.7938$ and 0.7778 for MB13B2 or KB2B1 respectively). These data suggest that environmental stresses, such as modelled microgravity, can impact the symbiont density within host tissues in a strain-specific manner.

Modelled microgravity stress altered the population structure within host crypt tissues

To determine whether the stress of modelled microgravity impacts the population structure of the squid light organ, hatchling squids were inoculated with two S-strains, ES114 and MB13B1, under both LSMMG and unit gravity conditions (Figure 4). The ratio between the two strains was assessed by plating and a shift in the population was observed (Figure 4A). The relative competitive edge of the wild-type-strain ES114 increased under modelled microgravity compared to normal gravity conditions. Although this shift was not statically significant, we explored whether there were differences in the localisation of the S-strains within the different crypt spaces.

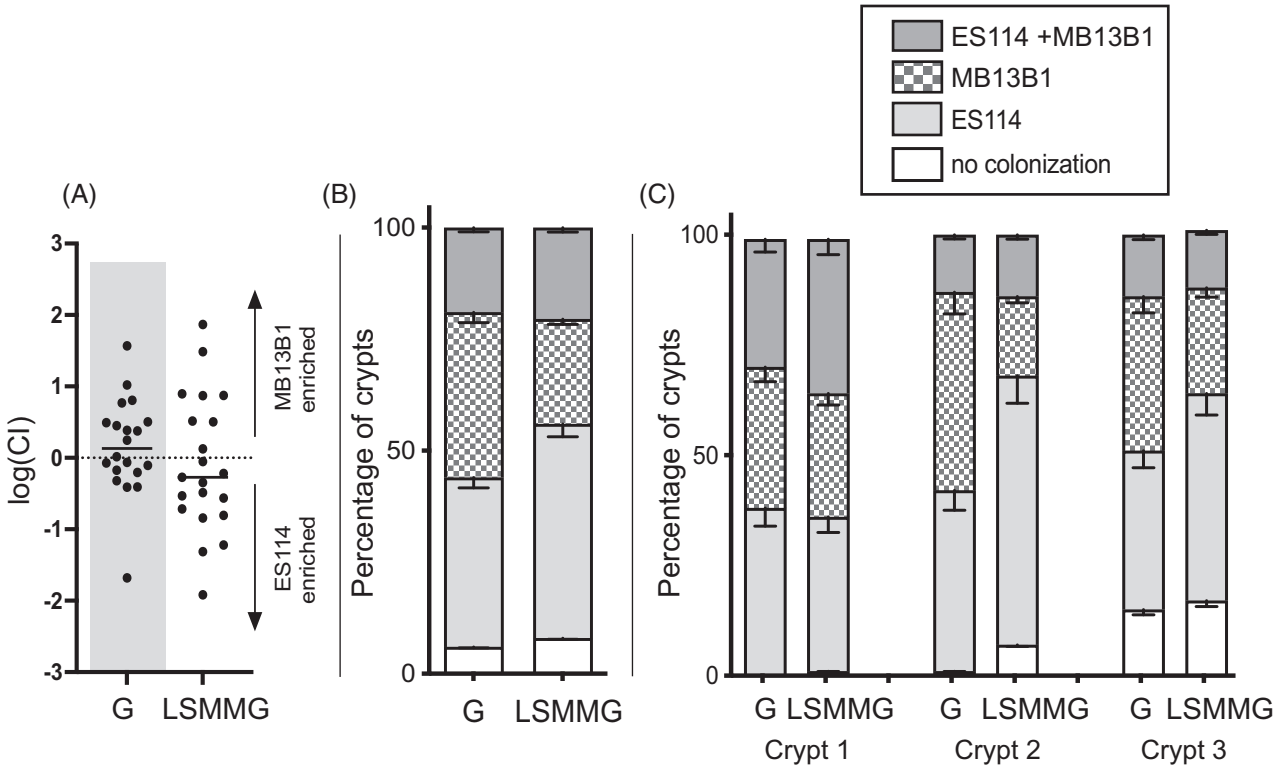

FIGURE 4 Colonisation and localisation of sharing *Vibrio fischeri* strains within host tissues under modelled micro- and unit gravity. All animals were co-colonised with ES114 and MB13B1 and incubated in HARVs under low-shear modelled microgravity (LSMMG) and gravity (G) conditions for 24 h. (A) The competitive index (CI) (i.e., the ratio of ES114 to MB13B1), indicated that there was a slight, but not significant enrichment in ES114 under the modelled microgravity conditions. Each dot corresponds to the log(CI) of a co-colonised squid light organ with a minimum of 20 biological replicates across three technical replicates. (B, C) Confocal microscopy analysis of the GFP and RFP labelled sharing strains to determine the strain content of each crypt of co-colonised animals. The crypts were colonised either by ES114 (light grey), MB13B1 (checkered), both strains (dark grey) or not colonised (white). The graphs indicate the mean percentage for each of the crypt contents and the error bars give the 95% confidence interval. (B) The total percentages of strain colonisation of total light organ suggest that ES114 had a competitive edge over MB13B1 under the stress of modelled microgravity. (C) Percentage of different sharing strains within each of the three crypt spaces from 39 squid under gravity conditions and 44 squid under LSMMG conditions across three technical replicates. GFP, Green Fluorescent Protein; RFP, Red Fluorescent Protein.

At hatching, the three independent crypt spaces on each side of the light organ vary in size as they are in different developmental states of maturity (Figure 1A) (Montgomery & McFall-Ngai, 1993; Sycuro et al., 2006) and, as a consequence, the concentration of bacteria in each crypt may be different. To examine whether the strains within each crypt change in response to the stress of modelled microgravity, confocal microscopy was used to observe all six light organ crypt spaces of squid co-inoculated with S-strains that contained either a GFP- or RFP-reporter. A total of 44 co-colonised squid were examined under LSMMG conditions, whereas 39 were examined under unit gravity controls. When examining the total light organ there was a significant increase in the abundance of the wild-type ES114 compared to MB13B1 under LSMMG conditions (Figure 4B); in addition, individual crypts also exhibited differences in strain localisation (Figure 4C).

In Crypt 1, the most developmentally mature of the crypt spaces, there was a significant decrease in the single colonisation of MB13B1 under LSMMG conditions and a corresponding increase in the proportion of Crypt 1 that were co-colonised with both S-strains (Figure 4C). Modelled microgravity stress also appeared to impact Crypt 2, with a majority (61%, 54 out of 88 observed) of the Crypt 2 spaces in LSMMG-treated animals exhibiting only single ES114 colonisation compared to gravity controls (41%, 32 out of 78 observed). Interestingly in Crypt 2, there were more uncolonised crypts in modelled microgravity-stressed animals compared to gravity controls (Figure 4C).

For Crypt 3, the least mature of the crypt spaces, a shift was also observed under microgravity conditions in which the proportion of ES114-colonised crypts increased, while that of MB13B1-colonised crypts decreased. Altogether, these results show that under the stress of modelled microgravity, crypt epithelial spaces are preferentially colonised by ES114 in comparison to MB13B1, which interestingly is a better coloniser under natural environmental conditions (Bongrand et al., 2016). This LSMMG-induced effect was strongest

with the less developmentally mature Crypts 2 and 3 (Figure 4C).

Bacterial aggregation under modelled microgravity stress

Since bacterial aggregation has been linked to the efficiency of host light organ colonisation by multiple studies (Koehler et al., 2019; Mandel et al., 2009; Rotman et al., 2019), we assessed whether mutants with altered aggregation phenotypes would be impacted by the stress of modelled microgravity. Two mutants of the symbiosis regulator RscS were used, with one having the *rscS*$^{-}$ gene absent (ΔRscS; Tischler et al., 2018) and the other having the gene mutated, resulting in the mutant being a hyper aggregator (RscS**; Marsden et al., 2017). Hatchling squid paralarvae were inoculated with either single strains or co-inoculations of the RscS mutants and the wild-type sharing strain ES114 and plated after 24 h. CFU per light organ were compared and results indicated that under LSMMG conditions, wild-type ES114 populations were at lower numbers than in gravity controls (*$p = 0.0203$); however, no significant differences were observed between the number of bacteria in squid that were singly colonised for either aggregation mutant under LSMMG or gravity conditions (Figure 5A). Additionally, when squid were co-inoculated with ES114 and the hyper aggregator, the latter was enriched in the light organ suggesting it outcompeted the wild type (Figure 5B). As expected, the strain harbouring a deletion of RscS was outcompeted by the wild-type strain. Those two results were seen under both growth conditions, suggesting that the stress of modelled microgravity does not alter the colonisation of the light organ by these aggregation mutant strains.

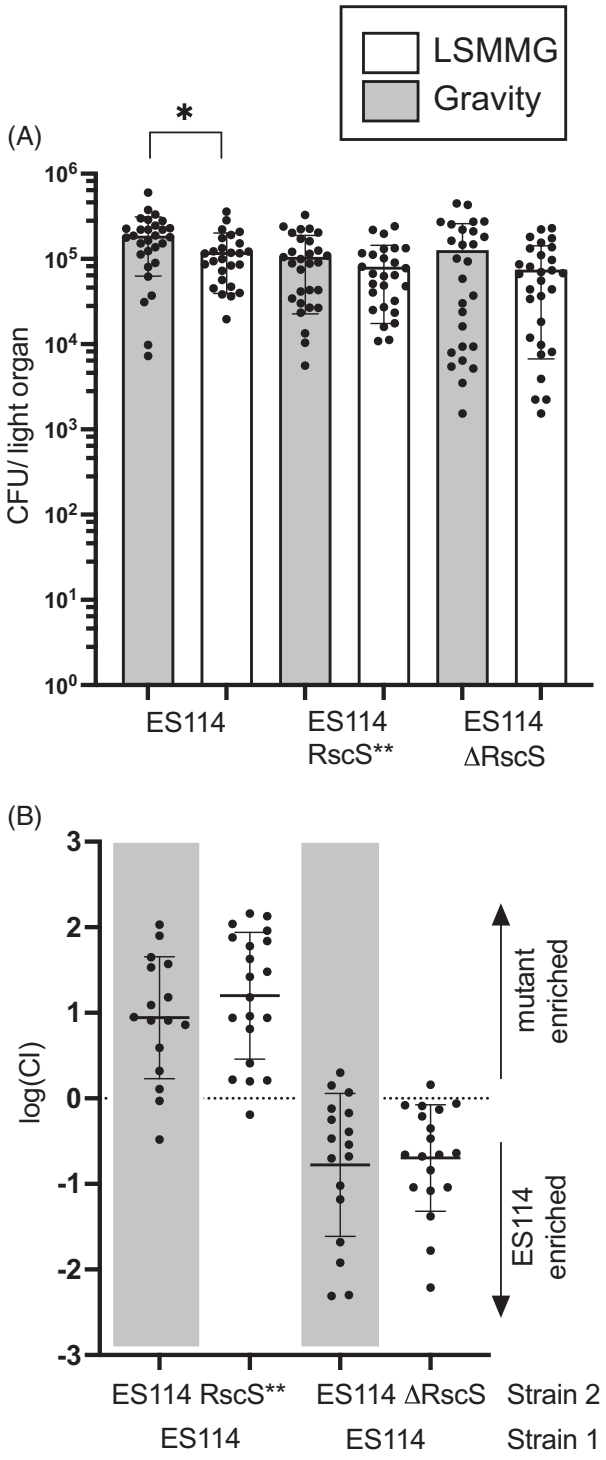

FIGURE 5 Colonisation of juvenile squid with wild-type ES114 and bacterial aggregation mutants stress under modelled micro- and unit gravity for 24 h. (A) Average of colony-forming units (CFU) present in each squid (i.e., each dot represents one light organ) colonised by a single strain of either ES114, the hyper-aggregator ES114 RscS**, or the non-aggregator ES114 ΔRscS under either gravity (grey bar) or low-shear modelled microgravity (LSMMG, white bar) conditions. For each condition, a minimum of 26 squid were analysed across three technical replicates. The error bars indicate the standard deviation. A Mann–Whitney–Wilcoxon *U* test, which was used to compare unit gravity with LSMMG-treated animals, indicated that the CFU per light organ were significantly different for ES114 (*$p \leq 0.1$) (B) The competitive index (CI), or the ratio of mutant to ES114, was determined for animals co-inoculated with the wild-type and either the hyper-aggregator ES114 RscS**, or the non-aggregator ES114 ΔRscS under either gravity (grey bars) or LSMMG (white bars) conditions. Each dot correspond to the log(CI) of a co-colonised squid. For each condition 31 squids in three replicates were co-inoculated by ES114 and a mutant, and 16 to 20 squids ending up co-colonised. The remaining squid were singly colonised (i) by ES114 RscS** in the ES114/ES114 RscS** competitions, and (ii) by ES114 in the ES114/ES114 RscS-competitions.

DISCUSSION

In this study, we evaluated the impact of a novel environmental stressor on the population structure of a horizontally transmitted symbiont. To this end, we colonised the squid *E. scolopes* with either one or two

strains of *V. fischeri* under modelled microgravity or unit gravity conditions. To our knowledge, this is the first description of the initial assembly and structure of a microbial symbiotic population in an animal host under modelled microgravity. Previously studies have been focused on the effect of modelled microgravity either on bacterial growth in culture (Kim et al., 2013; Mora et al., 2019; Su et al., 2021) or on the change to an already established microbial community in a host (Garrett-Bakelman et al., 2019). Here, we showed that under modelled microgravity (i) principle colonisation behaviours previously observed between *V. fischeri* strains were maintained, (ii) fewer CFUs were present in a colonised light organ for some S-strains, (iii) the ratio between strains in a co-colonised squid differed due to a differential partitioning of the strains within the crypts of the light organ and (iv) differences in aggregation were not seen and did not influence the establishment of the symbiosis.

First, we demonstrated that the S and D behaviours previously described in *V. fischeri* (Bongrand et al., 2016) were maintained in modelled microgravity. However, when singly colonised, the CFU per light organ of S-strain-colonised squids were significantly lower under modelled microgravity, suggesting that the environmental stress of altered gravity conditions negatively impacted the resulting symbiont density, a factor that can affect the protective benefits of a symbiosis (Drew & King, 2022). The loss of density of a symbiont population within the light organ under a given stress can be a function of the symbiont's response, the host's response, or a combination of both. However, the loss of density observed should not negatively affect the symbiosis since the bacteria still reach a cell density allowing bioluminescence (McCann et al., 2003). In addition, the S-strains' densities in LSMMG are comparable to the cell density observed when squids were colonised with the D-strain MB13B2. Overall, we hypothesised that this loss of bacterial density might relieve the squid from the stress of hosting the symbiont population without altering whether a symbiosis was maintained.

Interestingly, the growth of *V. fischeri* in culture shows the opposite response; that is, a bacterial culture is denser after 24 h under modelled microgravity conditions compared to unit gravity (Grant et al., 2014). Therefore, our data suggest that under modelled microgravity stress, symbiont density in the light organ is regulated not only by bacterial growth but also by the host squid. However, the impact of this host–microbe interaction is strain specific, with reduced symbiont density observed under microgravity only exhibited in host's colonised by S-strains and not D-strains. There is currently no clear mechanism to explain this response and further experiments will be necessary to understand it and determine whether the host deploys this strain-behaviour-specific effect when exposed to other environmental stresses.

We also demonstrated that under modelled microgravity there was a shift in the strain composition within the crypts of light organs co-colonised by two S-strains. We could discern no pattern in the strain distribution within crypts co-colonised by any two strains (Figure S1). Interestingly, the difference is mainly observed in Crypt 2, to a lesser extent in Crypt 3, and only marginally in Crypt 1. We also observed that microgravity conditions resulted in Crypt 2 being more often uncolonised and more crypts colonised by the typically-less competent coloniser, ES114, compared to MB13B1. The crypts of the light organ mature at different times during host development, with Crypt 1 being the most mature at hatching and the development of Crypt 2 and Crypt 3 continuing post-hatching (Essock-Burns et al., 2020; Sycuro et al., 2006). Taken together, these results lead us to hypothesise that the stress of microgravity induced a delay in the colonisation trajectory of Crypt 2 and Crypt 3, which could potentially benefit a slower coloniser (Bongrand & Ruby, 2019a). An appealing hypothesis would be that, when the crypt becomes prepared to take up a symbiont, both the fast S-strain and the slow S-strain may have already arrived at the crypt, increasing the opportunity that the slower strain will be present and able to compete for entry. These results highlight another way in which the physical environment has an important impact on the establishment of symbiosis.

Finally, we showed that modelled microgravity has no apparent effect on the colonisation of squids by different mutants defective in the steps leading to symbiont aggregation outside the light-organ pores. This aggregation process is potentiated by the bacterium's production of exopolysaccharide encoded by the *syp* genes, (Shibata et al., 2012) and regulated by the histidine kinase RscS (Yip et al., 2006), which is required for efficient colonisation of the squid (Koehler et al., 2019; Mandel et al., 2008). These results suggest that defects in aggregation were not exacerbated under modelled microgravity. Nevertheless, we cannot say that microgravity has no influence on the aggregation state. For example, aspects related to the timing of its appearance, its shape, or its robustness, only that whether the host still became colonised to a similar degree as wild-type conditions remain unexplored. In light of the previously described results, we conclude that the aggregation itself may not have a strong effect on the eventual population structure in the light organ.

In summary, this study highlights the consequences that environmental stress can have on establishing a symbiont population within a mutualistic association. And perturbing the initial colonisation can lead to potential long-lasting consequences for the stability and health of both the symbiosis and the host. Additional study is still needed to determine the mechanisms underlying how different environmental stresses can impact the symbiont and host separately, as well as the

interactions between the partners that are critical to maintaining a beneficial mutualism.

AUTHOR CONTRIBUTIONS

Clotilde Bongrand: Conceptualization (lead); formal analysis (lead); methodology (lead). **Jamie S. Foster:** Conceptualization (supporting); funding acquisition (supporting); investigation (supporting); resources (lead); supervision (equal); writing – original draft (supporting); writing – review and editing (equal).

ACKNOWLEDGEMENTS

We thank the Kennedy Space Center Microgravity Simulation Support Facility, especially Jeff Richards and Ye Zhang for their help and use of the stereo- and confocal microscopes. We also thank Karen Visick for the strains KV4366 and KV6533. The work was supported by NASA Space Biology grants 80NSSC19K0138 and 80NSSC18K1465 awarded to Jamie S. Foster.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated as part of this study is available within this manuscript and figures.

ETHICS STATEMENT

All cephalopod procedures received prior approval by IACUC and the approval number and the statement is located in the experimental procedure section of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Bongrand, C. & Foster, J.S. (2023) Modelled microgravity impacts *Vibrio fischeri* population structure in a mutualistic association with an animal host. *Environmental Microbiology*, 1–11. Available from: https://doi.org/10.1111/1462-2920.16522