Competitive interactions between food-borne pathogens within mixed-species biofilms.

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Abstract

Within nature bacteria primarily exist as biofilms; polymicrobial aggregates surrounded by a protective matrix. Biofilms are more tolerant to antimicrobials than their planktonic counterparts due to their ability to produce growth-arrested persister cells. Many infections have a biofilm component that makes treatment difficult and results in recurrent, chronic infections. Biofilms often comprise multiple species which promotes cooperation and competition between cells that results in rapid adaptation to an environment. Competition in multispecies biofilms is high due to limited space and resources and bacteria can employ a variety of mechanisms to inhibit or kill competitors. This study used a biofilm evolution model, using glass beads as a substrate for biofilm formation, to identify interactions between a food isolate of *E. coli* (EC166 of ST10) and three *Salmonella* strains. *S.* Typhimurium and *S.* Enteritidis growth was inhibited in the presence of *E. coli* EC166 biofilms however, all three strains survived with *E. coli* EC166 under planktonic conditions, suggesting a biofilm-dependent mechanism of inhibition. When added to pre-formed *E. coli* EC166 biofilms, growth of all three *Salmonella* strains was reduced suggesting an active mechanism of inhibition, induced by the presence of *Salmonella*, rather than general competition for resources. Furthermore, immediately after inoculation and passaging, all three *Salmonella* species were more abundant than *E. coli* EC166 in a multispecies biofilm, demonstrating that *Salmonella* initially colonises the bead model and that *E. coli* EC166 may employ an active mechanism of growth inhibition at a later stage of biofilm formation. Genomic analysis identified a type VI secretion system, including the toxic effector protein RhsD, within the *E. coli* EC166 genome which may be responsible for reduced *Salmonella* growth in the presence of *E. coli* EC166. Together, these results suggest that *E. coli* EC166 actively inhibits or kills *S.* Typhimurium and *S.* Enteritidis.

Introduction

A biofilm is an assembly of microorganisms living as a multicellular aggregate, surrounded by a self-produced matrix. In nature, most bacteria exist within biofilms; this mode of life is distinct from planktonic growth and offers protection from environmental stressors and antimicrobials (1–4). Biofilms are ubiquitous across Earth and can be found in seawater, groundwater, soil, and ocean sediment where they drive biogeochemical cycles of various elements (1,5). These diverse communities are exploited by humans and used for bioprocesses including: bioremediation to treat polluted environments; biofertilizers to increase crop yield; and production of biofuel as a source of renewable energy (6–9). Biofilms are significant in human and animal health and chronic infections generally contain a biofilm component in which the infection develops slowly and induces an adaptive inflammatory response, but is not cleared by the immune system (10–12). Due to their low susceptibility to antibiotics, these infections are incredibly difficult to treat and are associated with high mortality, particularly in patients with immunodeficiencies (13). A key feature of biofilms is their emergent properties; features that are not predictable from studying planktonic cells. These include production of an extracellular matrix, communication between cells, and rapid adaptation to new environments which collectively protect and enhance biofilms. We must study and understand these properties to effectively treat or remove biofilms and prevent their associated morbidity.

Initiation of biofilm formation

To initiate biofilm formation, motile planktonic bacteria must adhere to a biotic or abiotic surface. Within the environment, bacteria can attach to rocks and pipes but during infections bacteria typically attach to each other to form a self-contained aggregate. Some species can attach to surfaces within the body including indwelling devices or prostheses, and to epithelial cells in the gut and urinary tract (14,15). Initial attachment is reversible and mediated by physiochemical forces of the surface and bacterial envelope; if the attractive forces outweigh the repulsive forces, then reversible binding will occur (5). The cell envelope can comprise flagella—filamentous structures that induce cell motility and explore surface topography—as well as proteinaceous adhesins including pili, fimbriae, and curli that aid reversible binding. For example, the *E. coli* adhesin FimH is crucial for attachment of cells to human epithelia via FimH-mannose bonds. This occurs via a catch-bond which promotes attachment of cells under high shear stresses associated with the gastrointestinal and urinary tract of humans (16,17).

Following attachment, cells begin to divide and form microcolonies as the biofilm matures. Maturation occurs in response to an increase in the second messenger molecule cyclic diguanylate monophosphate (c-di-GMP), the major regulator of biofilm formation (18). c-di-GMP is synthesised by diguanylate cyclases (DGCs) and broken down by phosphodiesterases (PDEs) and high levels of c-di-GMP reduce motility and promote a sessile lifestyle. For example, the *Burkholderia cenocepacia* protein RpfR has both DGC and PDE activity and mutations in *rpfR* that reduce activity of the PDE domain to prevent c-di-GMP breakdown, resulting in larger aggregates, increased matrix production, and increased biofilm formation (18).

The biofilm matrix: polysaccharides and proteins

Following irreversible attachment, bacteria multiply and form microcolonies (Figure 1). These cells continue to adhere to the surface and/or each other and secrete extracellular matrix to surround and protect the growing biofilm. Production of extracellular matrix makes biofilms distinct from planktonic cells by immobilising the bacteria, providing mechanical stability, and promoting communication. The matrix is generally composed of hydrated exopolysaccharides (EPS), proteins, extracellular DNA (eDNA), and lipids, however the abundance of each component varies between species, conditions, and the stage of biofilm formation (2,19). For example, *Pseudomonas aeruginosa* secretes high levels of polysaccharides to the surrounding matrix including Psl which promotes cell-cell attachments to stabilise the biofilm in the early stage of formation (20). As the biofilm matures, Psl accumulates at the periphery and aids in dispersal of cells (20). *P. aeruginosa* also produces the polysaccharide alginate which protects cells from the environment. Colvin *et al*. (21) showed that alginate is overproduced by clinical isolates from the lungs of cystic fibrosis patients however is not required during biofilm formation *in vitro* demonstrating the role of the environment in matrix composition. One group demonstrated that alginate present in *P. aeruginosa* biofilms forms complexes with cationic antimicrobial peptides to reduce their diffusion via electrostatic and hydrophobic interactions. Peptides were aggregated and inactivated before they could reach cells within the biofilm (22–24). Conversely, more recent studies identified antimicrobial peptides that are able to diffuse freely through the extracellular matrix and are effective against enterohemorrhagic *E. coli* and *Staphylococcus aureus* (25,26). This must be considered when designing antimicrobial peptides for treatment of biofilms and a balance between antimicrobial activity and permeability must be reached.

Proteins are secreted to the matrix to stabilise and protect the biofilm, such as amyloids; highly aggregated proteins that aid in adhesion and facilitate host-bacterial interactions (27,28). One key example are curli fimbriae (produced by *Enterobacteriaceae*) that bind to extracellular matrix proteins and host cells. Curli are important in biofilms and curli-deficient mutants have reduced biofilm formation (29,30). In *Salmonella enterica* biofilms, the extracellular matrix is primarily composed of the polysaccharide cellulose and curli fimbriae which stabilise and protect the biofilm and aid in colonisation of host cells (31). Studies have shown that environmental conditions impact production of cellulose and curli. Activity of the cellulose promoter *agfD* changes depending on the surrounding oxygen concentration, temperature, and nutrient availability which significantly changes the composition of the matrix (32). Curli production is also altered depending on condition; Srinandan *et al*. (33) showed that at low shaking conditions, expression of the *csgD* was significantly higher than under high shaking, resulting in increased curli production (33).

The biofilm matrix: extracellular DNA

Extracellular DNA is released into the matrix by lysed cells or by living cells and disrupting eDNA can reduce biofilm formation (34,35). eDNA is important for stabilising the biofilm but is also protective; its negative charge sequesters positively charged antibiotics, such as aminoglycosides, to reduce entry to cells and susceptibility to the antibiotic (36). eDNA also chelates divalent cations, such as Mg^{2+}, which induces the PhoPQ two-component system in *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium. This system modifies lipopolysaccharide on the bacterial envelope resulting in reduced outer membrane permeability to antimicrobials (37,38). Interestingly, a recent study investigating *in vivo P. aeruginosa* biofilms found eDNA from host leukocytes protected the biofilm by forming a protective second matrix structure outside of the biofilm (39). Alhede *et al*. (39) showed host DNA and bacterial eDNA form a physical shield to protect bacteria from tobramycin and host immune cells.

Thanabalasuriar and colleagues also showed that *in vivo P. aeruginosa* biofilms are protected from antibiotics and immune cells, this time by a layer of host neutrophil extracellular trap formation (40). It would be interesting to investigate the role of host DNA, in addition to bacterial DNA, as a protective factor in biofilm formation and treatment.

Biofilms cause recurrent infections

The paradigm of biofilm formation is that biofilms develop into organised 3D structures composed of mushrooms and channels (41). However, *in vivo* biofilms are generally smaller, self-contained aggregates, that do not form complex structures and rarely adhere to host cells (42–44). Instead, these biofilms continually re-seed and lead to chronic, recurrent infections. For example, recalcitrant infections caused by resistant *B. cenocepacia* and *P. aeruginosa* are the major cause of death in cystic fibrosis patients; treatment with antibiotics allows these species to form biofilms whilst protective commensal species are lost (45,46). Similarly, Staphylococci spp. can form biofilms on indwelling devices such as catheters and cannulas which can develop into bloodstream infections and sepsis (46,47).

Persister Cells

A major concern about biofilms is their ability to form transiently multi-drug resistant persister cells which are associated with relapse of many bacterial infections (Figure 1). Persisters are a heterogeneous sub-population of bacteria that undergo a phenotypic change to become growth-arrested when stress (such as antibiotics, low oxygen, or low nutrients) is applied (48–50). As antibiotics target growth mechanisms, persisters are intrinsically tolerant and can re-establish an infection following the removal of susceptible cells (51). This results in relapse of infections and continual use of antibiotics can deplete protective microbiota and select for antibiotic-resistant populations (52). Miyaue *et al.* (53) showed that in *E. coli*, more persister cells were produced when grown as a biofilm compared to under liquid planktonic conditions and were maintained at high numbers up to four weeks after biofilm removal and fresh broth (53). Persister cells are formed using class II toxin-antitoxin systems. These encode a stable toxin that inhibits essential cellular functions resulting in quiescence, and a labile antitoxin that is degraded under cellular stress, such as low oxygen or nutrients, and DNA damage. The toxin accumulates under stress to induce a persister state and on removal of the stress, the antitoxin can neutralise the toxin and growth can resume (54,55). This was first identified in *E. coli* containing the *hip* operon which encodes the toxin HipA (a serine-protein kinase that phosphorylates tRNA inhibiting protein synthesis) and antitoxin HipB (a DNA-binding protein that forms a complex with HipA and inhibits its activity) that leads to production of persister cells (55,56).

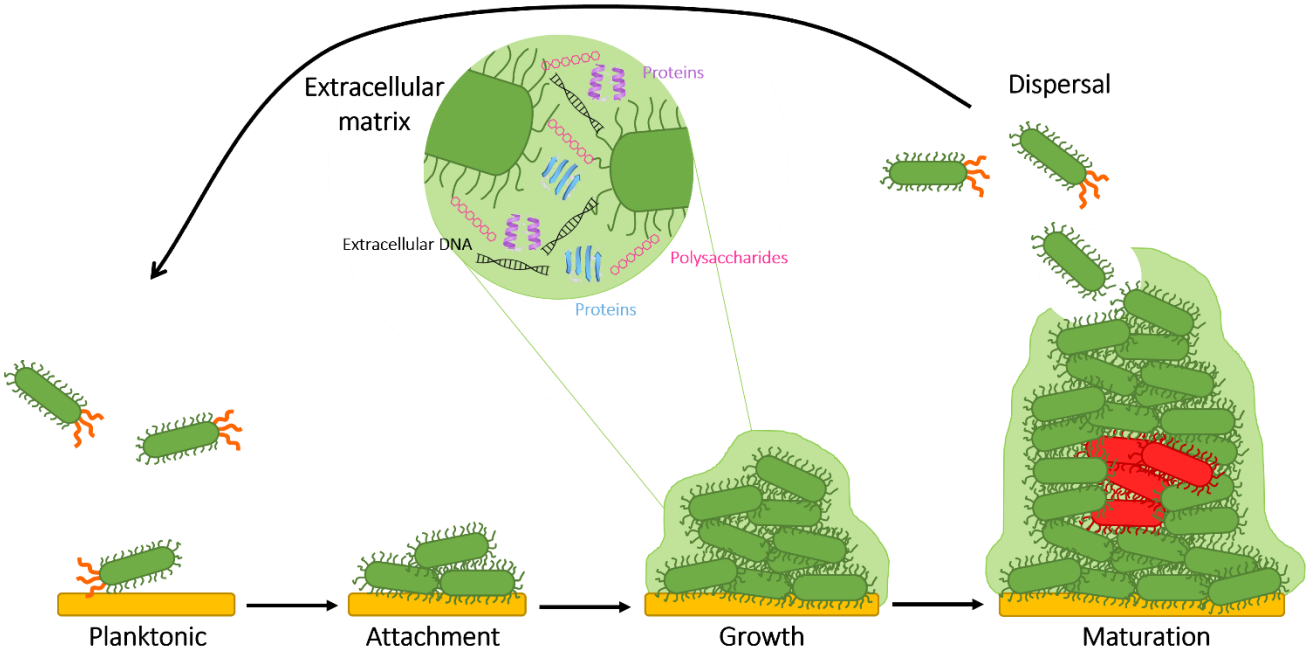

Figure 1. The Biofilm Lifecycle

Biofilm formation begins with attachment in which planktonic bacteria (green) use flagella (orange) to move towards and sense a surface (yellow). Bacteria attach to a surface using adhesins which also adhere the bacteria to each other; cells become sessile and downregulate flagella. The bacteria begin to grow and form microcolonies and start to produce an extracellular matrix (zoomed circle). This extracellular matrix contains extracellular DNA (black), proteins (purple and blue), and polysaccharides (pink) that together provide mechanical stability and protect the biofilm from desiccation. The biofilm matures by growing and producing more extracellular matrix and some cells can become persisters (red) that are growth arrested and inherently tolerant to antibiotics. Bacteria disperse from the biofilm and return to a planktonic lifecycle before forming a new biofilm.

Communication in biofilms: quorum sensing

The intimate nature of biofilms permits communication between members which occurs via quorum sensing—regulation of gene expression in response to changes in the density of the bacterial community (4). Quorum sensing allows the biofilm to act as a whole, making biofilms distinct from planktonic cells which do not communicate with others (57). Communication is mediated by autoinducer molecules which are released by bacteria and bind to cognate receptors on their own surface and the surface of surrounding cells. This binding triggers changes in relevant gene expression which results in altered protein production (58). Quorum sensing is crucial to biofilm formation and several studies show that quorum sensing inhibitors prevent biofilm formation (59). In *E. coli* biofilms, the autoinducer-2 analogue isobutyl-DPD significantly inhibits maturation of biofilms and another autoinducer-2 analogue, phenyl-DPD clears pre-formed biofilms (60). As well as cooperative communication in biofilms, quorum sensing can also be used for competition by invading cells and unwanted cells. For example, *Vibrio cholerae* uses quorum sensing to activate a type IV secretion system that injects toxins into surrounding cells. Cells without immunity to these toxins, (i.e. other species) will be killed and only *V. cholerae* will remain (61). This is also seen in *P. aeruginosa* which has three type IV secretion systems controlled by quorum sensing that provide an advantage in biofilm formation (62,63).

Biofilms drive diversity

Biofilms are physiologically heterogeneous and comprise cells in different growth states with varying roles, making the bacteria resilient beyond what is possible in a monoculture of very similar cells. Biofilms are often composed of multiple strains/species. These characteristics are possible due to the dynamic nature of biofilms which can change in response to the environment and adapt quickly to survive in unfavourable conditions. Intraspecific diversity (changes in one species that results in individual ecotypes) is common in biofilms; cells of the same species can have differential gene expression and metabolite production to promote growth and survival of the biofilm. For example, Lee *et al*. (64) showed that growth of monospecies biofilms of *P. aeruginosa, Pseudomonas protegens*, and *Klebsiella pneumoniae* led to morphotypic variants of all three species that differed from the wild-type strains in attachment, motility, and siderophore production.

A study by Poltak *et al.* (65) investigated adaptation of *B. cenocepacia* biofilms. After 1050 generations, *B. cenocepacia* diversified into three distinct colony morphologies; Studded (S type), Ruffled (R type), and Wrinkly (W type) which produced different levels of biofilm and attached to biotic and abiotic surfaces in distinct ways. Following this, the group used a biofilm bead model to study competition between the early and late populations (66). To study space as a limiting resource, additional beads were used. Only the early population showed a higher yield with an additional bead, indicating that space is limiting only in early morphotypes. The group also looked at two phenotypes (biofilm formation and growth rate). In the early populations no relationship between biofilm output and growth rate was seen, however, in the late population, the S morphotype had a significant increase in growth rate but also produced less biofilm than its early predecessor. Mutations in c-di-GMP may be drivers of this diversification; in the early population, the R morphotype had a mutation in *rpfR* which likely prevented action of the PDE domain so reduced breakdown of c-di-GMP, causing more biofilm formation (66).

Biofilms are inherently drug resistant

Bacterial research is typically carried out using liquid media promoting planktonic growth, however, most bacterial infections comprise a biofilm element. It is vital that we investigate biofilms to understand how we can best treat infections, particularly those with an antimicrobial resistance (AMR) component. Many studies show that planktonic and biofilm cells behave differently and highlight the importance of understanding biofilms to ensure correct treatment. A recent study investigated the impact of three antibiotics, at sub-inhibitory concentration, on biofilms and planktonic cultures. Biofilms rapidly adapted to each antibiotic in a step-wise manner and mutations were dependent on the antibiotic used. Furthermore, these mutations were different to those identified in planktonically-grown cells, indicating the importance of studying biofilms in addition to planktonic cells (67). A difference between biofilms and planktonic cells was also seen in *Acinetobacter baumannii* which causes recalcitrant infections in the lungs of cystic fibrosis individuals (68). Furthermore, two recent studies, looking at medieval remedies, found that although some remedies prevented the growth of planktonic cultures, little to no effect was seen in biofilms (69,70).

Bacteria can become resistant via resistance genes which can be acquired through horizontal gene transfer which allows rapid adaptation to an environment and occurs via the lateral movement of DNA (71,72). Mechanisms of horizontal gene transfer include transduction, transformation, and conjugation. The most clinically-relevant mechanism of horizontal gene transfer is conjugation of plasmids, transmissible elements that move between closely or distantly related species to

disseminate virulence and resistance genes (73). Plasmids carry a wide variety of resistance genes, one of the most problematic are those encoding extended-spectrum-β-lactamases (ESBLs) which break down β-lactam antibiotics including penicillins, carbapenems, and third generation cephalosporins, rendering the bacteria resistant (74,75). Alarmingly, a 50% increase in *Enterobacteriaceae* infections caused by bacteria harbouring an ESBL has occurred in the last decade (76).

Plasmid conjugation in biofilms

Conjugation enables the movement of plasmids and requires cell-to-cell contact facilitated by a type IV secretion system (T4SS) (77). Conjugative plasmids encode proteins that prepare the DNA and assist its movement, as well as proteins that make up the conjugation machinery. Conjugation occurs in three major steps: processing the DNA; recruiting the DNA complex to the T4SS; and moving the DNA complex through the T4SS (77).

General consensus is that conjugation is high in biofilms creating hotspots for horizontal gene transfer, arguing that the proximity of cells in biofilms permits plasmid transfer (78–81). However, this is not widely reported in the literature and many studies show plasmid conjugation physically promotes biofilm formation, as opposed to biofilms encouraging plasmid dissemination (82–84). For example, Burmølle *et al*. (85) showed the pOLA52 plasmid, which encodes a multidrug efflux pump and a β-lactamase, enhances biofilm formation in *Enterobacteriaceae.* pOLA52 encodes a type 3 fimbriae which promotes attachment to initiate biofilm formation, in a variety on environments, and aids in its dissemination. Reisner *et al*. (86) studied environmental isolates of *E. coli* and compared these to a K-12 lab strain that could not form a biofilm. When the environmental isolates were grown with K-12 strains, both formed biofilms. When an IncF plasmid was inserted into K-12 strain, the cells formed biofilms and when the original K-12 strain with no plasmid was grown with this, both formed biofilms. The group used plasmid exclusion to identify that the conjugation machinery was important in biofilm formation. Two plasmids of the same Inc group were inserted into the K-12 strain and when these were grown together, conjugation did not occur due to exclusion and biofilm formation was lost (81,86).

Some studies show that oxygen and nutrient gradients within biofilms result in areas of slow growing or growth arrested cells that limit plasmid movement (87). Krol *et al*. (88) showed that oxygen gradients limit plasmid movement within *E. coli* biofilms; plasmid transfer occurred only within a narrow zone at the air interface where oxygen concentration was high. These results suggest that biofilms have hotspots in which plasmid transfer is higher. Lilley *et al*. (89) showed that size and activity of a population determine how well a plasmid can establish; if the population is not dividing, plasmids will not move through the population. This was also shown in *Pseudomonas putida* biofilms; non-growing cells did not undergo plasmid transfer, even when subject to a high nutrient concentration (90). Interestingly, the group also showed that *P. putida* biofilms were subject to spatial constraints that prevented plasmid invasion, rather than supporting it as previous studies claimed.

It is still unclear whether plasmid transfer is increased or decreased in biofilms compared to in planktonic cultures and thus whether biofilms can promote plasmid dissemination. In 1999, Hausner *et al*. (91) reported that conjugation frequency in biofilms was higher than expected and higher in biofilms with increased nutrient concentration, however they did not use planktonic cultures as a comparison. Another study from 1999 investigated plasmid conjugation in biofilms but compared this to planktonic conditions. Under planktonic conditions, where continual mixing and movement of cells is permitted, numbers of transconjugants increased with time until all recipient cells contained a

plasmid. In biofilm experiments, position of cells was fixed and number of transconjugants increased upon addition of donor cells. However, transfer stopped once all recipients within close proximity of the donor cells had received the plasmid and overall, no significant difference was seen between conjugation frequency (92). A more recent study (93) simulated infection with *S.* Typhimurium, harbouring an MDR plasmid, followed by antibiotic treatment in a chicken gut model to study the movement of the plasmid from *S.* Typhimurium to *E. coli*. The plasmid was transferred to seven out of nine *E. coli* clones and these transconjugants remained in the model even following removal of the antibiotic. However, there remains a lack of up-to-date research investigating the rate of plasmid movement in biofilms, particularly multispecies biofilms, compared to their planktonic counterparts.

Multispecies biofilms

Most biofilm research has been carried out on monospecies cultures; however, many biofilms contain more than one species of bacteria or fungi and archaea. More recent studies aim to investigate the complexity of multispecies biofilms. These interactions are critical and govern spatial organisation of strains to induce cooperation or competition in biofilms. For example, biofilm biomass of the respiratory tract pathogens *A. baumannii* and *P. aeruginosa* increased by 26-fold and 102-fold, respectively, when grown in a multispecies biofilm with *Stenotrophomonas maltophilia*, compared to in a monospecies biofilm (94). Furthermore, biofilm formation was enhanced in a four-species mixed biofilm compared to monocultures in species isolated from food processing environments (95). These interactions exemplify the importance of understanding the properties of an infection—we must know what strains/species are present in a biofilm and what their characteristics are. This will allow us to be specific to effectively treat infections or remove biofilms from the environment where needed.

Cooperation in biofilms

In biofilms, cooperation enhances survival of the species involved and can allow cells to survive in conditions they would die in if alone (96). Co-aggregation is an important facet of cooperation and allows different species to attach to one another to stabilise the biofilm and protect all species involved (97). This is key in formation of oral biofilms which develop sequentially—primary colonisers adhere to the substratum to form microcolonies that subsequent colonisers can adhere to (98,99). Co-aggregation using curli is important in the gastrointestinal tract and cross-seeding of curli subunits between species of the gut microbiota increases surface attachment of cells and facilitates biofilm formation (100). For example, when *E. coli* O157:H7 is grown with *S.* Typhimurium, curli expression makes both species more tolerant to biocides (30,101).

Cooperation in multispecies biofilms can be synergistic which may increase abundance of one or more species and can reduce susceptibility to antimicrobials (102–106). Schwering *et al.* (104) investigated isolates of *E. coli*, *P. aeruginosa,* and *Enterobacter colaceae* from water sources and the ability of chlorine to eradicate monospecies compared with multispecies biofilms. Within multispecies biofilms, a chlorine concentration of 50-300-fold higher than for monospecies biofilms was required, exemplifying the need to be aware of biofilm composition and be able to deal with the worst case with regards to disinfection. Enhanced tolerance to disinfectants was also found in *Listeria monocytogenes* and *Lactobacillus plantarum* multispecies biofilms (103). Additionally, Lopes *et al.* (107) found that species common in cystic fibrosis patients have higher biomass and less susceptibility to a variety of antibiotics—including tobramycin, ciprofloxacin, cefotaxime, and chloramphenicol—when grown in a multispecies biofilm with *P. aeruginosa*.

Cooperation also means that one species can solely provide resistance for the whole biofilm. A study by Lee *et al*. (64) found that when grown as monospecies biofilms, *P. aeruginosa, P. protegens,* and *K. pneumoniae* were more susceptible to SDS and tobramycin. However, multispecies biofilms were resistant to both SDS and tobramycin. *P. aeruginosa* encodes a secreted SDS hydrolase (SdsA1) which can degrade and metabolise SDS within the biofilm. Additionally, *P. protegens* produces aminoglycoside-modifying enzymes that break down tobramycin and offer a community benefit. When the three species were grown as monocultures and exposed to tobramycin, only *P. protegens* survived showing the importance of different roles within a multispecies biofilm and how important it is to know which species are present as a species may be resistant to antimicrobial without possessing a resistance mechanism itself.

Competition in biofilms

Biofilms also promote competition for space and resources which can encourage character displacement in which a sub-group of cells from the same species create and occupy a new niche to reduce competition within that environment. Metabolic cross-feeding is important in character displacement; rather than using the same nutrient, some cells use metabolic products of other cells, creating a heterogeneous biofilm (108–110). This can occur in a monoculture, for example when *E. coli* is grown on glucose, some cells produce acetate due to incomplete oxidation of the glucose and other cells utilise this acetate in place of glucose (111,112). This reduces competition for nutrients and clears waste products. In multispecies biofilms, metabolic cross-feeding is common and promotes survival of multiple species in the biofilm. Henson *et al*. (113) reported extensive cross-feeding in a biofilm model of three gut commensals: *Bacteroides thetaiotaomicron*, *E. coli*, and *Faecalibacterium prausnitzii*. The study showed that production of succinate and acetate by *B. thetaiotaomicron* and *E. coli* and the consumption of these metabolites by *F. prausnitzii* is essential and loss of this relationship results in the loss of *F. prausnitzii* from the biofilm. Furthermore, production of ethanol by *E. coli* and its consumption by *B. thetaiotaomicron* was also essential and loss of this relationship led to loss of *F. prausnitzii* and *B. thetaiotaomicron* from the biofilm. Without a specific niche and the ability to use one another's metabolites, this multispecies biofilm could not grow.

Interference competition occurs via the production of molecules that prevent growth of other cells (114,115). This can occur at attachment for example, *Bacillus subtilis* and *Bacillus licheniformis* produce biosurfactants that prevent biofilm formation of *E. coli* and *Staphylococcus aureus* by breaking the surface tension of water to prevent colonisation (116). This method of preventing biofilm formation is seen in other species, including human pathogens and could be exploited to remove biofilms for treatment (117–119). Ramsey *et al*. (120) found that H_2O_2 produced by the commensal *Streptococcus gordonii* actually benefits the opportunistic pathogen *Aggregatibacter actinomycetemcomitans. A. actinomycetemcomitans* responds to H_2O_2 by inducing expression of the outer membrane protein ApiA that protects it from the innate immune system, enhancing survival. Furthermore, *S. gordonii* also produces L-lactate which, when metabolised by *A. actinomycetemcomitans*, increases pathogenicity of the latter (121). This allows cooperation mentioned previously and growth of cells together, making infections harder to treat.

Bacterial interactions shape biofilms

Within biofilms, competition is facilitated by bacterial interactions that result in killing or growth inhibition of competitors (122,123). Bacteria employ a range of methods to harm or kill other cells which can occur between different strains of the same species, or between different species. This competition is common in biofilms in which bacteria are in close proximity and competing for limited resources and space. An example of this is in the gut where interactions shape the gut microbiota and can prevent invasion by pathogenic strains which causes the gut microbiota to stay consistent over time (124–126). Attacking cells can secrete toxins (including small molecule antibiotics and antimicrobial peptides), tailocins (protein complexes that punch holes in closely-related strains), and phages that infect surrounding cells (123). These interactions can be contact-dependent, in which the host cell must physically interact with the target cell, or contact-independent, in which molecules are released into the surrounding environment. Contact-dependent mechanisms include production of toxins that are released directly into host cells and can occur through type IV secretion systems, type VI secretion systems, and contact-dependent growth inhibition (Figure 2). Contact-independent mechanisms release small molecules, proteins, tailocins, and phages into the extracellular milieu which diffuse to surrounding cells (Figure 3).

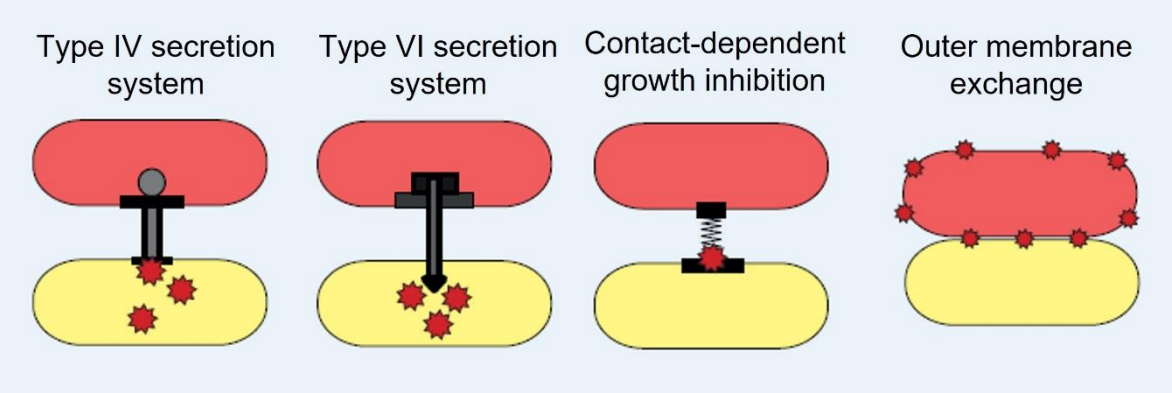

Figure 2. Contact-dependent bacterial interactions

Type IV secretion systems are found in Gram-positive and Gram-negative bacteria and allow transport of DNA and proteins directly from one cell to another. Translocation occurs via production of a pilus which transports proteins and DNA into target cells by active transport. Type VI secretion systems are found in Gram-negative species and move proteins from the cytoplasm of the host cell directly into the target cell. The type VI secretion system acts like a syringe and has a contractile sheath with a pointed tip that punches a hole into the target cell for effector proteins to move through. Contact-dependent growth inhibition uses a type V secretion system and involves a two-protein system in which one protein anchors the host and target cells together and the second protein contains a toxic domain to inhibit growth of the target cell. Outer membrane exchange involves presentation of toxic proteins on the surface of the host cell which bind to the outer membrane of target cells. Figure adapted from (123).

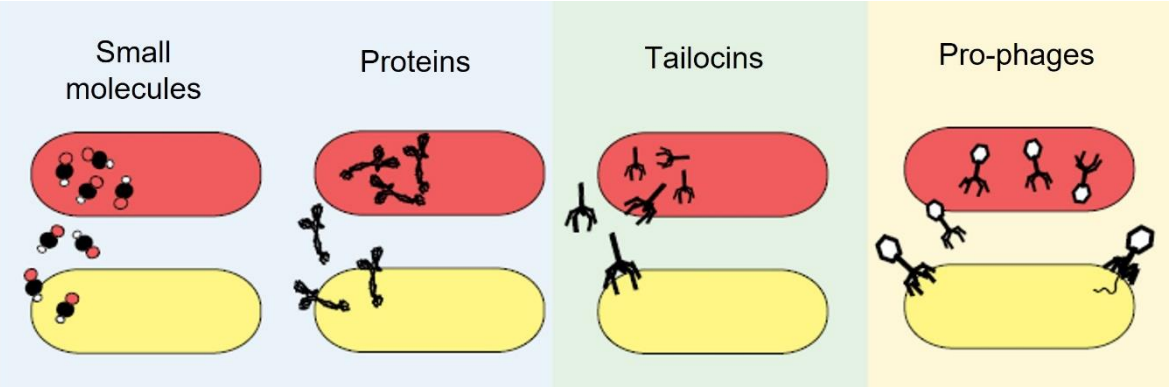

Figure 3. Contact-independent bacterial interactions

Small molecule toxins include antimicrobial peptides that are smaller than 10 kDa and are released from the host cell into the surrounding environment. These can then enter target cells to have their effect, often via diffusion due to their small size. Protein toxins bigger than 10 kDa are often released via cell lysis but due to their size require more specialised mechanisms to enter target cells. These proteins must bind to the target cell membrane and be actively taken up by the cell. Tailocins are derived from bacteriophages however do not have the DNA-containing capsid portion. Tailocins are released into the extracellular environment and punch holes in the membranes of surrounding cells. Pro-phages are integrated within the bacterial genome and upon activation are transcribed and released into the environment to bind to and enter cells that do not contain the same pro-phage DNA. Phages kill via cell lysis which results in the phage spreading further. Figure adapted from (123).

Bacteriocins

Bacteriocins are produced by Gram-positive and Gram-negative bacteria and are protein or peptide toxins that can exhibit antibacterial activity on cells of closely-related or the same species (127). A computational study modelled how bacteriocin production changes within biofilms and demonstrated that production is favoured when competitors are in close proximity and intermediately related (as opposed to highly or distantly) (128). Bacteriocins are frequently produced by commensals within the mammalian gut to prevent colonisation by pathogens. A study by Šmajs investigated bacteriocins produced by commensal *E. coli* isolated from the gut of two distinct populations; one from Brazil in 1978 and one from Czech Republic in 2004 and found that despite differences in population, 50% of *E. coli* isolates produced bacteriocins in both cases (129). *E. coli* produces two classes of bacteriocins: colicins and microcins (130,131). Colicins function by binding to outer membrane receptors of target cells and use them to translocate into the cytoplasm where they have a range of toxic effects including nuclease activity, depolarisation of the membrane, and inhibition of protein synthesis (130). Microcins are smaller than colicins, usually a few amino acids in length and can enter the cell by mimicking essential nutrients such as siderophores. Once inside the cell, microcins bind to and inhibit essential enzymes and can interact with the inner membrane resulting in death of the target cell (131).

Within the gut microbiota, commensal *E. coli* strains produce bacteriocins that can prevent colonisation of pathogenic bacteria. A study by Toshima *et al.* took isolates of gut microbes from 303 individuals and found that 52 isolates had inhibitory activity against enterohaemorrhagic *E. coli* O157 (132). Furthermore, Zihler *et al*. tested bacteriocin-producing *E. coli* against 68 non-typhoidal *Salmonella* isolates from clinical cases (133). These strains of *E. coli* produced four bacteriocins: microcin 24, microcin B17, colicin G, and colicin H. Strains producing microcin 24 were able to inhibit the growth of all 68 isolates and those producing microcin B17 inhibited 94%. Strains inhibiting colicin

H/G inhibited 48% and 21% of *Salmonella* isolates, respectively which included the 6% not inhibited by microcin B17. Another study demonstrated that colicin E1 produced by *E. coli* was also inhibitory against the pathogen *L. monocytogenes* (134). Bacteriocins can also be used as therapies to reduce colonisation of the gut by pathogenic bacteria (135,136). These studies illustrate how commensal species protect the host from pathogenic species, however it would be interesting to see whether these bacteriocins inhibit pathogenic bacteria within a biofilm.

P. aeruginosa also frequently produces bacteriocins, including pyocins which are classified into R, S, and F type. R-type can only kill other *P. aeruginosa* cells however S- and F-type can kill cells of other species (137). Pyocins kill cells by forming pores in the target cell membrane which results in depolarisation of the membrane and loss of essential processes resulting in cell lysis. Pyocin-producing strains are insensitive to their own pyocin so are protected from killing (138). In patients with cystic fibrosis, *P. aeruginosa* forms biofilms which contain competing strains that can use pyocins to interact with one another. Oluyombo *et al*. (137) found that R-pyocin producing strains were dominant in cystic fibrosis which may explain why certain strains dominate in cystic fibrosis (137). The group also found that pyocins have inhibitory activity against mature *P.* aeruginosa biofilms so could be used as treatments.

Type VI secretion systems

Another common and well-studied mechanism of bacterial interaction is the type VI secretion system (T6SS). These are present in Gram-negative species and are involved in a range of interactions between bacteria, which can be antagonistic and involved in competition in biofilms. T6SSs inject their effector proteins directly into a target cell, rather than relying on diffusion and uptake. Effectors can be simple single domain or large multi domain proteins that have a range of roles. Effector proteins target essential components of the target cell including the cell wall in which effector proteins degrade peptidoglycan (139). For example, in *P. aeruginosa,* the effector protein Tse1 cleaves between the second and third amino acid of the peptidoglycan peptide side chain (140). Also an effector protein of *P. aeruginosa*, Tse3 cleaves between *N*-acetylmuramic acid and *N*-acetylglucosamine within the peptidoglycan backbone (141). Tse1/Tse3 therefore lead to break down of the cell wall and subsequently cell lysis and death. However, in order to prevent death of own cells, immunity proteins Tsi1/Tsi3 are transported to the periplasm of *P. aeruginosa* to block the active sites of Tse1/Tse3 respectively (140). T6SS effectors can also target the cell membrane, for example in *V. cholerae,* VasX disrupts the membrane potential of target cells that do not possess the complementary immunity protein, leading to lysis and death of susceptible cells (142). Nucleic acid is also a target of T6SS effectors which can act as nucleases to cleave phosphodiester bonds present between nucleotides within DNA (139). T6SSs can also be used for killing own cells that are phage-infected, to prevent spread of the phage, non-cooperators, or for remodelling biofilms. Overall, T6SSs are important in biofilms for maintaining a functional, cooperative population, and preventing invasion of the biofilm by competitors.

The T6SS is composed of three sections: a membrane complex, a baseplate complex, and a tail complex (Figure 4) (143,144). The membrane complex is embedded in the inner/outer membranes of the host cell and works in a one-step mechanism in which effector proteins are translocated directly from the host cell cytoplasm into the cytoplasm of the target cell. The membrane complex is composed of Tss proteins which anchor the complex into the inner membrane and connect it to the baseplate via cytoplasmic loops of their component proteins. The baseplate is composed of TssK, TssF, TssG, and

TssE which form a wedge-like complex, six of which polymerise around a VgrG trimer. The baseplate is bound to the tail complex which extends into the cytoplasm and is formed of an inner tube made of repeating units of Hcp and an outer sheath made of repeating units of TssB/TssK (Figure 4). Upon activation, the tail complex contracts to push VgrG up and out of the cell where it can contact the target cell and punch a hole in its outer membrane. The effector proteins are bound to the VgrG trimer so are translocated into the target cell when VgrG punches a hole through the membrane (Figure 4) (143–145).

T6SSs are involved in competition between biofilm cells and are frequently used in the gut microbiota by commensals to prevent colonisation of pathogens and by pathogens to overcome commensals and establish an infection (124). Wexler *et al*. (146) used mathematical modelling to predict that within *Bacteroidetes* alone, 60-600 billion effector transmission events occur each day (146). Furthermore, commensal, non-toxic *Bacillus fragilis*, uses a T6SS to limit acquisition of other strains of *B. fragilis* or strains that are enterotoxigenic to protect the gut microbiota and allow persistence of a limited number of strains (125,147). Alternatively, T6SSs can also be used by pathogens to aid in colonisation and to establish an infection. MacIntyre *et al*. (148) showed that a strain of *V. cholerae* constitutively expressing a T6SS was able to significantly reduce the survival of enterohaemorrhagic and enteropathogenic *E. coli* strains but when the T6SS was non-functional, the *E. coli* could survive (148). Furthermore, Sana *et al*. (149) demonstrated in a mouse gut model that enteropathogenic *S.* Typhimurium requires a T6SS, encoded within *Salmonella* pathogenicity island-6, to kill commensal bacteria and efficiently establish itself in the gut (149). Overall, T6SSs are important to ensure biofilms are stable and cooperative communities and that threats to the biofilm are removed.

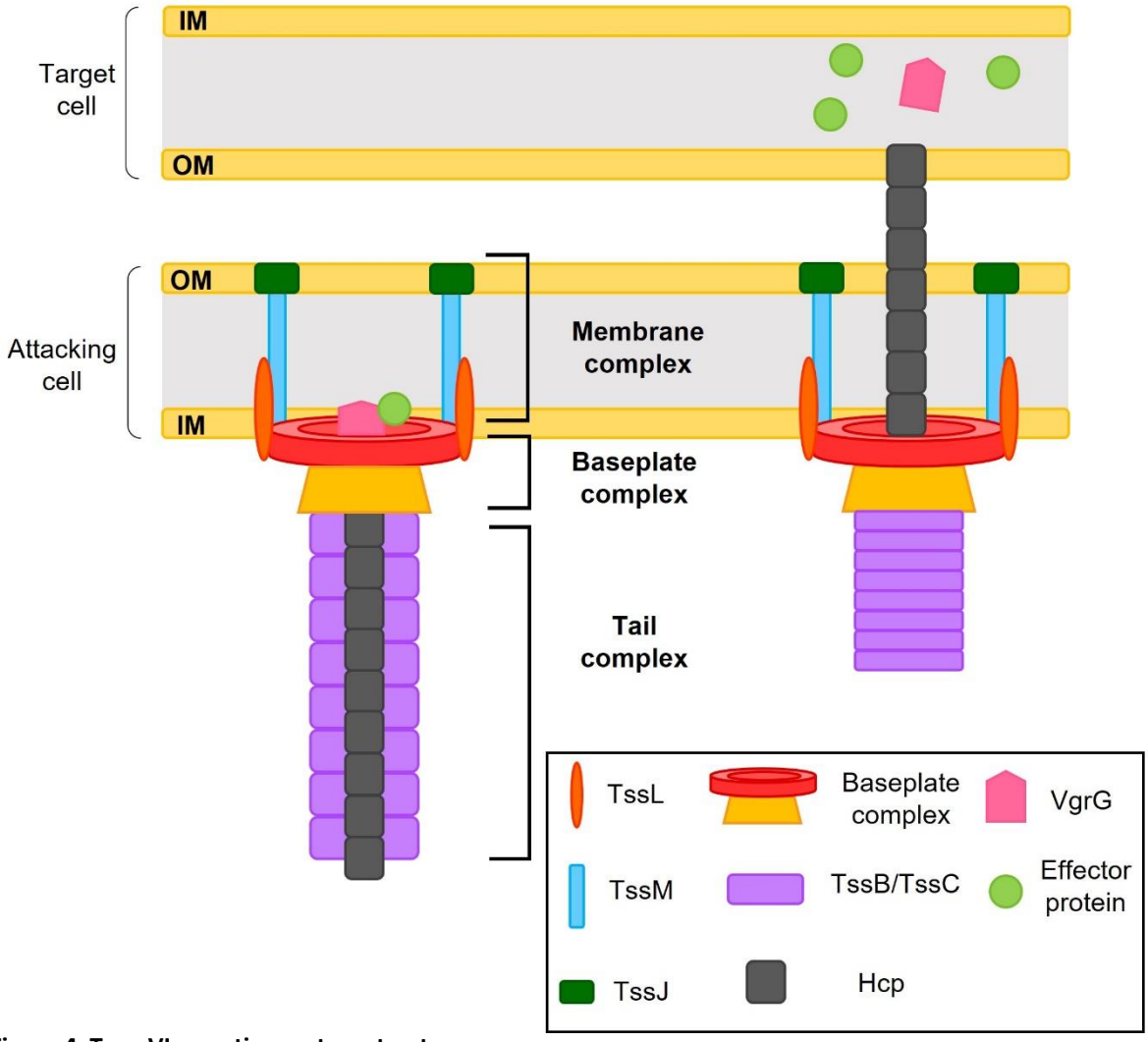

Figure 4. Type VI secretion system structure

The type VI secretion system (T6SS) sits in the membrane of the attacking cell and punches a hole through the target cell. The T6SS is made up of a membrane complex containing the proteins TssL, TssM, and TssJ which anchor the T6SS into the membrane. The membrane complex is bound to the baseplate complex, which is made up of TssK, TssF, TssG, and TssE that form a complex which polymerises around VgrG. The tail complex is bound to the baseplate and is made up of repeating units of TssB/TssC, which form the outer sheath of the tail, and Hcp repeats that form the inner sheath of the tail. Upon activation, the tail complex contracts to push VgrG up and out of the cell and through the neighbouring cell membrane. Effector proteins are bound to VgrG so are taken through the membranes of the attacking cell and into the target cell to impart their toxic effects.

Aims/objectives/hypotheses

The aim of this project was initially to investigate how plasmids move through multispecies biofilms and whether antibiotic stress impacts conjugation. However, after identifying an *E. coli* isolate with inhibitory activity against *S.* Typhimurium, I decided to follow this up for the remainder of the year. Therefore, my new aim is to identify how a food isolate of *E. coli* (ST10) prevents the growth of *Salmonella.* To do this, I will carry out the biofilm model experiment with two strains of *S.* Typhimurium and one strain of *S.* Enteritidis to identify whether the effect is present across strains and serovars. The biofilm model will allow us to observe the impact of *E. coli* EC166 on the life cycle of *Salmonella* biofilms*,* rather than just at one time point. I will expose *Salmonella* to *E. coli* EC166 single species biofilms and *E. coli* with *Salmonella* in multispecies biofilms to identify whether pre-formed biofilms prevent the growth of *Salmonella* and whether *Salmonella* must be present within the biofilm to induce this effect. I will calculate the abundance of *E. coli* EC166 and *Salmonella* biofilm cells over time using the biofilm model and finally use the BioFlux to observe where *E. coli* EC166 and *Salmonella* cells grow within relation to one another within biofilms.

I will use these experiments to test the hypothesis that *E. coli* EC166 inhibits the growth of *Salmonella* spp., when grown as a biofilm.

Methods

Bacterial strains and culture conditions

Escherichia coli (*E. coli*), *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) were used to investigate interactions between members of a mixed-species biofilm. For initial studies, I used *E. coli* BW25113, a well-characterised lab strain with no plasmids and a full collection of knockouts. However as this is a lab-adapted strain, I also used the *E. coli* food isolate EC18PR-0166 (EC166) of ST10 to study multispecies biofilms in the context of the food chain. EC166 was provided as a kind gift from the Mather group following a food survey conducted by Quadram Institute Biosciences. EC166 is a sequence type 10, a common sequence type in food (according to the food survey), and clinically important, thus a good candidate for a representative of the food chain and potential pathogens. *E. coli* EC166 also contains *lacI* and *lacZ* thus can be used for blue/white selection. For multispecies biofilms, *E. coli* BW25113 was grown with *S.* Typhimurium 14028S containing *lacI* and *lacZ* chromosomally integrated for blue/white selection. For multispecies biofilms, *E. coli* EC166 was grown with *S.* Typhimurium 14028S (STM2), *S.* Typhimurium SL1344 (STM1), and *S.* Enteritidis PT4 P125109 (STM14). These strains were chosen as these are well-characterised, common reference strains.

All strains were routinely cultured using LB broth and LB agar. LB broth was prepared with NaCl unless stated otherwise. Cultures were prepared from glycerol stocks stored at -80°C (*E. coli*) and -20°C (*Salmonella*). Within the biofilm model, beads were washed in sterile, filtered phosphate buffered saline (PBS) to remove planktonic cells. To distinguish between *E. coli* and *Salmonella,* blue-white screening was used. Cells were plated onto LB agar, supplemented with 5-Bromo-4C-Chloro-Inodlyl β-D-Galactopyranoside (X-GAL) at 40 μg/mL and isopropyl β-D-1-thiogalatopyranos (IPTG) at 1 mM. X-GAL was prepared in dimethyl sulfoxide, and IPTG (ThermoFisher) was prepared in distilled water. All media and reagents were supplied by Sigma-Aldrich unless stated otherwise.

Table 1. Species and strains

Identifier	Species/strain	*lac* operon	Fluorescent tag
BW25113	*E. coli*	No	No
EC166	*E. coli*	Yes	No
STM51	*S.* Typhimurium	Yes	No
STM2	*S.* Typhimurium	No	No
STM1	*S.* Typhimurium	No	No
STM14	*S.* Enteritidis	No	No
STM95	*S.* Typhimurium	No	Yes, *mplum*

Biofilm evolution model

To induce formation of biofilms, bacteria were grown in glass universal tubes containing 5 mL of LB broth without NaCl. Each tube contained three sterile 6 mm soda lime glass beads used as a substrate for biofilm formation; one bead for passaging, one bead to count biofilm cells, and one bead spare. For each experiment, *E. coli* and *Salmonella* were grown together and independently, and four technical

replicates were carried out per experiment. The model was inoculated with 50 μL of overnight liquid culture containing relevant species; for multispecies biofilms, 50 μL of each species was added to the same tube. LB broth containing no beads was inoculated with 50 μL of relevant culture; for multispecies, 50 μL of each species was added to the same tube, as planktonic controls. Tubes were incubated horizontally, shaking at 60 rpm to provide gentle agitation and induce biofilm formation.

To identify the impact of low temperature on multispecies biofilms, tubes were passaged every 48 hours and all tubes were incubated at 15°C. To assess the ability of two strains to survive together, tubes were passaged every 24 hours and incubated at 30°C for optimal biofilm formation. To passage the experiment, one bead from each tube was removed using a sterile loop (5 μL) and washed in 1mL of PBS per well of a 24-well cell culture plate for five minutes by gentle shaking, before being transferred to fresh LB broth without NaCl, containing three sterile beads. To passage the planktonic controls, 50 μL from each tube was transferred to fresh LB broth.

To quantify biofilm and planktonic cells, colony forming units (CFU) per bead or per mL, respectively, was calculated. For biofilm cells, one bead from each tube was removed using a sterile loop (5 μL), washed in PBS to remove planktonic cells, and transferred to an Eppendorf tube containing 1 mL of fresh PBS. Each Eppendorf tube was vortexed for 2 minutes to remove biofilm cells from the bead. Serial dilutions from 10^{-1} to 10^{-6}, using the supernatant from the vortexed bead, were performed for biofilm cells, all dilutions were plated. Serial dilutions from 10^{-1} to 10^{-9}, using cells from the liquid phase of universal tubes containing beads for planktonic cells, or from planktonic control tubes, were performed and dilutions 10^{-4} to 10^{-9} were plated. Blue-white colony screening was used to identify CFU of each species. For each dilution, 10 μL of cells was plated onto LB agar, supplemented with X-GAL (40 μg/mL) and IPTG (1 mM).

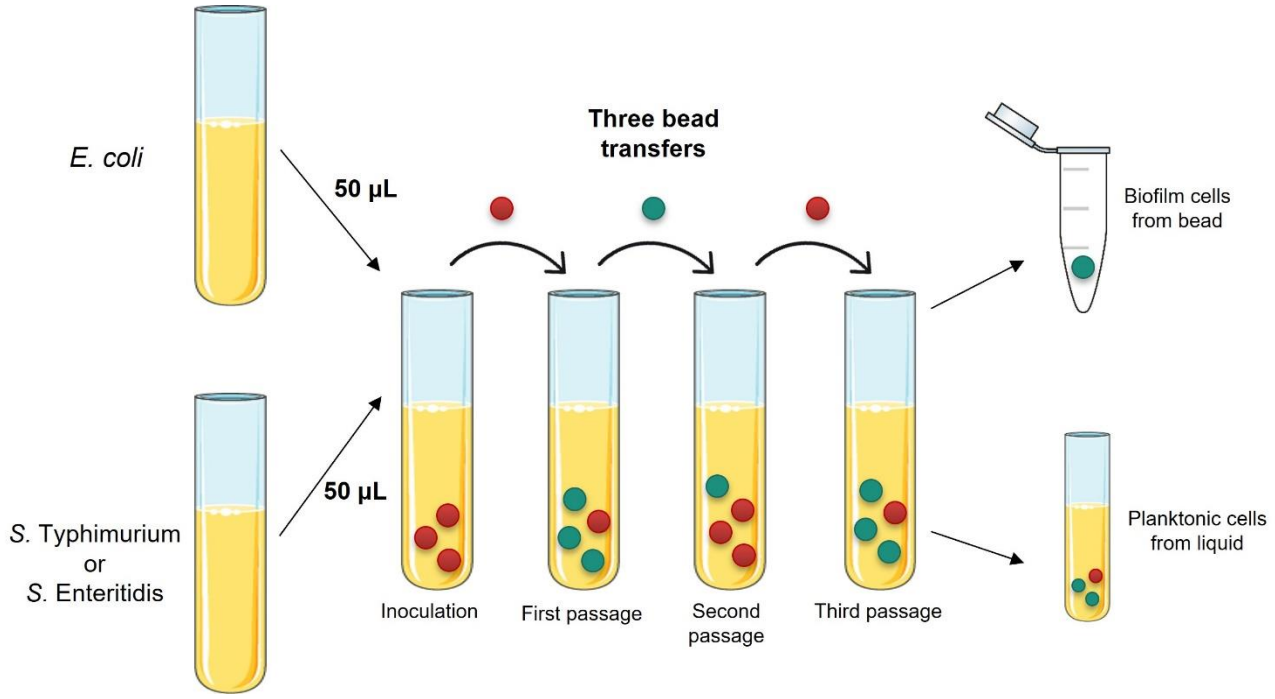

Figure 5. Biofilm evolution model

Glass beads were used as a substrate for biofilm attachment. Each tube was inoculated with 50 μL of relevant culture; multispecies biofilms were inoculated with 50 μL of *E. coli* and *S.* Typhimurium or *E. coli* and *S.* Enteritidis. To passage the cells, a bead was removed, washed in PBS, and added to fresh media. To count biofilm cells, a bead was removed and vortexed in PBS then the PBS was diluted and plated. To count planktonic cells, the liquid phase of the tube containing beads was diluted and plated.

Growth kinetics of *E. coli* and *Salmonella*

To determine growth rates of *E. coli*, *S.* Typhimurium STM2, *S.* Typhimurium STM1, and *S.* Enteritidis STM14, I assessed the growth kinetics of each strain. Overnight cultures of each strain (grown in LB broth at 37°C, 200 rpm) were diluted 1:100 in fresh LB broth and added to a 96-well microtiter plate with 16 technical replicates per strain. Using a microplate reader, the optical density at 600 nm was measured every 15 minutes for 24 hours at 37°C.

Biofilm evolution model: adding supernatant from final passage of the biofilm model

When EC166 and *S.* Typhimurium STM2 were grown together, *S.* Typhimurium STM2 was lost from the multispecies biofilm. To identify whether EC166 produced a diffusible product to prevent growth of *S.* Typhimurium STM2, *S.* Typhimurium STM2 was grown with EC166 supernatant from different conditions from the final passage of the biofilm model (Table 2). At 96 hours post inoculation, after one bead was removed for counting, the remaining beads were removed from each tube and the supernatant was obtained by centrifuging the tubes at 4000 rpm for 15 minutes. Supernatant from each condition was diluted 1:10 in LB broth and overnight cultures of *E. coli* and *S.* Typhimurium STM2 were added individually to each broth condition to make a 1:100 dilution of cells to broth + supernatant. The experiment was carried out in a microtiter plate and eight technical replicates for each condition were performed. Using a microplate reader, the optical density at 600 nm was measured every 15 minutes for 24 hours at 37°C.

Table 2. Supernatant conditions added to LB broth for growth curves

Growth condition	Species
Overnight liquid culture (planktonic alone)	*E. coli* alone
Final passage planktonic with beads	*E. coli* + *S.* Typhimurium
Final passage planktonic with beads	*E. coli* alone
Final passage planktonic control	*E. coli* + *S.* Typhimurium
Final passage planktonic control	*E. coli* alone

Biofilm evolution model: adding pre-formed biofilms to planktonic *Salmonella*

To determine whether pre-formed *E. coli* biofilms could prevent the growth of planktonic *S.* Typhimurium, beads colonised with *E. coli* biofilms were added to *S.* Typhimurium. This was done using an adapted biofilm model in which 5mL of LB broth without NaCl containing four beads was inoculated with 50 μL of *E. coli* EC166 or 50 μL of *E. coli* EC166 and 50 μL of *S.* Typhimurium STM2. Two tubes of each condition were prepared; one to add to a 3-hour culture of *S.* Typhimurium and one to add to a 24-hour culture of *S.* Typhimurium. Single species and multispecies biofilms were used to determine whether the presence of *S.* Typhimurium was required to induce the potential inhibitory activity of *E. coli* EC166. Tubes were incubated horizontally at 30°C, shaking at 60 rpm to induce biofilm formation.

Unlike the biofilm model, beads were not passaged but were incubated for 24, 48, or 72 hours to identify whether the maturity of the biofilm impacted the ability to prevent *S.* Typhimurium growth.

At 24, 48, and 72 hours, three beads were removed from each tube and washed in 1 mL of PBS per well of a 24-well cell culture plate. Six beads from the multispecies condition and six beads from the single species *E. coli* EC166 condition were added to twelve individual tubes of LB broth (5 mL) inoculated with *S.* Typhimurium STM2. Three multispecies beads and three single species beads were added to *S.* Typhimurium that had been inoculated then incubated for 3 hours to identify the effect of biofilms on *S.* Typhimurium in exponential phase. Three multispecies beads and three single species beads were added to *S.* Typhimurium STM2 that had been inoculated then incubated for 24 hours to identify the effect of biofilms on *S.* Typhimurium in log phase. Both 3-hour and 24-hour cultures of *S.* Typhimurium STM2 were incubated with no beads added as controls and all *S.* Typhimurium cultures were incubated at 37°C, shaking at 60 rpm.

After addition of the beads, 3-hour and 24-hour cultures were incubated for 2 hours then counted and incubated for a further 22 hours before counting again. This was to identify whether the effect of *E. coli* EC166 on *S.* Typhimurium occurred at an early or late stage. To count the cells, 20 μL of the liquid phase of each tube was removed and diluted to 10^{-9} and 10 μL of each replicate was plated onto X-GAL (40 μg/mL) and IPTG (1 mM).

Adding one bead to planktonic cultures of *S.* Typhimurium STM2 did not show any difference between the growth of *S.* Typhimurium with biofilms compared to the controls. I therefore repeated the experiment but added five beads to 3-hour and 24-hour cultures of *S.* Typhimurium as described previously. However, I included an additional time point and added beads incubated for 24, 48, 72, and 96 hours.

Adding planktonic S*almonella* to pre-formed E. *coli* biofilms

To determine whether pre-formed biofilms could prevent the growth of *Salmonella,* biofilms were grown in 24-well cell-culture plates. To each well, 1 mL of LB broth without NaCl was added and inoculated with 25 μL of overnight *E. coli* EC166 or *E. coli* BW25113. Plates were incubated at 30°C, shaking at 60 rpm to induce biofilm growth. After 24 hours, media was removed with a 1 mL pipette without touching/disturbing the biofilm formed on the walls of each well and fresh LB broth and 25 μL of *Salmonella* were added to each well. To *E. coli* EC166, *S.* Typhimurium STM2, *S.* Typhimurium STM1, or *S.* Enteritidis STM14 were added and to *E. coli* BW25113, *S.* Typhimurium STM51 was added. Controls of 25 μL of each *Salmonella* strain alone were also included in duplicate, and the mixed-species were carried out in quadruplicate. The plate was incubated for a further 24 hours before media from each well was removed and diluted for plating and counting. *E. coli* and *Salmonella* were distinguished by blue-white screening. Cells from each well were diluted to 10^{-9} and 10 μL per replicate was plated onto X-GAL (40 μg/mL) and IPTG (1 mM).

Biofilm evolution model: *E. coli* and S*almonella* in mixed-species biofilms over time

The biofilm model provides a snapshot of which species are present on a bead at one time point every 24 hours, however I aimed to determine which species colonise the bead first and how the abundance of each species changes over time. To do this, I carried out the biofilm model whilst removing one bead for counting at 2, 4, 6, 8, and 24 hours post-inoculation of the model, as well as 2, 4, 6, 8, and 24 hours

post passage. As previously described, 5 mL of LB broth without NaCl was inoculated with 50 μL of *E. coli* EC166 and 50 μL of *S.* Typhimurium STM2, 50 μL of *S.* Typhimurium STM1, or 50 μL of *S.* Enteritidis STM14, all from overnight cultures prepared in LB broth. The experiment was carried out in quadruplicate and tubes were incubated horizontally at 30°C, shaking at 60 rpm.

At 2 hours post inoculation, one bead was removed from each tube and washed in 1 mL of PBS per well of a 24-well cell culture plate before biofilm cells were removed via vortexing for counting, as described previously. This was repeated at 4, 6, and 8 hours post inoculation of the model and at each time point, biofilm cells were diluted to 10^{-6} and 10 μL was plated for counting. At 24 hours post inoculation, two beads were removed from each tube and washed. One bead was transferred to fresh LB broth without NaCl containing five sterile beads and one bead was vortexed and counted. At 2 hours post passage, one bead was removed from each tube and washed in 1 mL of PBS per well of a 24-well cell culture plate before biofilm cells were removed via vortexing for counting. This was repeated at 4, 6, and 8 hours post passage and at each time point. To count biofilm cells, supernatant from vortexed Eppendorf tubes containing beads was diluted and 10 μL per replicate was plated onto X-GAL (40 μg/mL) and IPTG (1 mM).

Growing mixed-species biofilms using a bioflux system and fluorescence microscopy

To visualise *S.* Typhimurium and *E. coli* within a biofilm, I used a BioFlux system to promote the growth of multispecies biofilms for imaging. *S.* Typhimurium STM2 tagged with *mplum* (*S.* Typhimurium STM95) was used for imaging. To prepare the BioFlux plate, 250 μL of LB broth without NaCl was added to the outlet wells of each column of a 48-well BioFlux plate. The outlet wells were attached to the BioFlux which was run at 0.3 dyne for five minutes to allow the inlet and outlet wells to balance and media to enter the flow cells. The excess broth was removed from the outlet wells and 50 μL of overnight culture, diluted 1:100 in LB broth, was added to the inlet wells of each column. *E. coli* EC166 and *S.* Typhimurium STM95 were added at ratios of 1:1, 1:2, 1:3. 1:5, 2:1, and 5:1 (EC:STM respectively), and controls of *E. coli* EC166 and *S.* Typhimurium STM95 alone were used. Each condition was carried out in triplicate. The inlet wells of the plate were attached to the BioFlux which was ran at 3 dyne for 5 seconds to move bacteria into the flow cells. The plate was then left at room temperature for one hour for bacteria to adhere to the flow cells. Following this, 1 mL of LB without NaCl was added to the inlet wells which were then attached to the BioFlux and run at 0.3 dyne for 24 hours.

At 24 hours, the BioFlux plate was detached and the waste media from the outlet wells was removed. To each of the inlet wells, 1 mL of fresh LB without NaCl was added before attaching the wells to the BioFlux which was run at 0.3 dyne for a further 24 hours. The BioFlux plate was removed and the waste media from the outlet wells and excess media from the inlet wells were removed to reduce flow within the flow cells to allow for better imaging. Fluorescence microscopy was carried out using a Nikon Eclipse Ti Inverted Microscope. Images were captured at 20X magnification using an excitation of 590 nm to show *mplum* fluorescence within the *S.* Typhimurium STM95. Images were captured from the centre of each flow cell and the number of fluorescence particles was analysed using Fiji (ImageJ).

Genomic analysis

In order to identify the potential mechanism of *E. coli* EC166 inhibition of *Salmonella* growth, I analysed the *E. coli* EC166 genome. *E. coli* EC166 was sequenced and Spades (150) and Prokka (151) using a Galaxy installation was used for assembly and annotation. I used Artemis 18.1.0 (152) to search for

potential genes responsible for inhibition of *Salmonella*. I searched for secretion systems, bacteriocins, colicins, microcins, and individual genes conserved within secretion systems, e.g., *vgrG*. I also looked for islands with high or low GC content indicating these may have been acquired from other bacteria which is common for bacterial weapons. The amino acid sequence of hits were further analysed by using the protein-protein function in BLAST (153) to identify potential protein products.

Statistical analysis

CFU/mL and CFU/bead were calculated in Microsoft Excel using (number of colonies x dilution factor)/volume spotted. All data was plotted and statistically analysed using GraphPad Prism 9.

Results

I used a biofilm evolution model to investigate interactions between *E. coli* EC166 (a food isolate), and three common reference strains of *Salmonella* (two *S.* Typhimurium and one *S.* Enteritidis) in a mixed community. Glass beads acted as a substrate for biofilm attachment and at each passage, one bead was removed to continue the experiment allowing us to observe interactions over time. We also aimed to uncover the mechanism by which *E. coli* EC166 inhibits the growth of *Salmonella*. This was done by exposing *Salmonella* to supernatant taken from *E. coli* EC166 biofilms to identify whether a diffusible inhibitory product was present within the media. *Salmonella* was also exposed to pre-formed *E. coli* EC166 biofilms to determine whether the inhibitory effect was biofilm-specific. To visualise how *E. coli* EC166 and *Salmonella* exist in mixed biofilms, a BioFlux system was used to grow the mixed community and image biofilms.

E. coli BW25113 is not maintained in a multispecies biofilm with *S.* Typhimurium

Initially, I aimed to identify whether temperature influenced the maintenance of *E. coli* BW25113 and *S.* Typhimurium STM51 in a multispecies biofilm over time. The biofilm model was inoculated with equal volumes of *E. coli* BW25113 and *S.* Typhimurium STM51 and incubated at 15°C to assess the impact of cold on biofilms. This was to match a food processing environment which commonly use 10°C. In our case, 15°C was low enough to observe a temperature effect but still allowed some growth (Figure 6). The experiment was also carried out 30°C as a control to promote optimal biofilm formation for these species under these conditions (Figure 7). The experiment was passaged every 48 hours to allow sufficient time for the cells at 15°C to grow.

At 15°C, *E. coli* BW25113 was lost from the multispecies biofilm by 96 hours (the first passage), and by 192 hours (the final passage) in the single species biofilm (Figure 6A). At 15°C, under planktonic conditions, *E. coli* BW25113 was lost from the multispecies condition by 144 hours but remained throughout the experiment when grown as a single species biofilm (Figure 6B). *S.* Typhimurium was maintained in biofilms and planktonically in both single species and multispecies conditions at 15°C (Figure 6A). The CFU/bead and CFU/mL for *S.* Typhimurium in biofilm and planktonic conditions was similar between multispecies and single species conditions (Figure 6).

At 30°C, *E. coli* BW25113 was present within the single species and multispecies biofilm throughout the experiment, however in the multispecies condition, one of four replicates showed no growth at 48 and 96 hours (Figure 7A). *E. coli* BW25113 at 30°C survived in multispecies and single species planktonic conditions throughout the duration of the experiment however showed a lower CFU/bead or CFU/mL when in a mixed condition compared with alone (Figure 7B). At 30°C, *S.* Typhimurium STM51 was present in biofilms and planktonically in single species and multispecies conditions throughout the experiment (Figure 7). The CFU/bead and CFU/mL for *S.* Typhimurium STM51 was similar between multispecies and single species conditions in biofilm and planktonic conditions (Figure 7).

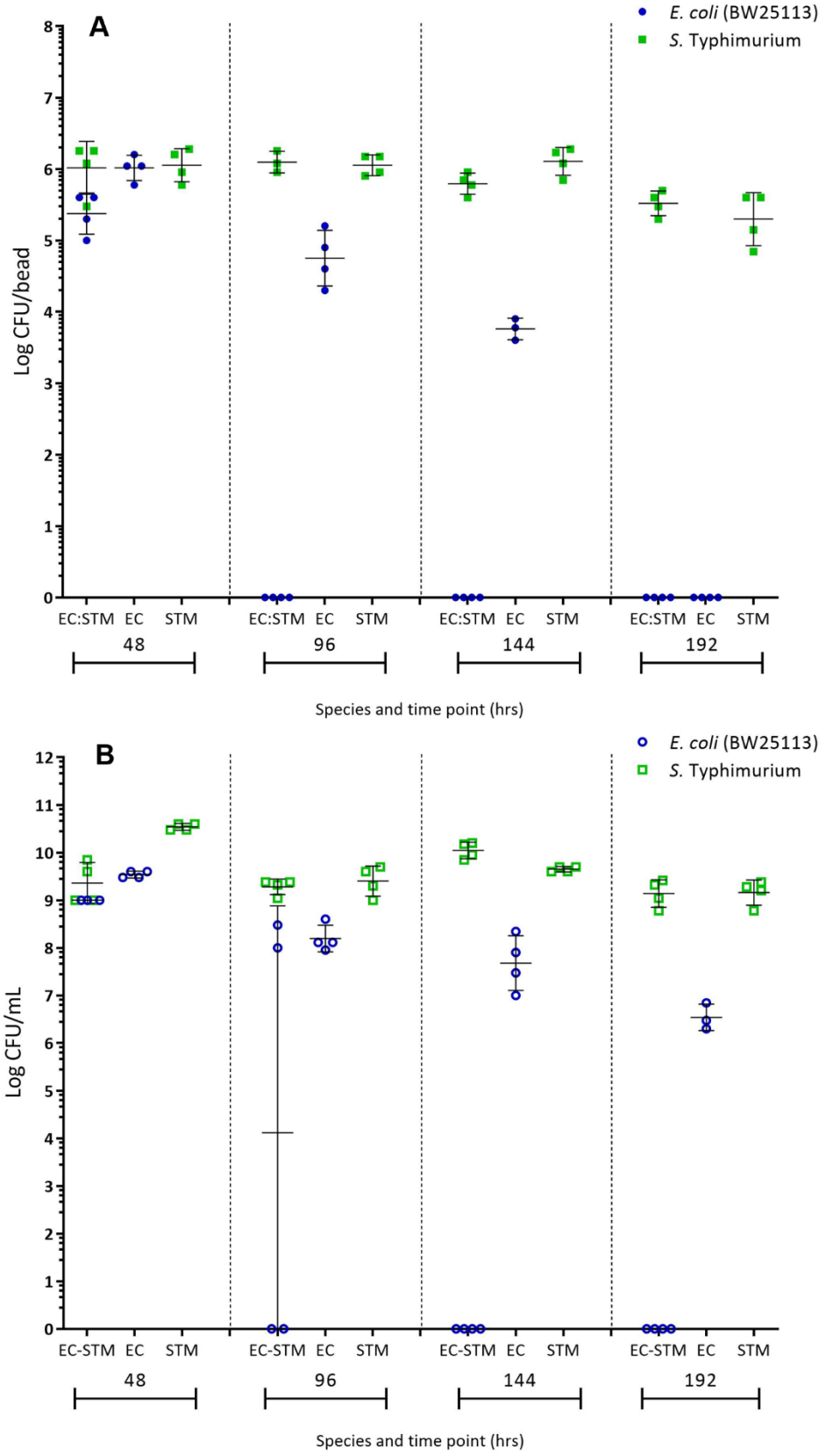

Figure 6. *E. coli* BW25113 is lost from biofilm and planktonic conditions when grown with *S.* Typhimurium at 15°C

E. coli BW25113 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated at 15°C and passaged every 48 hours. At each passage, a bead was removed from each condition and washed before biofilm cells (A) were removed for plating. Planktonic cells (B) were removed from the liquid phase of the tube for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal lines) and the error bars show +/- one standard deviation from the mean.

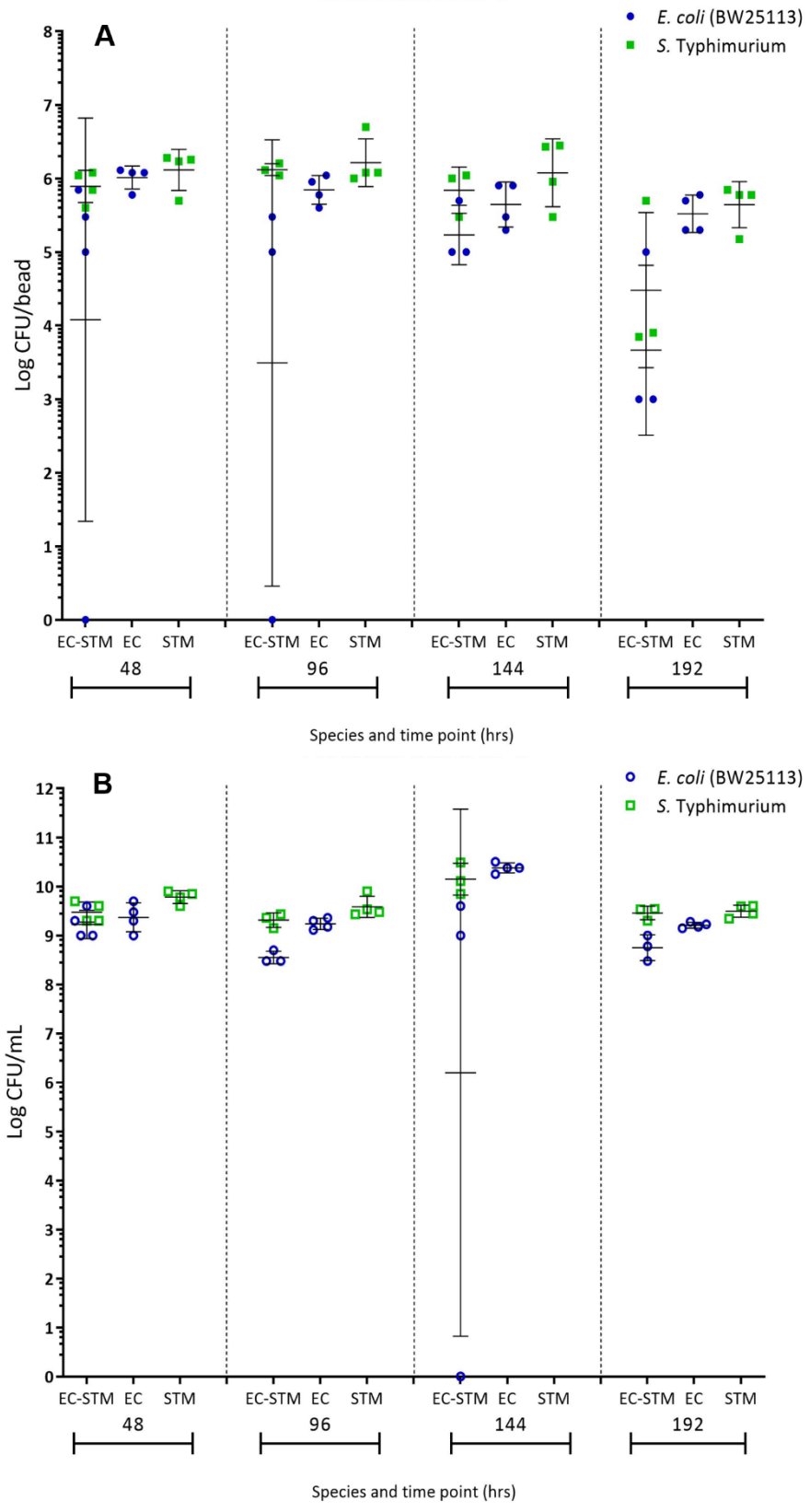

Figure 7. *E. coli* BW25113 is reduced in biofilm and planktonic conditions when grown with *S.* Typhimurium at 30°C. *E. coli* BW25113 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated at 30°C and passaged every 48 hours. At each passage, a bead was removed from each condition and washed before biofilm cells (A) were removed for plating. Planktonic cells (B) were removed from the liquid phase of the tube for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal lines) and the error bars show +/- one standard deviation from the mean.

E. coli EC166 outcompetes *S.* Typhimurium in a multispecies biofilm

As *E. coli* BW25113 was unable to persist in a multispecies biofilm with *S.* Typhimurium, I repeated the biofilm experiment using a different strain of *E. coli*. The food isolate *E. coli* EC166 (of ST10) isolated from a recent QIB food survey, was chosen to investigate whether *E. coli* and *Salmonella* could be maintained at the same abundance in a multispecies biofilm. *S.* Typhimurium STM2 was used as the *Salmonella* strain; this is the same strain as *S.* Typhimurium STM51 but without the lac operon as *E. coli* EC166 contains the *lac* operon already. The biofilm model was repeated as previously; incubated at 15°C and 30°C and passaged every 48 hours.

Contrast to the data with *E. coli* BW25113, *S.* Typhimurium STM2 was lost from the multispecies biofilm when *E. coli* EC166 was present. At 15°C in the multispecies biofilm, three out of four replicates showed no *S.* Typhimurium STM2 growth by 144 hours (Figure 8A), and *S.* Typhimurium STM2 was completely lost from the multispecies biofilm at 30°C by 192 hours (the final passage) (Figure 9A). However, at both temperatures *E. coli* EC166 was maintained throughout the experiment. Moreover, when *S.* Typhimurium STM2 was grown as a single species biofilm, all four replicates were maintained throughout the experiment at 15°C and 30°C.

Under planktonic conditions, *S.* Typhimurium STM2 could not grow in a mixed species culture at 15°C or 30°C (Figure 8B; Figure 9B). This contrasts with the previous results in which *E. coli* BW25113 was lost from multispecies biofilms but was able to grow planktonically at 30°C (Figure 6B; Figure 7B). When grown alone, *S.* Typhimurium STM2 was able to form biofilms and was maintained planktonically throughout the experiment at both temperatures. Furthermore, data from growth with *E. coli* BW25113 demonstrates the ability of *S.* Typhimurium STM2 to form a multispecies biofilm.

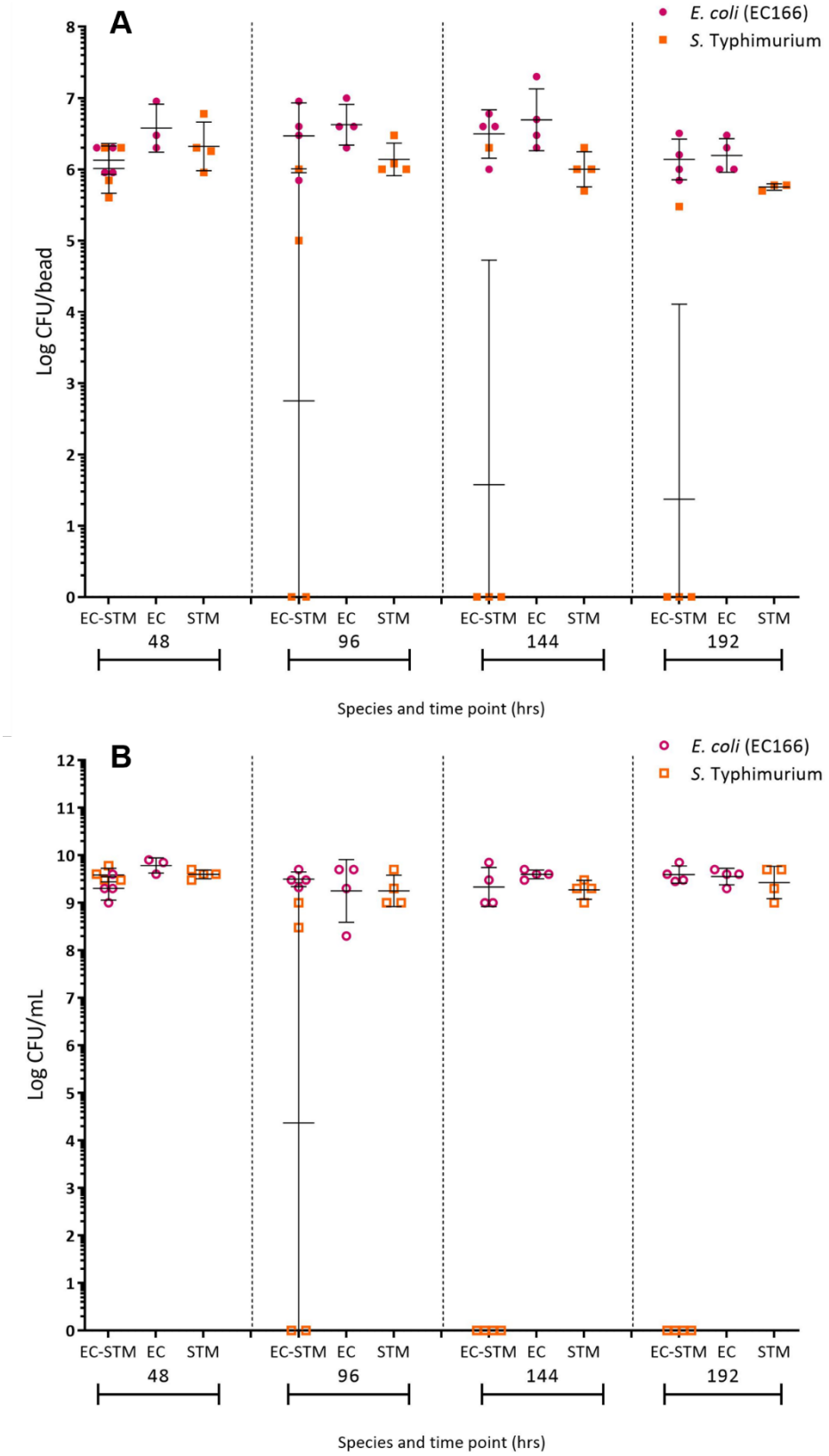

Figure 8. *S.* Typhimurium is lost from biofilm and planktonic conditions when grown with *E. coli* EC166 at 15°C.

E. coli EC166 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated at 15°C and passaged every 48 hours. At each passage, a bead was removed from each condition and washed before biofilm cells (A) were removed for plating. Planktonic cells (B) were removed from the liquid phase of the tube for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal lines) and the error bars show +/- one standard deviation from the mean.

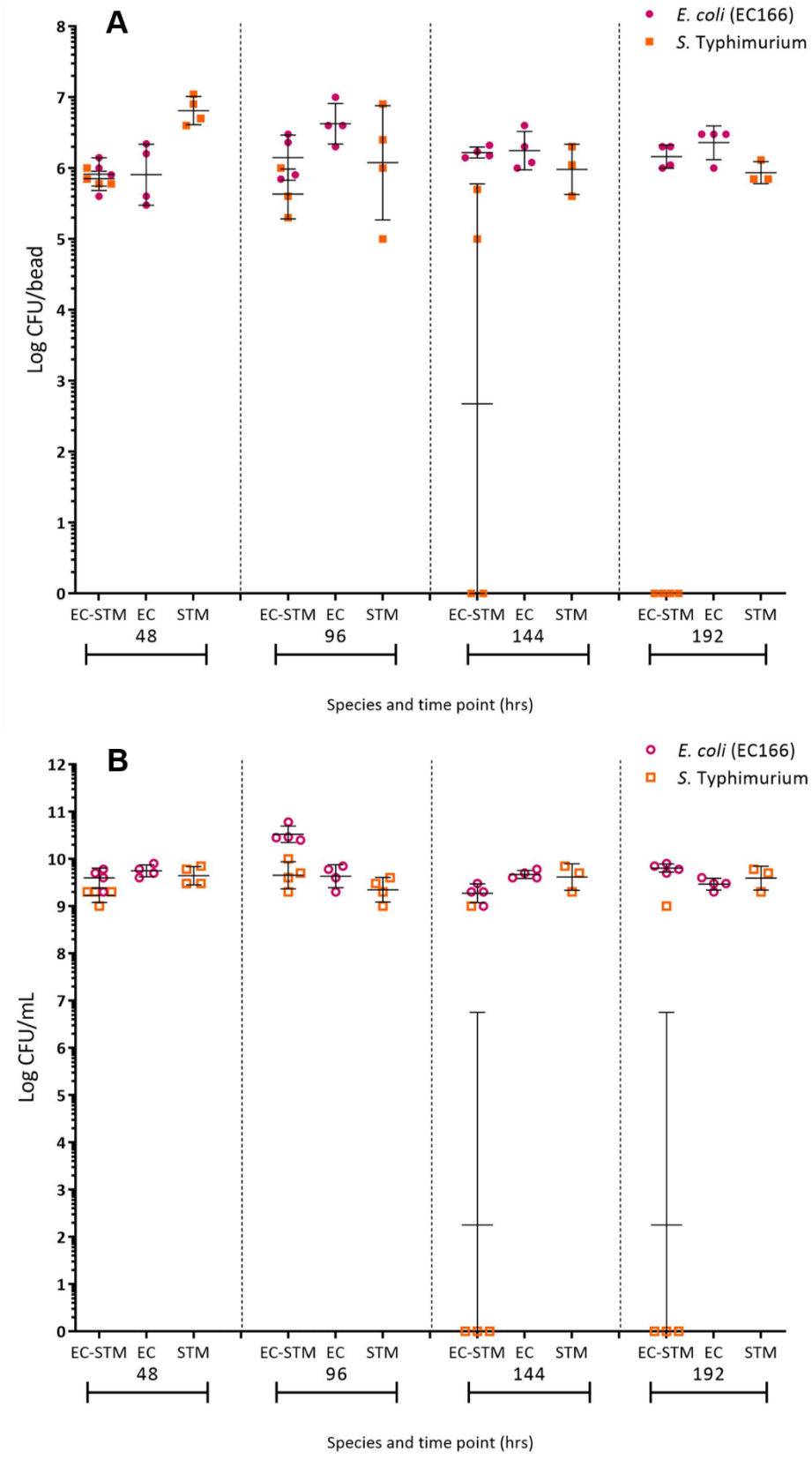

Figure 9. *S.* Typhimurium is lost from biofilm and planktonic conditions when grown with *E. coli* EC166 at 30°C.

E. coli EC166 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated at 15°C and passaged every 48 hours. At each passage, a bead was removed from each condition and washed before biofilm cells (A) were removed for plating. Planktonic cells (B) were removed from the liquid phase of the tube for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal lines) and the error bars show +/- one standard deviation from the mean.

***E. coli* BW25113 is lost from multispecies biofilm but not planktonically**

To confirm previous results, I repeated the biofilm evolution experiment at 30°C but passaged the beads every 24 hours to determine whether a shorter interval also prevented multispecies biofilm formation. I also used another planktonic control to determine whether reduction in planktonic growth in the *E. coli* EC166 experiment was biofilm dependent. The additional planktonic controls were grown with no beads but incubated under the same conditions as tubes containing beads (30°C, 60 rpm) . I carried out the experiment using *E. coli* EC166 with *S.* Typhimurium STM2 and *E. coli* BW25113 with *S.* Typhimurium STM51 as a control as previously *E. coli* BW25113 was lost in biofilm conditions but grew planktonically in the liquid phase, however *S.* Typhimurium STM2 was lost under both conditions when grown with *E. coli* EC166.

As previously, *E. coli* BW25113 had reduced growth in a multispecies biofilm compared with a single species biofilm, however it was not completely lost from the multispecies biofilm; two out of four replicates remained (Figure 10). As some data was missing, a mixed-effects analysis was used to analyse the effects of *S.* Typhimurium STM51 in a multispecies (with *E. coli* BW25113) vs. single species biofilm and time point on the CFU/mL of *S.* Typhimurium STM51. This revealed that there was no significant interaction between effects of *S.* Typhimurium STM51 in a multispecies vs. single species biofilm and time point on CFU/mL of *S.* Typhimurium STM51 [$F(3, 22) = 0.084$, $p=0.489$)]. Simple main effects analysis showed that *S.* Typhimurium STM51 in a multispecies vs. single species biofilm had a significant effect on CFU/mL [$F(1, 22) = 8.500$, $p=0.008$], as did time point, [$F(1.54, 11.30) = 6.195$, $p=0.020$]. Post-hoc Šídák's multiple comparison test showed no significant difference between *S.* Typhimurium STM51 CFU/mL in a multispecies vs. single species biofilm at any time point (Figure 10).

I also performed a mixed effects analysis to analyse the effects of *E. coli* BW25113 in a multispecies (with *S.* Typhimurium STM51) vs. single species biofilm and time point on the CFU/mL of *E. coli* BW25113—data for 96 hours was missing so this time point was not included in the analysis. This revealed that there was no significant interaction between effects of *E. coli* BW25113 in a multispecies vs. single species biofilm and time point on the CFU/mL of *E. coli* BW25113 [$F(2, 16) = 0.851$, $p=0.444$]. Simple main effects analysis showed that *E. coli* BW25113 in a multispecies vs. single species biofilm did not have a significant effect on *E. coli* BW25113 CFU/mL [$F(1, 16) = 3.957$, $p=0.064$], neither did time point [$F(1.07, 8.56) = 1.251$, $p=0.298$]. Post-hoc Šídák's multiple comparison test showed no significant difference between *E. coli* BW25113 in a multispecies vs. single species biofilm at any time point (Figure 11).

E. coli BW25113 and *S.* Typhimurium STM51 were maintained in the multispecies and single species cultures of planktonic cells with and without beads until 72 hours (Figure 11). The planktonic cells from tubes containing beads showed no growth of *E. coli* BW25113 at the last time point, however I believe this should be repeated as this time point also showed no growth of *E. coli* BW25113 as a single species in biofilm or planktonic conditions (Figure 11).

Mixed effects analysis was used to analyse the effects of *S.* Typhimurium STM51 in the multispecies (with *E. coli* BW25113) planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM51 to determine whether the presence of biofilms had an impact on the maintenance of *S.* Typhimurium STM51 with *E. coli* BW25113. This revealed that there was no significant interaction between effects of planktonic *S.* Typhimurium STM51 in the multispecies planktonic condition with vs. without beads and the time point on the CFU/mL of *S.* Typhimurium STM51 [$F(3, 16) = 0.378$, $p=0.771$]. Simple main effects analysis showed that planktonic *S.* Typhimurium STM51 with vs. without beads did not have a significant effect on *S.* Typhimurium STM51

CFU/mL [F (1, 6) = 1.708, p=0.239], neither did time point [F (1.53, 8.18) = 1.410, p=0.288). Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM51 in multispecies planktonic conditions with vs. without beads at 72 hours (p=0.41) and 96 hours (p=0.47) (Figure 11).

I also used a mixed effects analysis to analyse the effects of planktonic *E. coli* BW25113 in the multispecies (with *E. coli* EC166) planktonic condition with vs. without beads and time point on the CFU/mL of *E. coli* BW25113 to determine whether the presence of biofilms had an impact on *E. coli* EC166 maintenance with *S.* Typhimurium STM51. This revealed that there was a significant interaction between effects of *E. coli* BW25113 in the multispecies condition with vs. without beads and time point on the CFU/mL of *E. coli* BW25113 [F (3, 16) = 881.2, p<0.0001]. Simple main effects analysis showed that *E. coli* EC166 in the multispecies planktonic condition with vs. without had a significant effect on *E. coli* BW25113 CFU/mL [F (1, 6) = 851.2, p<0.0001], as did time point [F (2.21, 11.78) = 976.6, p<0.0001). Post-hoc Šídák's multiple comparison test showed a significant difference between *E. coli* BW25113 in a multispecies vs. single species biofilm at 72 hours (p=0.049) and 96 hours (Figure 13).

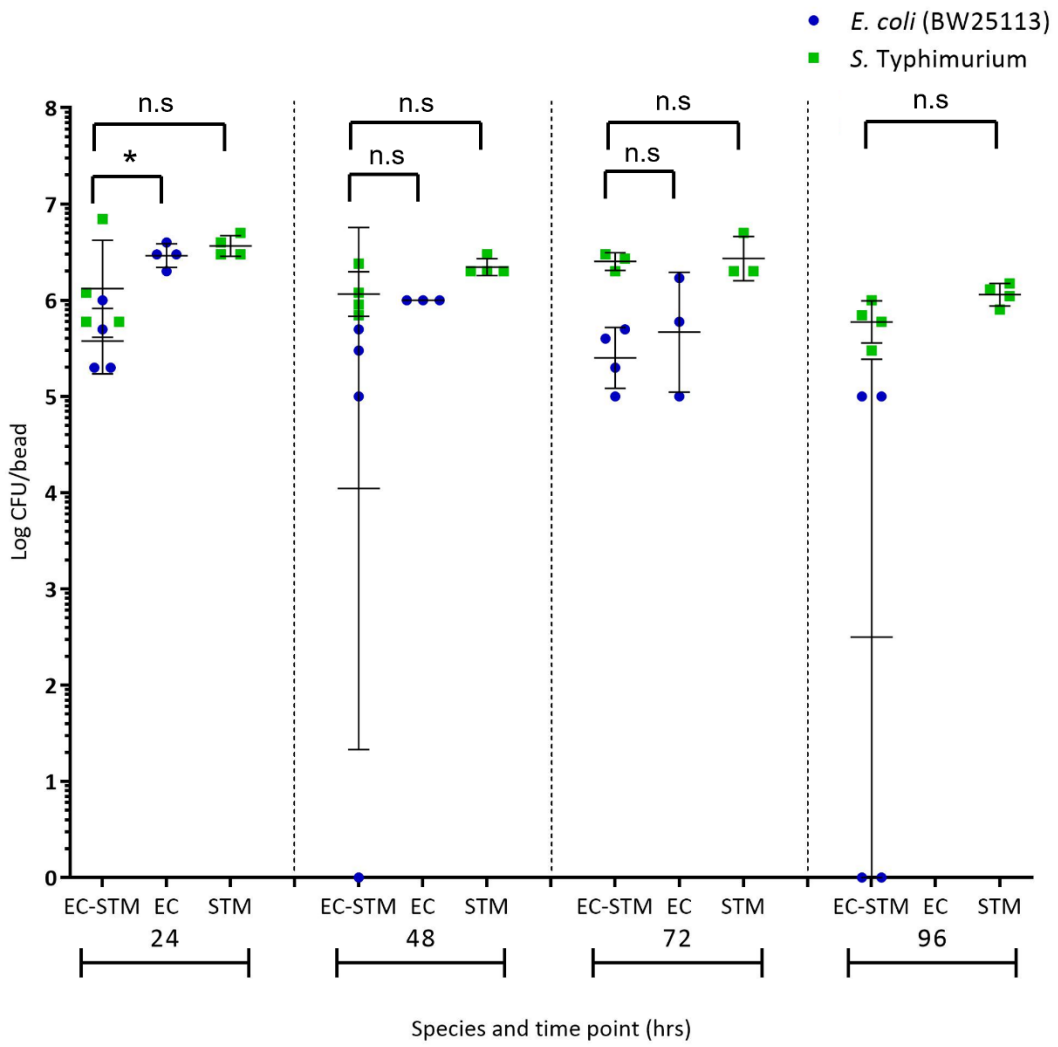

Figure 10. *E. coli* and *S.* Typhimurium biofilm cells in mixed species vs. single species biofilms over time.

E. coli BW25113 and *S.* Typhimurium STM51 were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated 30°C and passaged every 24 hours. At each passage, a bead was removed from each condition and washed before biofilm cells were removed for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal lines) and the error bars show +/- one standard deviation from the mean. A mixed effects analysis with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of *S.* Typhimurium STM51 and *E. coli* BW25113 in a multispecies vs. single species biofilm at each time point. Asterisks show a significant difference (* = $p \leq 0.05$), n.s = not significant.

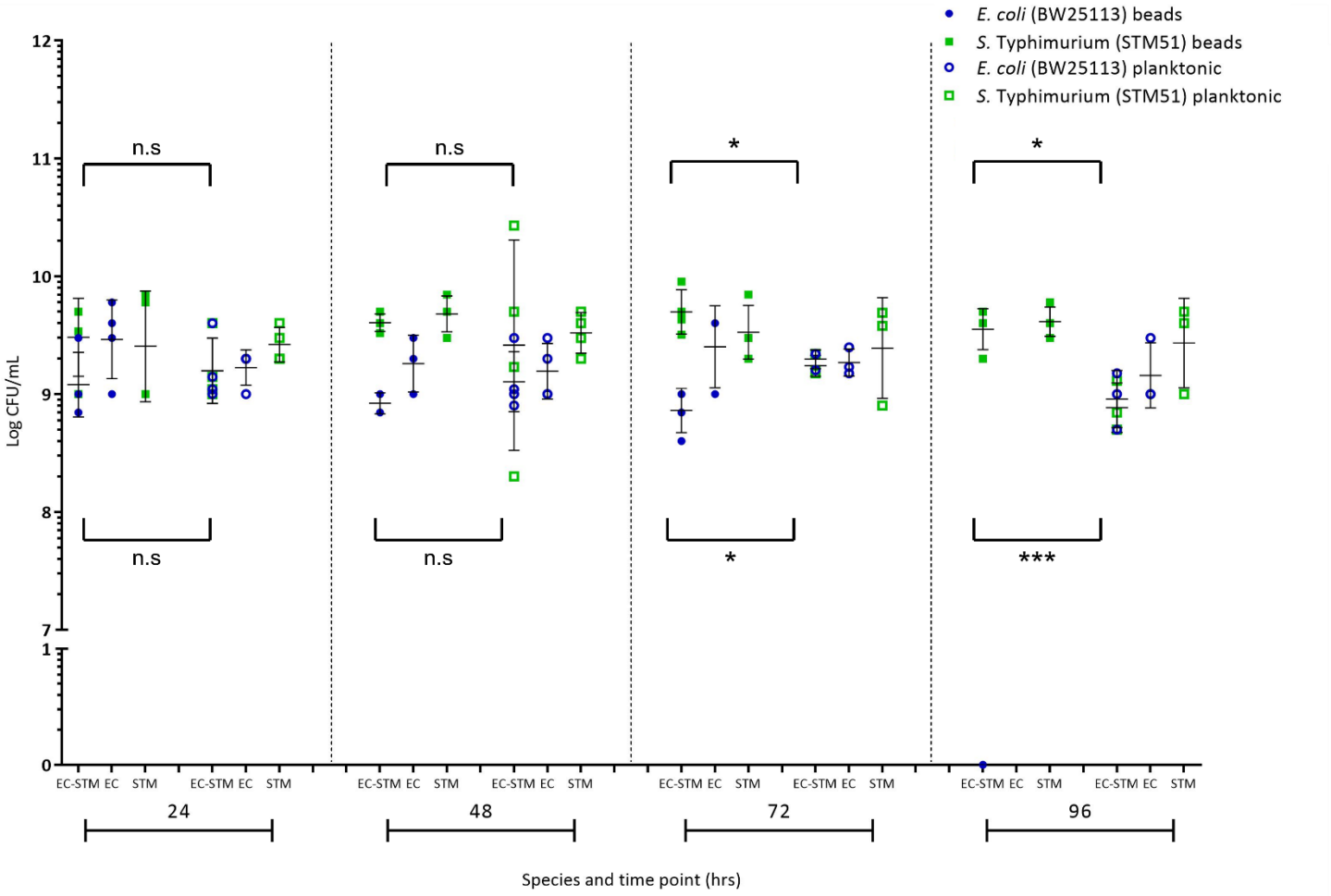

Figure 11. *E. coli* and *S.* Typhimurium planktonic cells in mixed species vs. single species biofilms over time.

E. coli BW25113 and *S.* Typhimurium STM51 were grown together (EC-STM) or individually (EC; STM) in LB broth without salt containing glass beads and passaged every 24 hours. Each condition was carried out in quadruplicate and cells were incubated at 30°C. At each passage, planktonic cells from the liquid phase of the tube were removed for plating (closed circles and squares). *E. coli* BW25113 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) in LB broth and passaged every 24 hours. At each passage, cells were taken for plating (open circles and squares). Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/bead. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A mixed effects analysis with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of planktonic *S.* Typhimurium STM51 and *E. coli* BW25113 in the multispecies condition with vs. without beads at each time point. Brackets above data show results for *S.* Typhimurium STM51, brackets below data show results for *E. coli* BW25113, (*=p≤0.05, ***=p≤0.001), n.s = not significant.

Reduced growth of *S.* Typhimurium STM2, with *E. coli* EC166 is biofilm-dependent

I carried out the biofilm evolution experiment at 30°C passaging every 24 hours using *E. coli* EC166 and *S.* Typhimurium STM2. In this case, *S.* Typhimurium STM2 was not completely lost from the multispecies biofilm, unlike with 48-hour passages, however half of the replicates showed no *S.* Typhimurium STM2 present in the multispecies biofilm by 72 hours (Figure 12). When grown in a single species biofilm, all four replicates of *S.* Typhimurium STM2 were maintained throughout the experiment (Figure 12). I performed a two-way analysis of variance (ANOVA) to analyse the effects of *S.* Typhimurium STM2 in a multispecies (with *E. coli* EC166) vs. single species biofilm and time point on the CFU/mL of *S.* Typhimurium STM2. This revealed that there was no significant interaction between effects of *S.* Typhimurium STM2 in a multispecies vs. single species biofilm and the time point on CFU/mL of *S.* Typhimurium STM2 [F (3, 18) = 2.410, p=0.101)]. Simple main effects analysis showed that *S.* Typhimurium STM2 in a multispecies vs. single species biofilm did have a significant effect on CFU/mL [F (1, 6) = 14.500, p=0.009], however time point did not [F (3, 18) = 2.243, p=0.119]. Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM2 CFU/mL in a multispecies vs. single species biofilm at 72 hours (p=0.009), and 96 hours (p=0.025) (Figure 12).

I performed a two-way ANOVA to analyse the effects of *E. coli* EC166 in a multispecies (with *S.* Typhimurium STM2) vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166. This revealed that there was a significant interaction between effects of *E. coli* EC166 in a multispecies vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166 [F (3,18) = 3.706, p=0.031]. Simple main effects analysis showed that *E. coli* EC166 in a multispecies vs. single species biofilm did not have a significant effect on *E. coli* EC166 CFU/mL [F (1,6) = 1.917, p=0.216], however time point did [F (1.951, 11.70) = 6.620, p=0.012]. Post-hoc Šídák's multiple comparison test showed no significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at any time point (Figure 12).

I also carried out planktonic controls containing no beads and compared these with planktonic cells taken from the liquid culture containing beads. Unlike in *E. coli* BW25113 in which both species remained, *S.* Typhimurium STM2 was lost from the multispecies condition but only when beads were present (Figure 13). Furthermore, *S.* Typhimurium STM2 was present as a single species in planktonic conditions with and without beads throughout the experiment (Figure 13).

A two-way ANOVA was used to analyse the effects of *S.* Typhimurium STM2 in the multispecies (with *E. coli* EC166) planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM2 to determine whether the presence of biofilms had an impact on the maintenance of *S.* Typhimurium STM2 with *E. coli* EC166. This revealed that there was a significant interaction between effects of planktonic *S.* Typhimurium STM2 in the multispecies planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM2 [F (3, 18) = 4.372, p=0.018]. Simple main effects analysis showed that planktonic *S.* Typhimurium STM2 with vs. without beads did not have a significant effect on *S.* Typhimurium STM2 CFU/mL [F (1, 6) = 5.128, p=0.064], however, time point did [F (1.74, 10.42) = 4.737, p=0.038). Post-hoc Šídák's multiple comparison test showed no significant difference between *S.* Typhimurium STM2 in multispecies planktonic conditions with vs. without beads at any time point (Figure 13).

I also used a two-way ANOVA to analyse the effects of planktonic *E. coli* EC166 in the multispecies (with *S.* Typhimurium STM2) planktonic condition with vs. without beads and time point on the CFU/mL of *E. coli* EC166 to determine whether the presence of biofilms had an impact on *E. coli* EC166 maintenance with *S.* Typhimurium STM2. This revealed that there was no significant interaction

between effects of *E. coli* EC166 in the multispecies condition with vs. without beads and time point on the CFU/mL of *E. coli* EC166 [F (3, 18) = 2.83, p=0.068]. Simple main effects analysis showed that *E. coli* EC166 in the multispecies planktonic condition with vs. without did not have a significant effect on *E. coli* EC166 CFU/mL [F (1, 6) = 1.89, p=0.218], however time point did have a significant effect [F (1.795, 10.77) = 5.55, p=0.024). Post-hoc Šídák's multiple comparison test showed no significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at any time point (Figure 13).

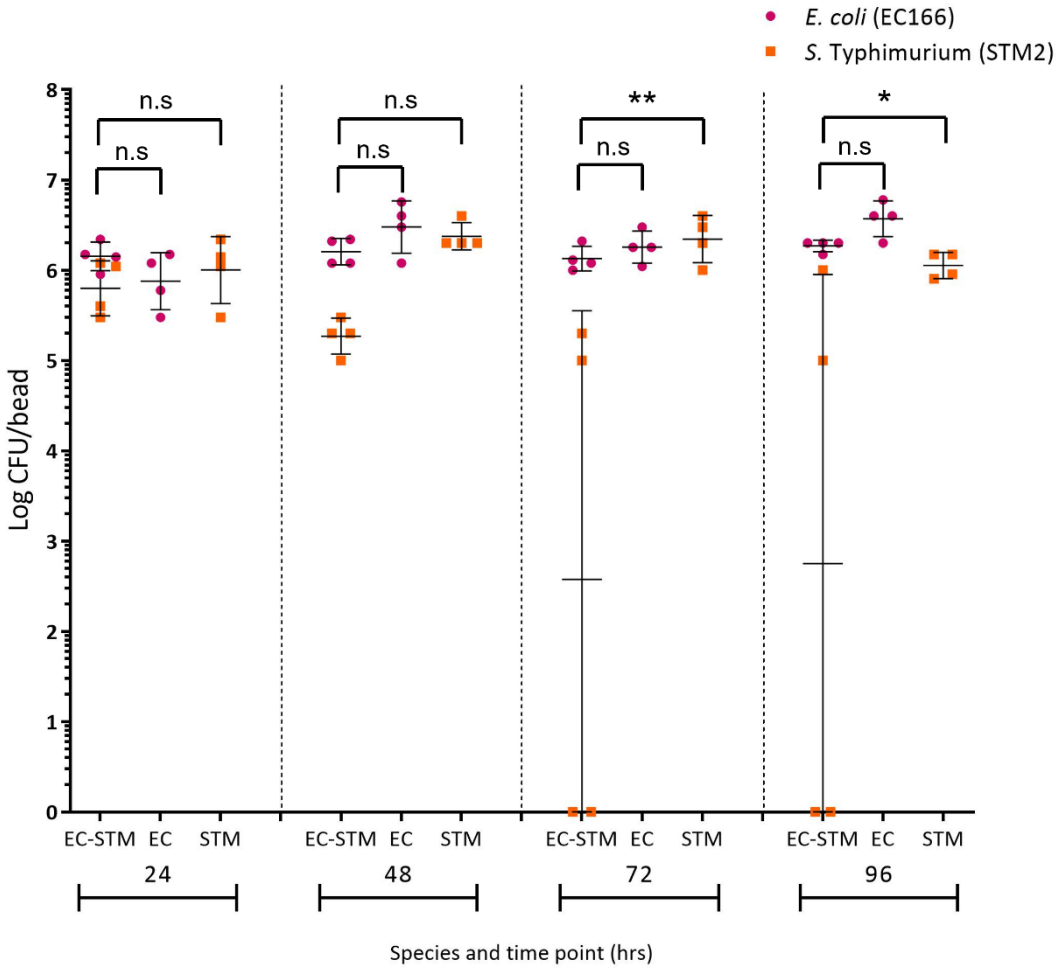

Figure 12. *E. coli* and *S.* Typhimurium STM2 biofilm cells in multispecies vs. single species biofilms over time.

E. coli EC166 and *S.* Typhimurium STM2 were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated 30°C and passaged every 24 hours. At each passage, a bead was removed from each condition and washed before biofilm cells were removed for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A two-way analysis of variance with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of *S.* Typhimurium STM2 and *E. coli* EC166 in a multispecies vs. single species biofilm at each time point. Asterisks show a significant difference (* = p≤0.05; ** = p ≤0.01), n.s = not significant.

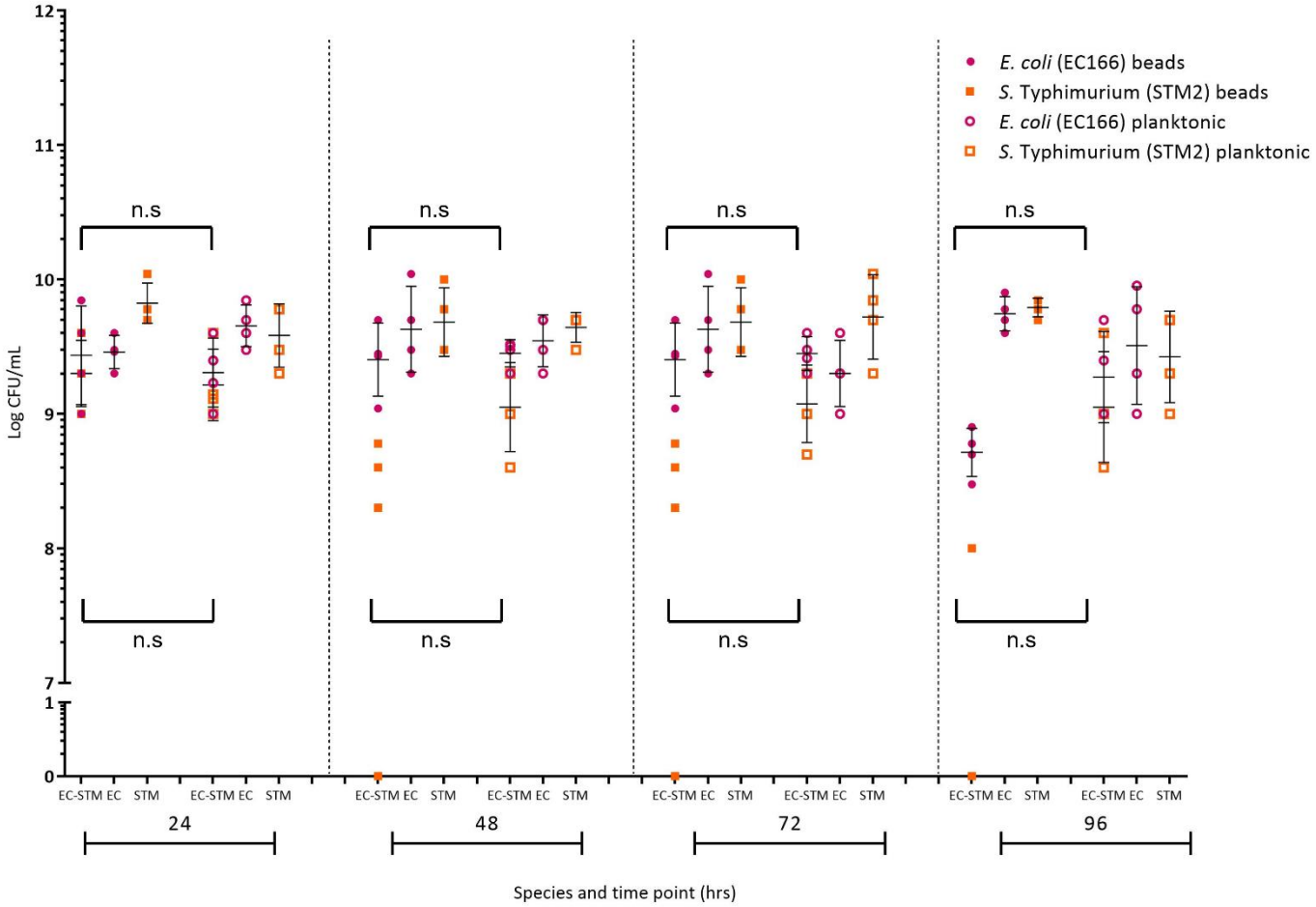

Figure 13. *E. coli* and *S.* Typhimurium STM2 planktonic cells in mixed species vs. single species biofilms over time.

E. coli EC166 and *S.* Typhimurium STM2 were grown together (EC-STM) or individually (EC; STM) in LB broth without salt containing glass beads and passaged every 24 hours. Each condition was carried out in quadruplicate and cells were incubated at 30°C. At each passage, planktonic cells from the liquid phase of the tube were removed for plating (closed circles and squares). *E. coli* BW25113 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) in LB broth and passaged every 24 hours. At each passage, cells were taken for plating (open circles and squares). Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/m. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A two-way analysis of variance with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of planktonic *S.* Typhimurium STM2 in the multispecies condition with vs. without beads at each time point. Brackets above data show results for *S.* Typhimurium STM2, brackets below data show results for *E. coli* EC166, n.s = not significant.

E. coli* EC166 inhibits multiple strains of *Salmonella

To investigate whether *E. coli* EC166 inhibition of *Salmonella* is strain specific, I carried out the biofilm model using another common reference strain of *S.* Typhimurium. I inoculated the biofilm with equal volumes of *E. coli* EC166 and *S.* Typhimurium STM1 (SL1344) and incubated at 30^{o}C, passaging every 24 hours as previously. In both single species and multispecies biofilm conditions *E. coli* EC166 was maintained throughout the experiment (Figure 14). In the multispecies condition, *S.* Typhimurium STM1 was completely lost from the biofilm by 72 hours however remained when grown in a single species biofilm, showing that this strain can form—and be maintained—in a biofilm (Figure 14).

I performed a two-way ANOVA to analyse the effects of *S.* Typhimurium STM1 in a multispecies (with *E. coli* EC166) vs. single species biofilm and time point on the CFU/mL of *S.* Typhimurium STM1. This revealed that there was a significant interaction between effects of *S.* Typhimurium STM1 in a multispecies vs. single species biofilm and time point on CFU/mL of *S.* Typhimurium STM1 [$F(3, 42) = 5.34$, $p=0.003$)]. Simple main effects analysis showed that *S.* Typhimurium STM1 in a multispecies vs. single species biofilm did have a significant effect on CFU/mL [$F(1, 14) = 336.1$, $p<0.0001$], as did time point [$F(1.06, 14.77) = 8.40$, $p=0.010$]. Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM1 CFU/mL in a multispecies vs. single species biofilm at 48 hours ($p<0.0001$), 72 hours ($p<0.0001$), and 96 hours ($p<0.0001$) (Figure 14).

I also performed a two-way ANOVA to analyse the effects of *E. coli* EC166 in a multispecies (with *S.* Typhimurium STM1) vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166. This revealed that there was no significant interaction between effects of *E. coli* EC166 in a multispecies vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166 [$F(3, 42) = 0.50$, $p=0.682$]. Simple main effects analysis showed that *E. coli* EC166 in a multispecies vs. single species biofilm did not have a significant effect on *E. coli* EC166 CFU/mL [$F(1, 14) = 0.73$, $p=0.407$], however time point did [$F(1.32, 18.44) = 8.79$, $p=0.005$]. Post-hoc Šídák's multiple comparison test showed no significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at any time point (Figure 14).

In both planktonic conditions, *E. coli* EC166 was maintained throughout the experiment when grown with and without *S.* Typhimurium STM1 (Figure 15). As with *S.* Typhimurium STM2, *S.* Typhimurium STM1 was only present in multispecies planktonic controls when no beads were present (Figure 15). In the presence of multispecies biofilms, planktonic *S.* Typhimurium STM1 was lost by 72 hours (Figure 15). However, when grown alone *S.* Typhimurium STM1 was maintained planktonically with and without beads until the end of the experiment.

A two-way ANOVA was used to analyse the effects of *S.* Typhimurium STM1 in the multispecies (with *E. coli* EC166) planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM1 to determine whether the presence of biofilms had an impact on the maintenance of *S.* Typhimurium STM1 with *E. coli* EC166. This revealed that there was a significant interaction between effects of planktonic *S.* Typhimurium STM1 in the multispecies planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM1 [$F(3, 42) = 7.59$, $p=0.0004$]. Simple main effects analysis showed that planktonic *S.* Typhimurium STM1 with vs. without beads had a significant effect on *S.* Typhimurium STM1 CFU/mL [$F(1, 14) = 35.64$, $p<0.0001$], as did time point [$F(2.58, 36.14) = 11.95$, $p<0.0001$). Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM1 in multispecies planktonic conditions with vs. without beads at 48 hours ($p=0.41$), 72 hours ($p=0.0009$), and 96 hours ($p=0.001$) (Figure 15).

I also used a two-way ANOVA to analyse the effects of planktonic *E. coli* EC166 in the multispecies (with *S.* Typhimurium STM1) planktonic condition with vs. without beads and time point on the CFU/mL of

E. coli EC166 to determine whether the presence of biofilms had an impact on *E. coli* EC166 maintenance with *S.* Typhimurium STM1. This revealed that there was no significant interaction between effects of *E. coli* EC166 in the multispecies condition with vs. without beads and time point on the CFU/mL of *E. coli* EC166 [F (3, 42) = 0.55, p=0.98]. Simple main effects analysis showed that *E. coli* EC166 in the multispecies planktonic condition with vs. without did have a significant effect on *E. coli* EC166 CFU/mL [F (1, 14) = 5.28, p=0.037], as did time point [F (1.15, 16.13) = 13.06, p=0.002). Post-hoc Šídák's multiple comparison test showed a significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at 24 hours (p=0.0008) and 72 hours (p=0.0002) (Figure 15).

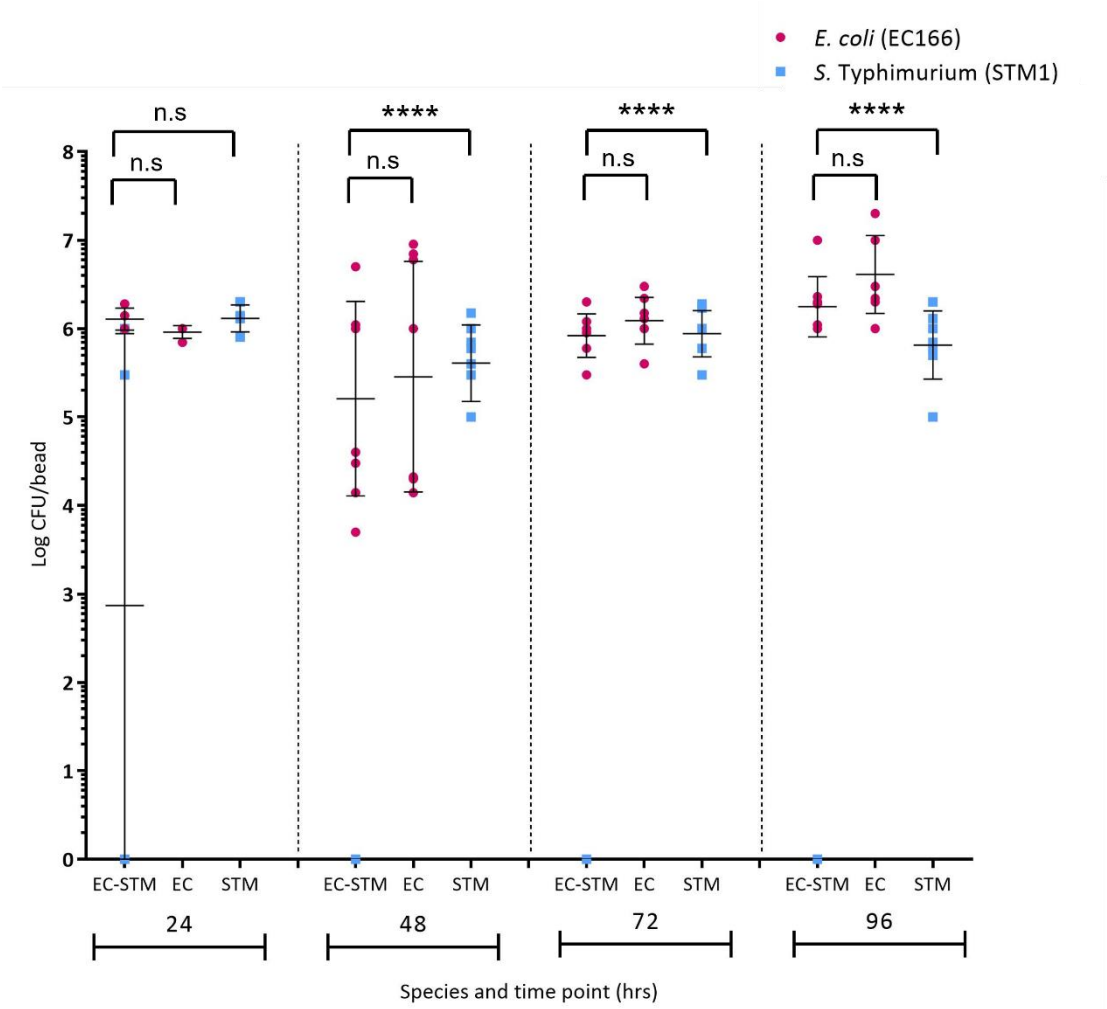

Figure 14. *E. coli* and *S.* Typhimurium STM1 biofilm cells in mixed species vs. single species biofilms over time.

E. coli EC166 and *S.* Typhimurium STM1 were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. The results show two experimental replicates in which each condition was carried out in quadruplicate and cells were incubated 30°C and passaged every 24 hours. At each passage, a bead was removed from each condition and washed before biofilm cells were removed for plating. Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A two-way analysis of variance with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of *S.* Typhimurium STM1 and *E. coli* EC166 in a multispecies vs. single species biofilm at each time point. Asterisks show a significant difference (**** = $p \leq 0.0001$), n.s = not significant.

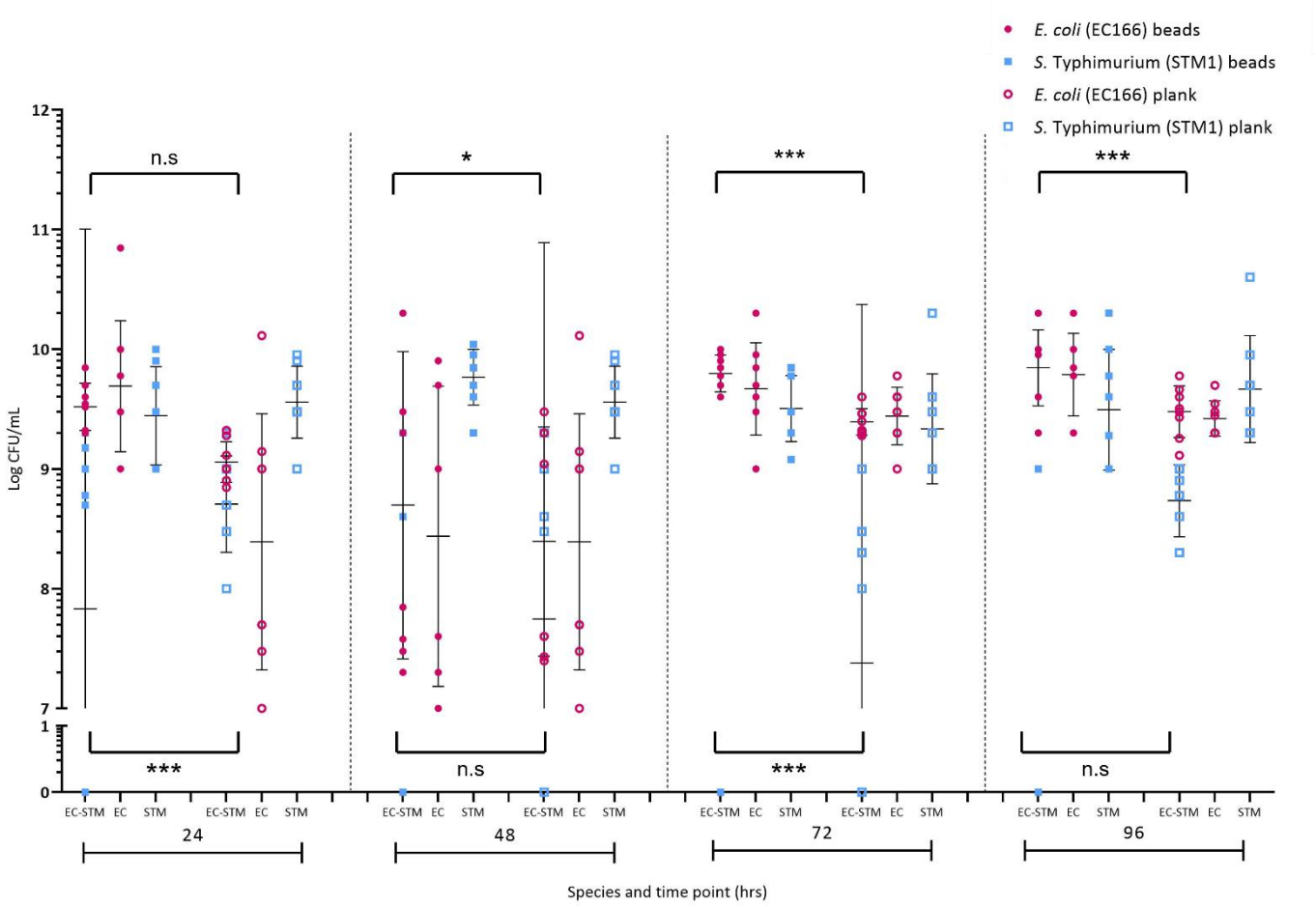

Figure 15. *E. coli* and *S.* Typhimurium STM1 planktonic cells in mixed species vs. single species biofilms over time.

E. coli EC166 and *S.* Typhimurium STM1 were grown together (EC-STM) or individually (EC; STM) in LB broth without salt containing glass beads and passaged every 24 hours. The results show two experimental replicates in which each condition was carried out in quadruplicate and cells were incubated at 30° C. At each passage, planktonic cells from the liquid phase of the tube were removed for plating (closed circles and squares). *E. coli* BW25113 and *S.* Typhimurium were grown together (EC-STM) or individually (EC; STM) in LB broth and passaged every 24 hours. At each passage, cells were taken for plating (open circles and squares). Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A two-way analysis of variance with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of planktonic *S.* Typhimurium STM1 in the multispecies condition with vs. without beads at each time point. Brackets above data show results for *S.* Typhimurium STM1, brackets below data show results for *E. coli* EC166, (* = p≤0.05; *** = p ≤0.001), n.s = not significant.

E. coli* EC166 inhibits multiple serovars of *Salmonella

To identify whether the ability of *E. coli* EC166 to reduce growth is serovar-specific, I carried out the biofilm model using a common reference strain, *S.* Enteritidis STM14 (PT4 P125109), another non-typhoidal strain of *Salmonella* that can cause infections in humans. The biofilm model was inoculated with *E. coli* EC166 and *S.* Enteritidis STM14 and incubated the model at 30°C, passaging every 24 hours. *S.* Enteritidis STM14 was lost from the multispecies biofilm condition by 72 hours, however remained in a single species biofilm until the end of the experiment, demonstrating that *S.* Enteritidis STM14 can form a biofilm under these conditions (Figure 16). As previously, *E. coli* EC166 was maintained in both single species and multispecies biofilm conditions throughout the experiment (Figure 16).

Due to missing data, I performed a mixed effects analysis to analyse the effects of *S.* Typhimurium STM14 in a multispecies (with *E. coli* EC166) vs. single species biofilm and time point on the CFU/mL of *S.* Typhimurium STM14. This revealed that there was a significant interaction between effects of *S.* Typhimurium STM14 in a multispecies vs. single species biofilm and time point on CFU/mL of *S.* Typhimurium STM14 [$F(3, 40) = 31.23$, $p<0.0001$)]. Simple main effects analysis showed that *S.* Typhimurium STM14 in a multispecies vs. single species biofilm had a significant effect on CFU/mL [$F(1, 14) = 311.30$, $p<0.0001$], as did time point [$F(1.51, 15.34) = 33.66$, $p<0.0001$]. Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM14 CFU/mL in a multispecies vs. single species biofilm at 24 hours ($p=0.0096$), 48 hours ($p=0.0032$), 72 hours ($p<0.0001$), and 96 hours ($p<0.0001$) (Figure 16).

I performed a two-way ANOVA to analyse the effects of *E. coli* EC166 in a multispecies (with *S.* Typhimurium STM14) vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166. This revealed that there was no significant interaction between effects of *E. coli* EC166 in a multispecies vs. single species biofilm and time point on the CFU/mL of *E. coli* EC166 [$F(3, 42) = 0.40$, $p=0.753$]. Simple main effects analysis showed that *E. coli* EC166 in a multispecies vs. single species biofilm did not have a significant effect on *E. coli* EC166 CFU/mL [$F(1, 14) = 0.24$, $p=0.634$], however time point did [$F(1.41, 19.70) = 9.55$, $p=0.003$]. Post-hoc Šídák's multiple comparison test showed no significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at any time point (Figure 16).

At 72 hours, no *S.* Enteritidis STM14 planktonic growth was present in tubes containing biofilms. However, *S.* Enteritidis STM14 was maintained in the multispecies planktonic control conditions throughout the experiment (Figure 17). Under both planktonic conditions, *E. coli* EC166 was maintained throughout the experiment alone and when grown with *S.* Enteritidis. Two-way ANOVA was used to analyse the effects of *S.* Typhimurium STM14 in the multispecies (with *E. coli* EC166) planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM14 to determine whether the presence of biofilms had an impact on the maintenance of *S.* Typhimurium STM14 with *E. coli* EC166. This revealed that there was a significant interaction between effects of planktonic *S.* Typhimurium STM14 in the multispecies planktonic condition with vs. without beads and time point on the CFU/mL of *S.* Typhimurium STM2 [$F(3, 42) = 29.25$, $p<0.0001$]. Simple main effects analysis showed that planktonic *S.* Typhimurium STM14 with vs. without beads had a significant effect on *S.* Typhimurium STM14 CFU/mL [$F(1, 14) = 259.40$, $p<0.0001$], as did time point [$F(1.03, 14.43) = 27.93$, $p<0.0001$). Post-hoc Šídák's multiple comparison test showed a significant difference between *S.* Typhimurium STM14 in multispecies planktonic conditions with vs. without beads at 48 hours ($p=0.023$), 72 hours ($p<0.0001$), and 96 hours($p<0.0001$) (Figure 17).

I also used a two-way ANOVA to analyse the effects of planktonic *E. coli* EC166 in the multispecies (with *S.* Typhimurium STM14) planktonic condition with vs. without beads and time point on the CFU/mL of

E. coli EC166 to determine whether the presence of biofilms had an impact on *E. coli* EC166 maintenance with *S.* Typhimurium STM14. This revealed that there was no significant interaction between effects of *E. coli* EC166 in the multispecies condition with vs. without beads and time point on the CFU/mL of *E. coli* EC166 [$F(3, 42) = 0.03$, $p=0.99$]. Simple main effects analysis showed that *E. coli* EC166 in the multispecies planktonic condition with vs. without had a significant effect on *E. coli* EC166 CFU/mL [$F(1, 14) = 14.32$, $p=0.002$], time point also had a significant effect [$F(1.51, 21.15) = 18.46$, $p<0.0001$). Post-hoc Šídák's multiple comparison test showed a significant difference between *E. coli* EC166 in a multispecies vs. single species biofilm at 24 hours ($p=0.0003$), 72 hours ($p=0.0018$), and 96 hours ($p=0.490$) (Figure 17).

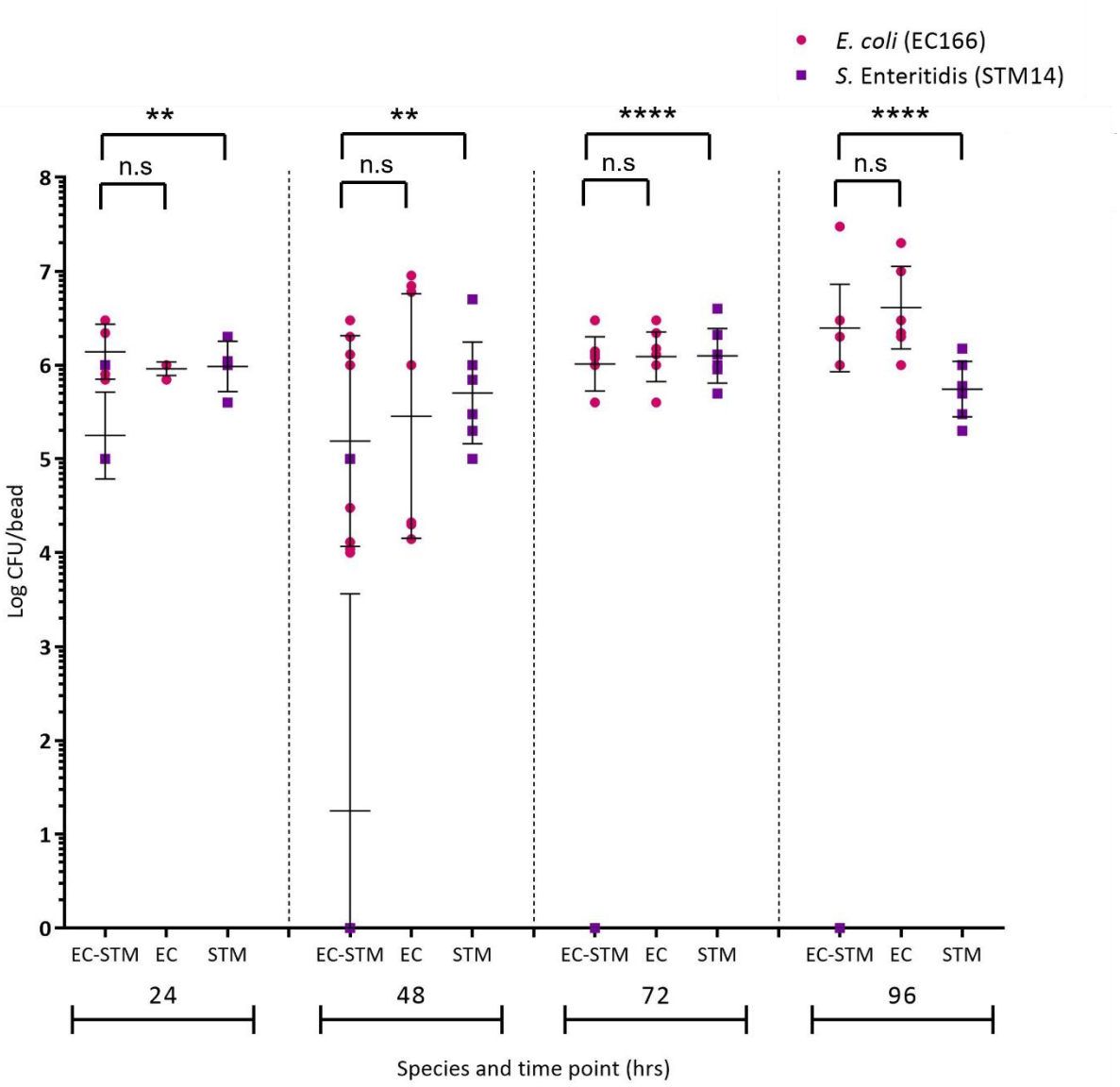

Figure 16. *E. coli* and *S.* Enteritidis biofilm cells in mixed species vs. single species biofilms over time.

E. coli EC166 and *S.* Enteritidis STM14 were grown together (EC-STM) or individually (EC; STM) on glass beads in LB broth without NaCl to form biofilms. The results show two experimental replicates in which each condition was carried out in quadruplicate and cells were incubated 30°C and passaged every 24 hours. At each passage, a bead was removed from each condition and washed before biofilm cells were removed for plating. Blue-white screening was used to count *E. coli* or *S.* Enteritidis in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or log CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A mixed effects analysis with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of *S.* Typhimurium STM14 and a two-way analysis of variance was used to identify significant differences between the CFU/mL of *E. coli* EC166 in a multispecies vs. single species biofilm at each time point. Asterisks show a significant difference (** = $p \leq 0.01$; **** = $p \leq 0.0001$), n.s = not significant.

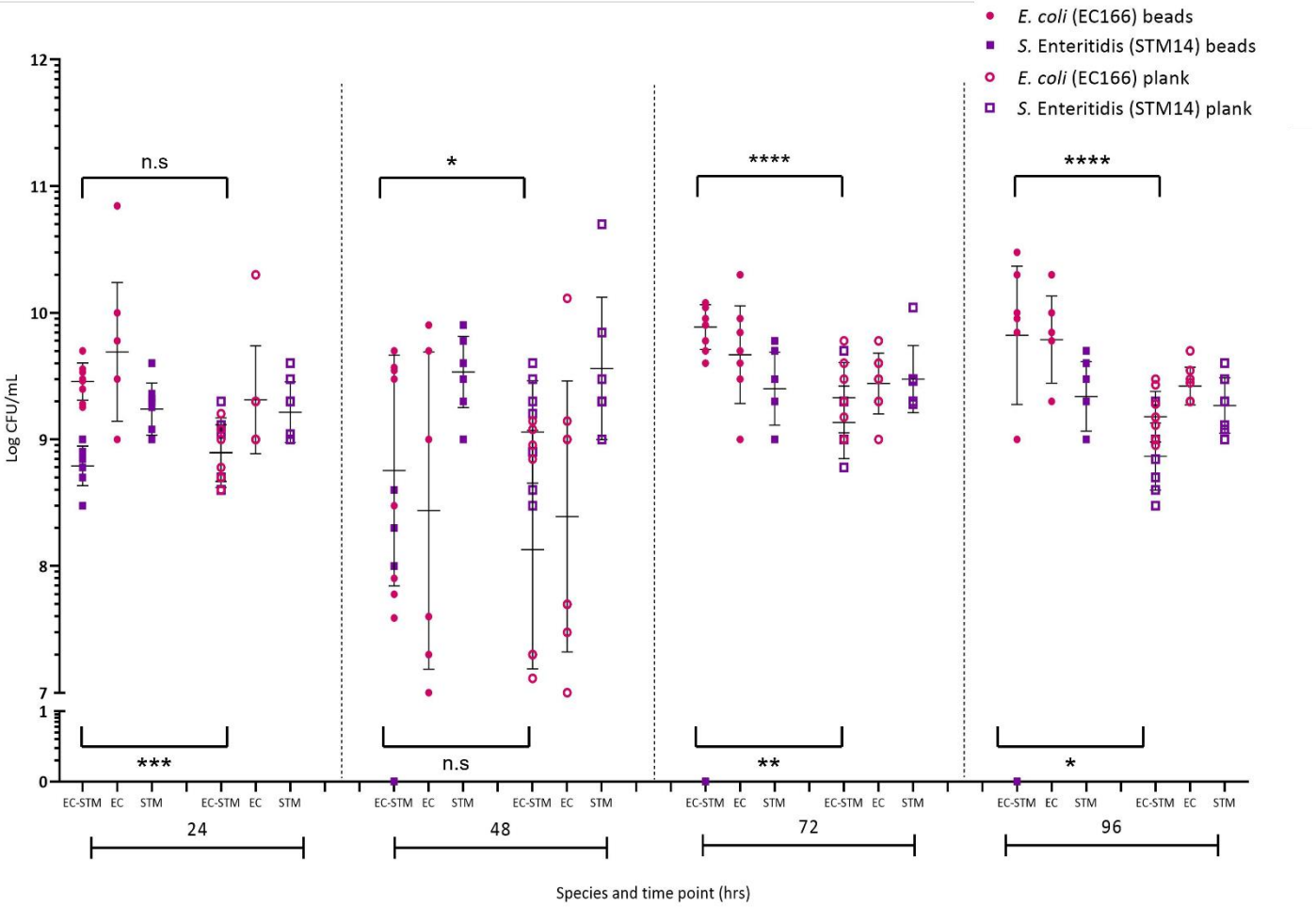

Figure 17. *E. coli* and *S.* Enteritidis planktonic cells in mixed species vs. single species biofilms over time.

E. coli EC166 and *S.* Enteritidis STM14 were grown together (EC-STM) or individually (EC; STM) in LB broth without salt containing glass beads and passaged every 24 hours. The results show two experimental replicates in which each condition was carried out in quadruplicate and incubated at 30°C. At each passage, planktonic cells from the liquid phase of the tube were removed for plating (closed circles and squares). *E. coli* BW25113 and *S.* Enteritidis were grown together (EC-STM) or individually (EC; STM) in LB broth and passaged every 24 hours. At each passage, cells were taken for plating (open circles and squares). Blue-white screening was used to count *E. coli* or *S.* Typhimurium in the mixed species conditions. Colony counts were used to calculate CFU/bead and CFU/mL. The graphs show results from each replicate, the mean log CFU/bead or CFU/mL (horizontal line) and the error bars show +/- one standard deviation from the mean. A two-way analysis of variance with post-hoc Šídák's multiple comparison test was used to identify significant differences between the CFU/mL of *S.* Typhimurium STM1 and *E. coli* EC166 in a multispecies vs. single species biofilm at each time point. Asterisks show a significant difference (* = p ≤0.05; ** = p ≤0.01; *** = p ≤0.001; **** = p ≤0.0001), n.s = not significant.

E. coli EC166 and *Salmonella* growth kinetics

To determine the growth rate of each species, the OD_{600} of each species was measured every 15 minutes over a 24-hour growth period. The growth curve of each species is plotted in figure 18 which shows that *E. coli* EC166 reaches an overall higher OD_{600} compared to any of the *Salmonella* strains. The average final OD_{600} of *E. coli* EC166 is 2.32 compared to the highest *Salmonella* strain (STM2) which was 2.12 (Figure 18). The generation time of each species was calculated and overall, *E. coli* EC166 had the shortest generation time with an average of 31.4 minutes compared with the shortest *Salmonella* (STM2) which was 49.1 minutes.

ANOVA using Dunnett's multiple comparisons test was carried out to identify whether there was a significant difference between the generation time of *E. coli* EC166 and any of the *Salmonella* strains. There was a statistically significant difference in generation time of *E. coli* EC166 and *S.* Typhimurium STM2 ($p<0.0001$, 95% confidence interval (CI) = -18.89 to -16.48). There was also a statistically significant difference in generation time of *E. coli* EC166 and *S.* Typhimurium STM1 ($p<0.0001$, 95% CI = -37.05 to -34.64). Finally, there was a statistically significant difference between *E. coli* EC166 and *S.* Typhimurium STM14 ($p<0.0001$, 95% CI = -36.17 to -33.75) (Figure 19).

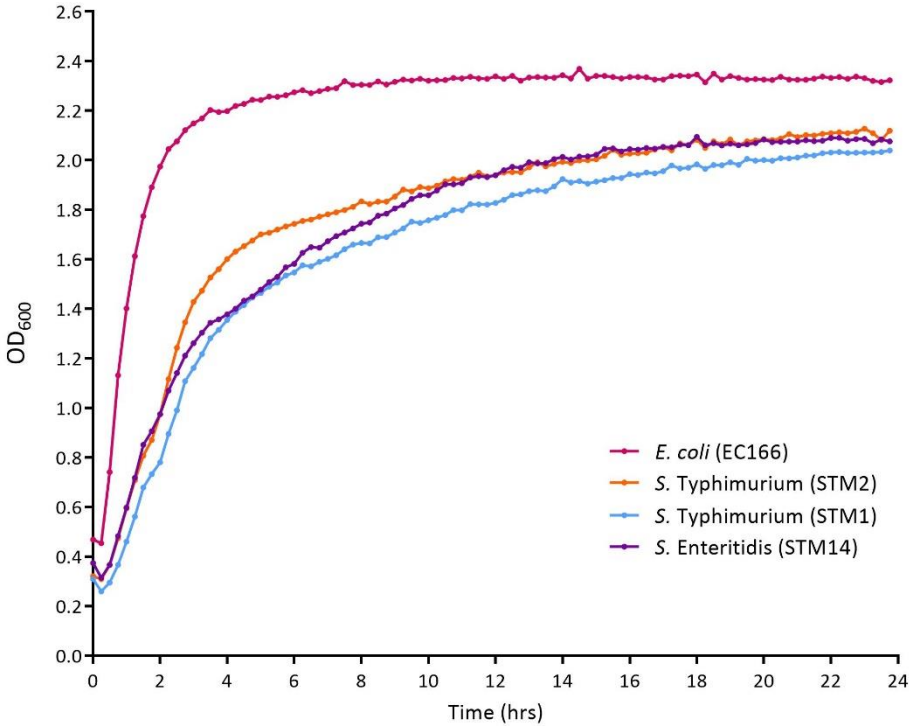

Figure 18. Growth curves of *E. coli, S.* Typhimurium, and *S.* Enteritidis.

Growth curves were plotted using the mean OD_{600} of 16 replicates measured every 15 minutes over 24 hours.

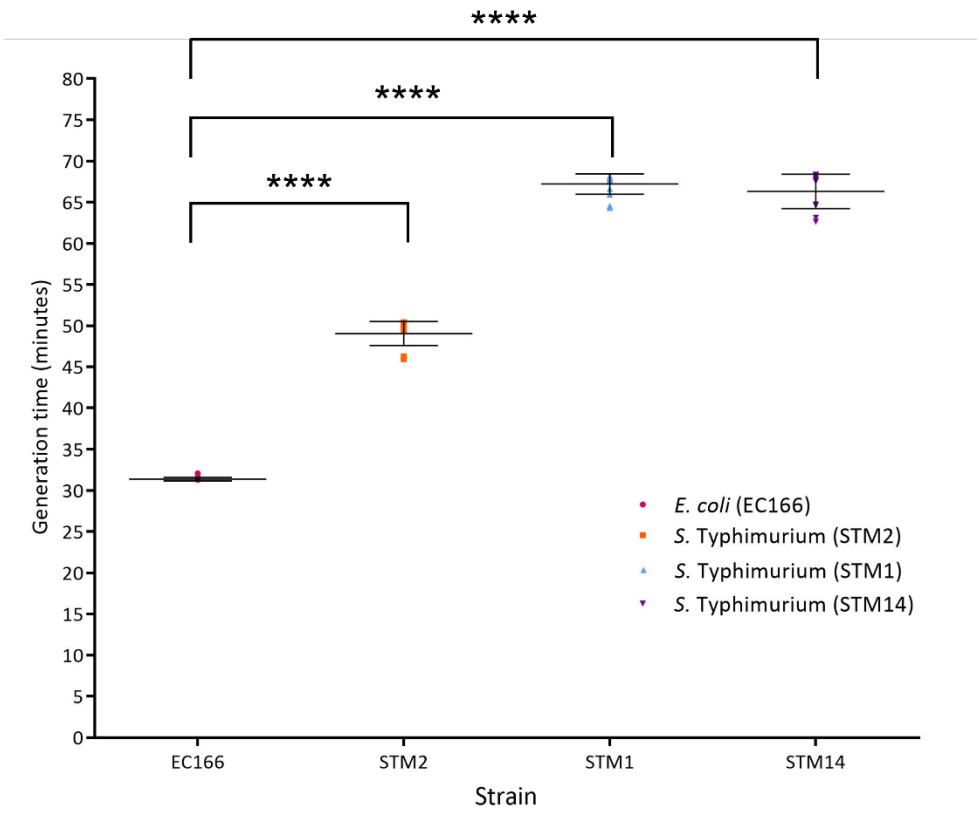

Figure 19. Growth rates of *E. coli, S.* Typhimurium, and *S.* Enteritidis.

Generation times were calculated from growth curve data using the OD_{600} of 16 replicates measured every 15 minutes over 24 hours. Error bars show the mean generation time and +/- one standard from the mean. One-way analysis of variance with Dunnett's multiple comparisons test was carried out to identify whether there was a significant difference between the means of the control (EC166) and *Salmonella* strains. Asterisks show a significant difference (**** = p≤0.0001).

Addition of cell-free supernatant changes growth of *E. coli* EC166

I aimed to investigate the mechanism behind reduced growth of *S.* Typhimurium STM2 in the presence of *E. coli* EC166 biofilms and whether *E. coli* EC166 produced a diffusible product that inhibits *Salmonella* growth. To do this I added cell-free supernatant from various conditions to cultures of *E. coli* EC166 and *S.* Typhimurium STM2; if the *E. coli* EC166 product was present within the supernatant it should prevent growth of *S.* Typhimurium STM but not itself. I collected supernatant by centrifuging relevant tubes (from the final passage of the biofilm model) and diluting the supernatant 1:10 in LB broth. Supernatant conditions included: a 24-hour liquid planktonic culture of *E. coli* EC166; the liquid phase of the multispecies biofilm; the liquid phase of the single species *E. coli* EC166 biofilm; multispecies planktonic without beads; and *E. coli* EC166 planktonic without beads. This would allow me to identify whether *E. coli* EC166 produced a product under just biofilm, just planktonic, or both conditions, and whether *S*. Typhimurium STM2 presence was required to induce the production. I added supernatant and grew the cells at 37°C for 24 hours whilst measuring the OD_{600} every 15 minutes to create growth curves (Figure 20; Figure 22).

E. coli EC166 showed no difference when grown in a multispecies compared with single species biofilm therefore I tested each supernatant condition against *E. coli* EC166 as a control. Furthermore, a bacteriocin produced by *E. coli* should not prevent its own growth. I calculated the generation time of *E. coli* EC166 under each condition and compared these to a control grown in LB broth without supernatant. I used a one-way ANOVA with Dunnett's multiple comparisons test to identify whether there was a significant difference between the control and each supernatant condition. The *E. coli* EC166 control compared with *E. coli* EC166 grown with supernatant from an overnight culture showed a significant difference in generation time ($p<0.0001$, 95% CI = -6.89 to -6.63). There was a significant difference in generation time between the control and *E. coli* EC166 growth with supernatant from the mixed biofilm condition ($p<0.0001$, 95% CI = -6.94 to -6.69). The generation time of *E. coli* EC166 with supernatant from the *E. coli* biofilm condition was significantly different to that of the control ($p<0.0001$, 95% CI = -7.06 to -6.80). There was a significant difference between the generation time of the control and *E. coli* EC166 growth with supernatant from the mixed planktonic condition ($p<0.0001$, 95% CI = -3.47 to -3.22). However, there was no significant difference between the generation time of the control and that of *E. coli* EC166 growth with supernatant from the *E. coli* planktonic condition ($p=0.993$, 95% CI = -0.11 to -0.15) (Figure 21).

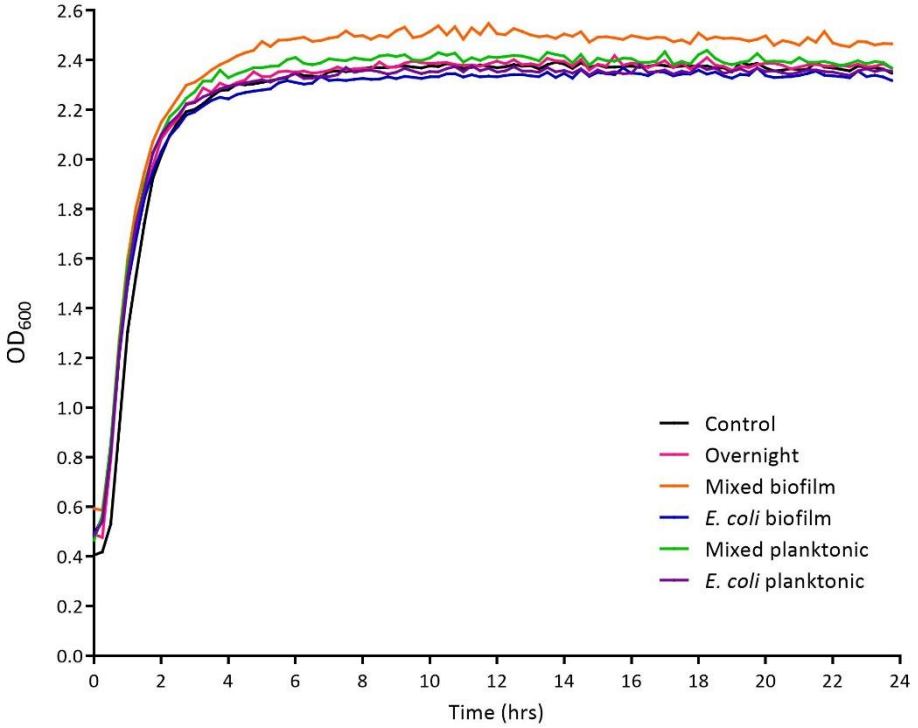

Figure 20. Growth curves of *E. coli* EC166 grown in broth supplemented with supernatant from different sources.

Growth curves were plotted using the mean OD_{600} of eight replicates measured every 15 minutes over 24 hours. *E. coli* EC166 was grown in 6 different conditions.

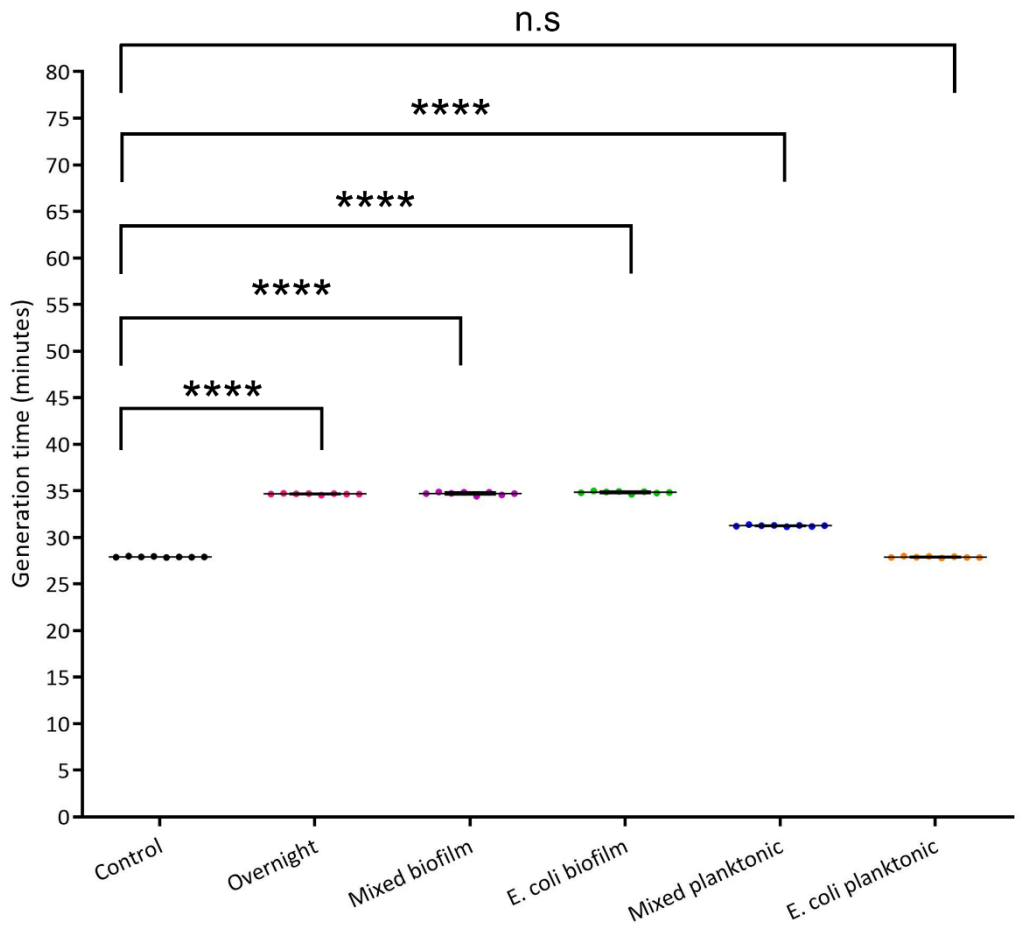

Figure 21. Growth rates of *E. coli* EC166 grown in broth supplemented with supernatant from different sources.

Generation times were calculated from growth curve data using the OD_{600} of eight replicates measured every 15 minutes over 24 hours. Error bars show the mean generation time and +/- one standard from the mean. 'Control' shows EC166 grown in LB broth alone. 'Overnight' shows EC166 grown in LB broth supplemented with 10% supernatant from an overnight culture of EC166. 'Mixed biofilm' shows EC166 grown in LB broth supplemented with 10% supernatant from the liquid phase of the biofilm model containing EC166 and *S.* Typhimurium at the fourth passage. '*E. coli* biofilm' shows EC166 grown in LB broth supplemented with 10% supernatant from the liquid phase of the biofilm model containing EC166 at the fourth passage. 'Mixed planktonic' shows EC166 grown in LB broth supplemented with 10% supernatant from cells grown planktonically (incubated at 30°C, 60 rpm) (EC166 + *S.* Typhimurium) at the fourth passage. '*E. coli* planktonic' shows EC166 grown in LB broth supplemented with 10% supernatant from cells grown planktonically (incubated at 30°C, 60 rpm) at the fourth passage. One-way analysis of variance using Dunnett's multiple comparisons test was carried out to identify a significant difference between the means of the Control and each supernatant condition. Asterisks show a significant difference (**** = $p \leq 0.0001$), n.s = not significant.

Addition of cell-free supernatant changes growth of *S.* Typhimurium STM2

I added supernatant to *S.* Typhimurium STM2 to identify whether any diffusible products were present within the supernatant that could reduce the growth of *S.* Typhimurium STM2 without the presence of active *E. coli* EC166 growth. *S.* Typhimurium STM2 grown in supernatant from planktonic cultures containing no beads showed the highest generation time compared with the control. This was the opposite to that seen in *E. coli* EC166 in which supernatant from these conditions resulted in lower generation time. However, it is possible that the waste present within the supernatant increased the generation time of *S.* Typhimurium therefore I would like to repeat this experiment with filtered cell-free supernatant.

A one-way ANOVA was used to determine with Dunnett's multiple comparisons test to identify whether there was a significant difference between the control and each supernatant condition. The *S.* Typhimurium STM2 control compared with *S.* Typhimurium STM2 grown with supernatant from an overnight culture showed a significant difference in generation time ($p<0.0001$, 95% CI = -3.42 to -2.98). There was a significant difference in generation time between the control and *S.* Typhimurium STM2 growth with supernatant from the mixed biofilm condition ($p<0.0001$, 95% CI = -6.71 to -6.28). The generation time of *S.* Typhimurium STM2 with supernatant from the *E. coli* biofilm condition was significantly different to that of the control ($p<0.0001$, 95% CI = -6.87 to -6.43). There was a significant difference between the generation time of the control and *S.* Typhimurium STM2 growth with supernatant from the mixed planktonic condition ($p<0.0001$, 95% CI = -19.24 to -9.79). Finally, there was a significant difference between the generation time of the control and that of *S.* Typhimurium STM2 growth with supernatant from the *E. coli* planktonic condition ($p<.0001$, 95% CI = -6.82 to -6.38) (Figure 23).

However, as a significant difference was also seen between the *E. coli* EC166 control and conditions, we cannot be sure that this shows *E. coli* EC166 supernatant inhibits *S.* Typhimurium STM2 growth. Therefore, we continued to investigate the mechanism of *E. coli* EC166 inhibition of each *Salmonella* species.

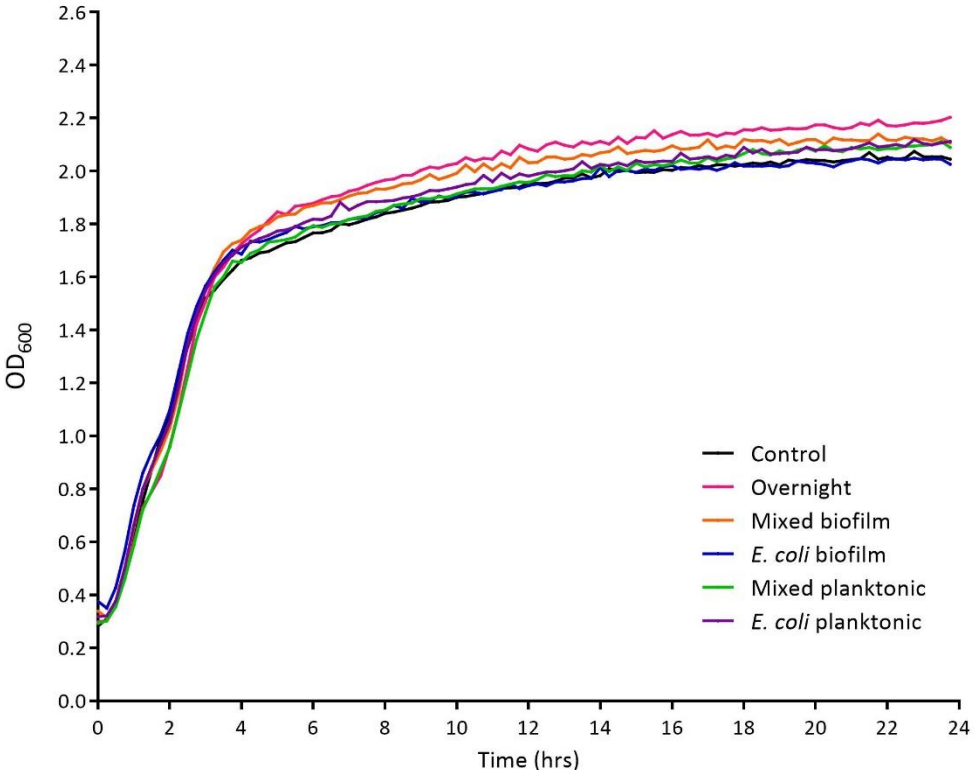

Figure 22. Growth curves of *S.* Typhimurium grown in broth supplemented with supernatant from different sources.

Growth curves were plotted using the mean OD_{600} of eight replicates measured every 15 minutes over 24 hours. *S.* Typhimurium was grown in 6 different conditions.

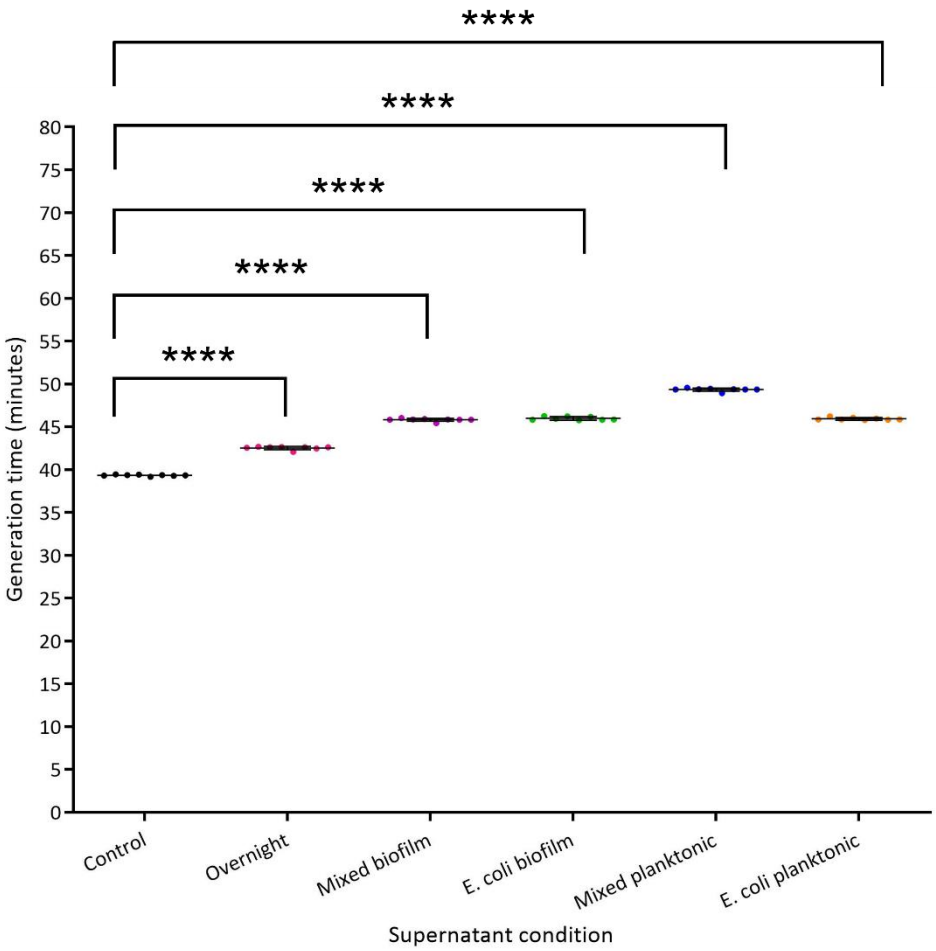

Figure 23. Growth rates of *S.* Typhimurium grown in broth supplemented with supernatant from different sources.

Generation times were calculated from growth curve data using the OD_{600} of eight replicates measured every 15 minutes over 24 hours. Error bars show the mean generation time and +/- one standard from the mean. *S.* Typhimurium was grown in 6 different conditions. 'Control' shows *S.* Typhimurium *grown* in LB broth alone. 'Overnight' shows *S.* Typhimurium grown in LB broth supplemented with 10% supernatant from an overnight culture of EC166. 'Mixed biofilm' shows *S.* Typhimurium grown in LB broth supplemented with 10% supernatant from the liquid phase of the biofilm model containing EC166 and *S.* Typhimurium at the fourth passage. '*E. coli* biofilm' shows *S.* Typhimurium grown in LB broth supplemented with 10% supernatant from the liquid phase of the biofilm model containing EC166 at the fourth passage. 'Mixed planktonic' shows EC166 grown in LB broth supplemented with 10% supernatant from cells grown planktonically (incubated at 30°C, 60 rpm) (EC166 + *S.* Typhimurium) at the fourth passage. '*E. coli* planktonic' shows EC166 grown in LB broth supplemented with 10% supernatant from cells grown planktonically (incubated at 30°C, 60 rpm) at the fourth passage. One-way analysis of variance by ranks with Dunnett's multiple comparisons test was carried out to identify whether there was a significant difference between the means of the Control (EC166) and each supernatant condition. Asterisks show a significant difference (**** = p≤0.0001).

Adding *E. coli* biofilms grown on beads does not reduce *S.* Typhimurium growth: one bead

To identify whether mature *E. coli* EC166 biofilms could reduce the growth of *S.* Typhimurium STM2, *E. coli* EC166 biofilms were grown on beads which were then added to planktonic *S.* Typhimurium STM2 cultures. Single species *E. coli* EC166 biofilms and multispecies *E. coli* EC166 and *S.* Typhimurium STM2 biofilms were grown on beads to determine whether *S.* Typhimurium STM2 must be present in a biofilm to induce the ability of *E. coli* EC166 to inhibit *Salmonella* growth. Biofilms were grown on beads for 24, 48, or 72 hours before being added to planktonic *S.* Typhimurium STM2 to identify whether the growth phase of *S.* Typhimurium STM2 was important. Controls of *S.* Typhimurium STM2 with no beads were also used. *S.* Typhimurium STM2 was inoculated 3 hours (Figure 24) or 24 hours (Figure 25) prior to addition of a bead. Following this, cells from the liquid phase were plated 2 or 24 hours after addition of the bead.

Planktonic S. *Typhimurium STM2 incubated for 3 hours prior to addition of a bead*

When planktonic *S.* Typhimurium STM2 was incubated for 3 hours prior to addition of a bead, no *E. coli* EC166 growth was observed at any time point in single species or multispecies conditions however the detection threshold was 10^8 CFU/mL for this experiment, therefore *E. coli* EC166 may have been present but undetected (Figure 24). *S.* Typhimurium STM2 growth was present in multispecies and single species biofilm conditions at all time points (Figure 24). A two-way ANOVA was carried out to analyse the effect of *E. coli* EC166 multispecies or single species biofilms and time point on the CFU/mL of *S.* Typhimurium STM2. This revealed that for the experiment incubated for 2 hours after addition of a bead, there was no significant interaction between the effects of *E. coli* EC166 multispecies or single species biofilms and time point on CFU/mL of *S.* Typhimurium STM2 [$F(4, 12) = 0.13$, $p=0.96$]. Similarly, no significant interaction was seen for the experiment incubated for 24 hours after addition of a bead [$F(4, 12) = 0.80$, $p=0.56$]. Tukey's multiple comparisons test found no significant difference between mean CFU/mL of *S.* Typhimurium STM2 in the control and mean CFU/mL of *S.* Typhimurium STM2 when grown with *E. coli* EC166 multispecies biofilms or *E. coli* EC166 single species biofilms for experiments incubated for 2 hours or 24 hours after addition of a bead at any time points ($p>0.05$ in all cases) (Figure 24).

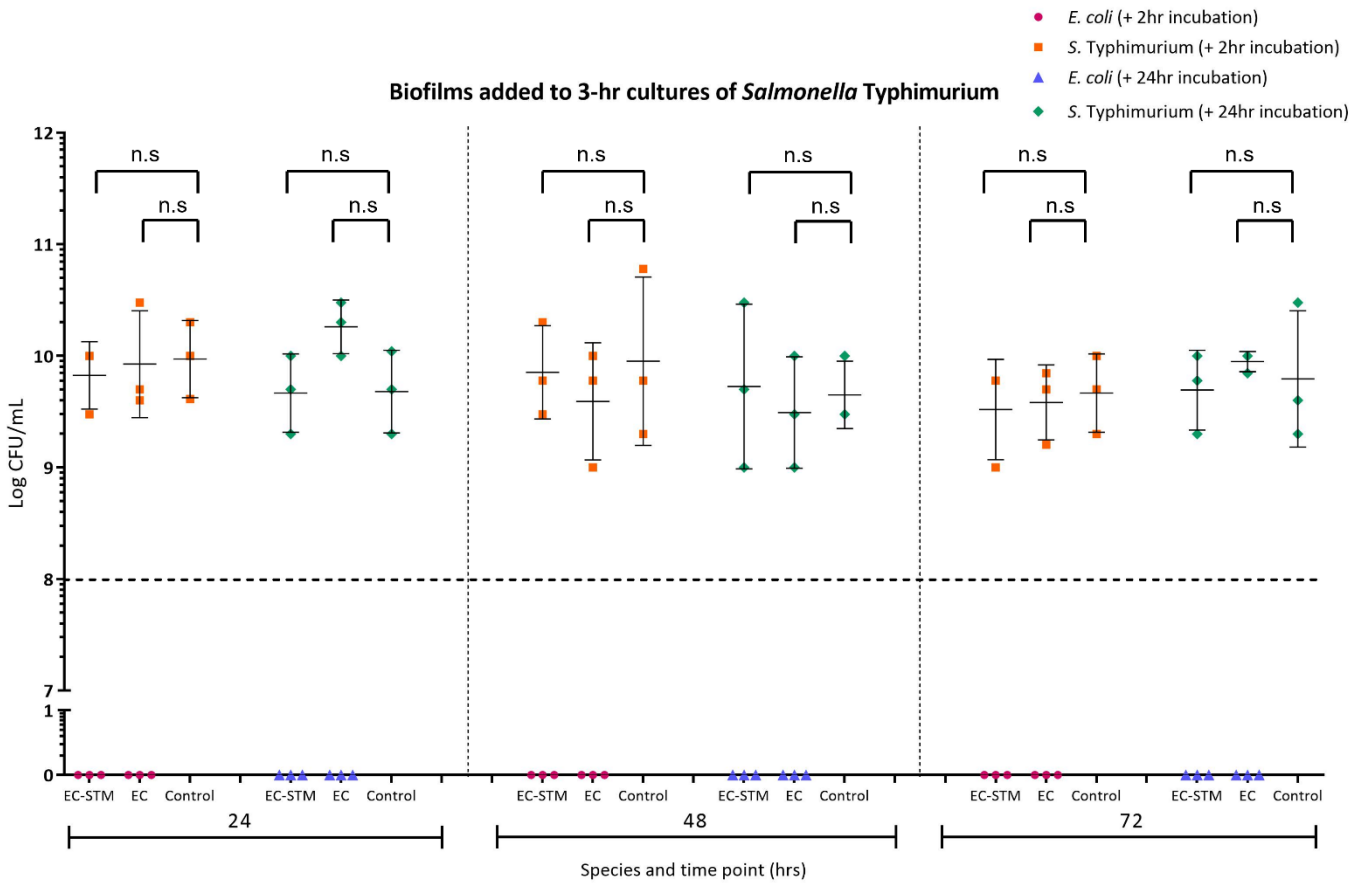

Figure 24. Adding biofilms to planktonic *S.* Typhimurium.

E. coli EC166, *S.* Typhimurium STM2 were grown on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in triplicate and cells were incubated at 30°C and passaged at 24 hours. Beads were inoculated with equal volumes of EC166 and Salmonella at 0 hours and at 24, 48, and 72 hours, one bead was taken and added to 3 hour or 24 hour planktonic cultures of *S.* Typhimurium. Planktonic *S.* Typhimurium + bead were incubated for 2 hours then 20 µL of planktonic cells were diluted and counted. Tubes were incubated for a further 22 hours and 20µL of planktonic cells were removed, diluted and counted again. *E. coli* and *S.* Typhimurium cells were distinguished using blue-white screening and colony counts were used to calculate CFU/mL. The graph shows results from each replicate, the mean log CFU/mL (horizontal lines), and the error bars show +/- one standard deviation from the mean. Tukey's multiple comparisons test was used to identify significant differences between the mean CFU/mL of *S.* Typhimurium STM2 in the control compared with that in the two biofilm conditions, n.s = not significant. Horizontal line shows the detection threshold (lowest CFU/mL detectable for this experiment).

Planktonic S. *Typhimurium STM2 incubated for 24 hours prior to addition of a bead*

E. coli EC166 growth was seen in single species and multispecies conditions when biofilms and *S.* Typhimurium STM2 were incubated together for 24 hours after addition of the 48-hour bead (Figure 25). *E. coli* EC166 was also present in the multispecies condition when biofilms were incubated for 72 hours before being added to planktonic *S.* Typhimurium STM2 (Figure 25). No *E. coli* EC166 growth was seen at any other time point, however the detection threshold for this experiment was 10^8 CFU/mL, therefore *E. coli* EC166 may have been present but undetected. Despite this, *S.* Typhimurium STM2 was present in all conditions (Figure 25). A two-way ANOVA was used to identify the effect of *E. coli* EC166 multispecies or single species biofilms and time point on the CFU/mL of *S.* Typhimurium STM2. This revealed that for the experiment incubated for 2 hours after addition of a bead, there was no significant interaction between the effects of *E. coli* EC166 multispecies or single species biofilms and time point on CFU/mL of *S.* Typhimurium STM2 [$F(4, 12) = 0.752$, $p=0.58$]. No significant interaction was seen for the experiment incubated for 24 hours after addition of a bead [$F(4, 12) = 0.737$, $p=0.58$. Tukey's multiple comparisons test found no significant difference between mean CFU/mL of *S.* Typhimurium STM2 in the control and mean CFU/mL of *S.* Typhimurium STM2 when grown with *E. coli* EC166 multispecies biofilms or *E. coli* EC166 single species biofilms for experiments incubated for 2 hours or 24 hours after addition of a bead at any time points ($p>0.05$ in all cases) (Figure 25).

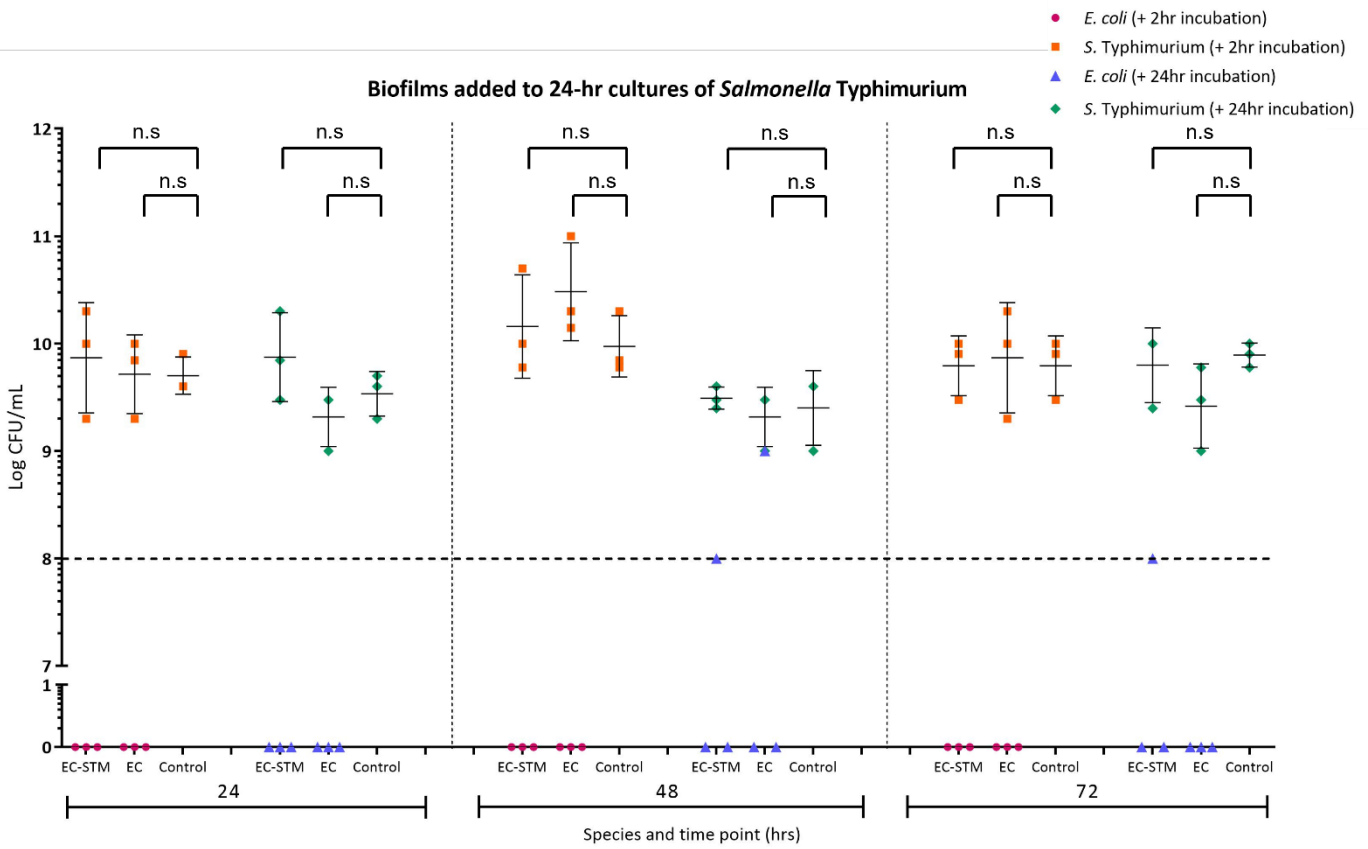

Figure 25. Adding biofilms to planktonic *S.* Typhimurium.

E. coli EC166, *S.* Typhimurium STM2 were grown on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in triplicate and cells were incubated at 30°C and passaged at 24 hours. Beads were inoculated with equal volumes of EC166 and Salmonella at 0 hours and at 24, 48, and 72 hours, one bead was taken and added to 3 hour or 24 hour planktonic cultures of *S.* Typhimurium. Planktonic *S.* Typhimurium + bead were incubated for 2 hours then 20 μL of planktonic cells were diluted and counted. Tubes were incubated for a further 22 hours and 20 μL of planktonic cells were removed, diluted and counted again. *E. coli* and *S.* Typhimurium cells were distinguished using blue-white screening and colony counts were used to calculate CFU/mL. The graph shows results from each replicate, the mean log CFU/mL (horizontal lines), and the error bars show +/- one standard deviation from the mean. Tukey's multiple comparisons test was used to identify significant differences between the mean CFU/mL of *S.* Typhimurium STM2 in the control compared with that in the two biofilm conditions, n.s = not significant. Horizontal line shows the detection threshold (lowest CFU/mL detectable for this experiment).

Adding *E. coli* biofilms grown on beads does not reduce *S.* Typhimurium growth: five beads

Addition of one bead to *S.* Typhimurium STM2 did not reduce the growth of *S.* Typhimurium STM2 therefore, the experiment was repeated using five biofilm beads. The experiment was repeated in the same way but with an additional time point in which biofilms were incubated for 96 hours prior to being added to planktonic *S.* Typhimurium STM2.

Planktonic S. *Typhimurium STM2 incubated for 3 hours prior to addition of beads*

Addition of beads to 3-hour *S.* Typhimurium STM2 cultures resulted in growth of *S.* Typhimurium STM2 in all conditions at all time points (Figure 26). No *E. coli* EC166 was seen when multispecies or single species biofilms were added to *S.* Typhimurium STM2 and incubated for 2 hours. However, *E. coli* EC166 was observed when biofilms were incubated with *S.* Typhimurium STM2 for 24 hours in a multispecies at 24 and 96 hours and in single species at 96 hours. The detection threshold for this experiment was 10^6 CFU/mL, therefore *E. coli* EC166 may have been present at lower than detectable numbers. The CFU/mL of *S.* Typhimurium STM2 grown with multispecies and single species biofilms was higher than controls at 24 and 96 hours therefore I would repeat this experiment.

A two-way ANOVA was carried out to analyse the effect of *E. coli* EC166 multispecies or single species biofilms and time point on the CFU/mL of *S.* Typhimurium STM2. When planktonic *S.* Typhimurium STM2 was incubated for 2 hours after addition of the bead, there was no significant interaction between the effects of multispecies or single species *E. coli* EC166 biofilms and time point on CFU/mL of *S.* Typhimurium STM2 [$F(6, 18) = 0.835$, $p=0.56$]. No significant interaction was seen when planktonic *S.* Typhimurium STM2 was incubated with beads for 24 hours prior to counting [$F(6, 18) = 1.826$, $p=0.150$]. Tukey's multiple comparisons test found no significant difference between mean CFU/mL of *S.* Typhimurium STM2 in the control and mean CFU/mL of *S.* Typhimurium STM2 when grown with *E. coli* EC166 multispecies biofilms or *E. coli* EC166 single species biofilms for experiments incubated for 2 hours or 24 hours after addition of a bead at any time points ($p>0.05$ in all cases) (Figure 26).

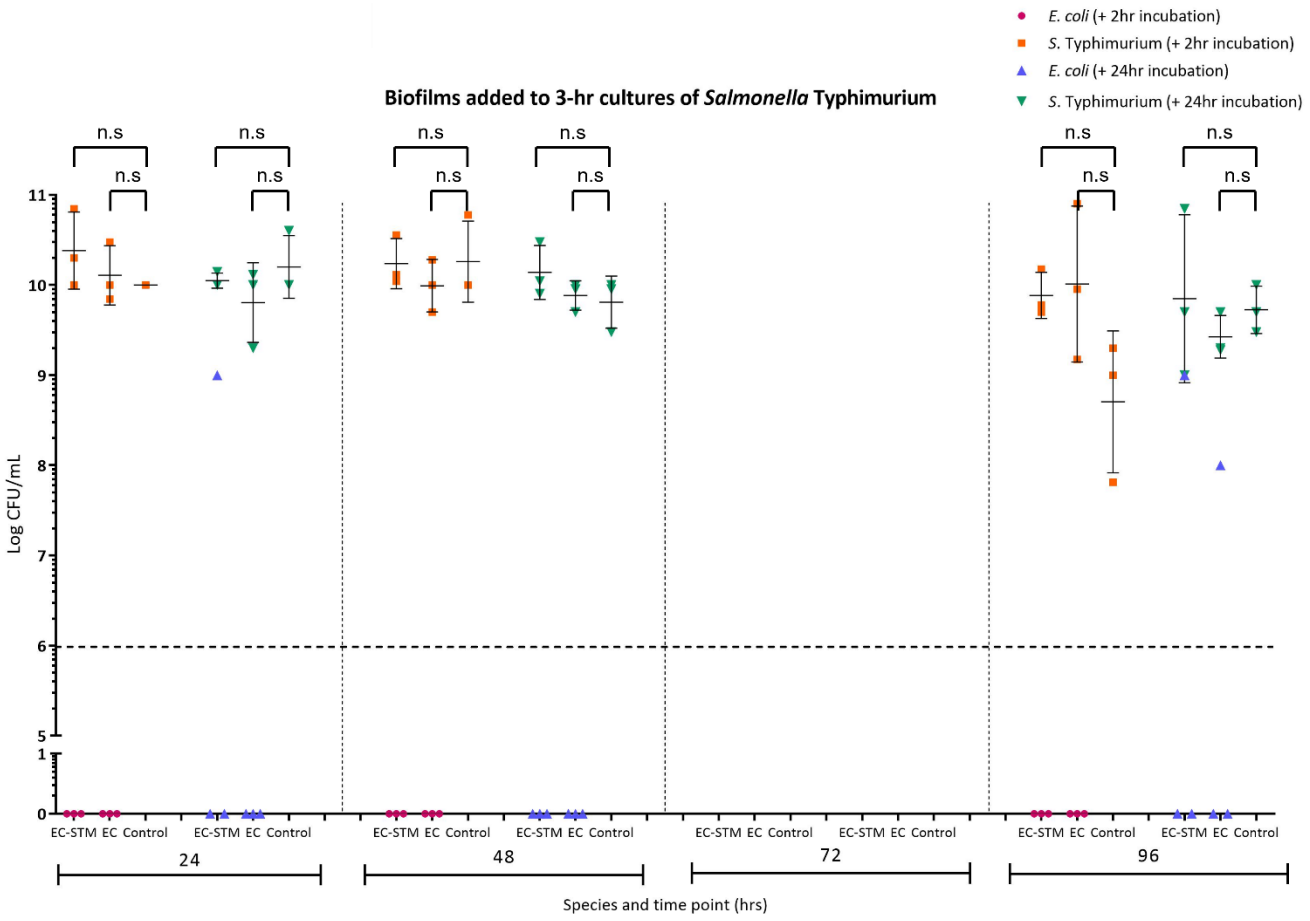

Figure 26. Adding biofilms to planktonic *S.* Typhimurium.

E. coli EC166, *S.* Typhimurium STM2 were grown on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in triplicate and cells were incubated at 30°C and passaged at 24 hours. Beads were inoculated with equal volumes of EC166 and Salmonella at 0 hours and at 24, 48, and 72 hours, five beads were taken and added to 3 hour or 24 hour planktonic cultures of *S.* Typhimurium. Planktonic *S.* Typhimurium + bead were incubated for 2 hours then 20 μL of planktonic cells were diluted and counted. Tubes were incubated for a further 22 hours and 20 μL of planktonic cells were removed, diluted and counted again. *E. coli* and *S.* Typhimurium cells were distinguished using blue-white screening and colony counts were used to calculate CFU/mL. The graph shows results from each replicate, the mean log CFU/mL (horizontal lines), and the error bars show +/- one standard deviation from the mean. N.B. data from 72 hours is missing. Tukey's multiple comparisons test was used to identify significant differences between the mean CFU/mL of *S.* Typhimurium STM2 in the control compared with that in the two biofilm conditions, n.s = not significant. Horizontal line shows the detection threshold (lowest CFU/mL detectable for this experiment).

Planktonic S. *Typhimurium STM2 incubated for 24 hours prior to addition of beads*

When *S.* Typhimurium STM2 was incubated with multispecies or single species biofilms for 24 hours before addition of beads, S. Typhimurium was seen at every time point (Figure 27). However, at 72 and 96 hours, controls were lower than multispecies and single species therefore, this should therefore be repeated (Figure 27). *E. coli* EC166 was present when biofilms and *S.* Typhimurium STM2 were incubated for 24 hours before counting in multispecies conditions at 48, 72, and 96 hours and in single species conditions at 48 and 72 hours. *E. coli* EC166 may have been present in the media at undetectable levels of the detection threshold for this experiment was 10^6 CFU/mL. A two-way ANOVA was used to identify the effect of *E. coli* EC166 multispecies or single species biofilms and time point

on the CFU/mL of *S.* Typhimurium STM2. This revealed that for the experiment incubated for 2 hours after addition of a bead, there was no significant interaction between the effects of *E. coli* EC166 multispecies or single species biofilms and time point on CFU/mL of *S.* Typhimurium STM2 [F (4, 12) = 2.371, p=0.11]. No significant interaction was seen for the experiment incubated for 24 hours after addition of a bead [F (4, 12) = 0.427, p=0.79]. Tukey's multiple comparisons test found no significant difference between mean CFU/mL of *S.* Typhimurium STM2 in the control and mean CFU/mL of *S.* Typhimurium STM2 when grown with *E. coli* EC166 multispecies biofilms or *E. coli* EC166 single species biofilms for experiments incubated for 2 hours or 24 hours after addition of a bead at any time points (p>0.05 in all cases) (Figure 27).

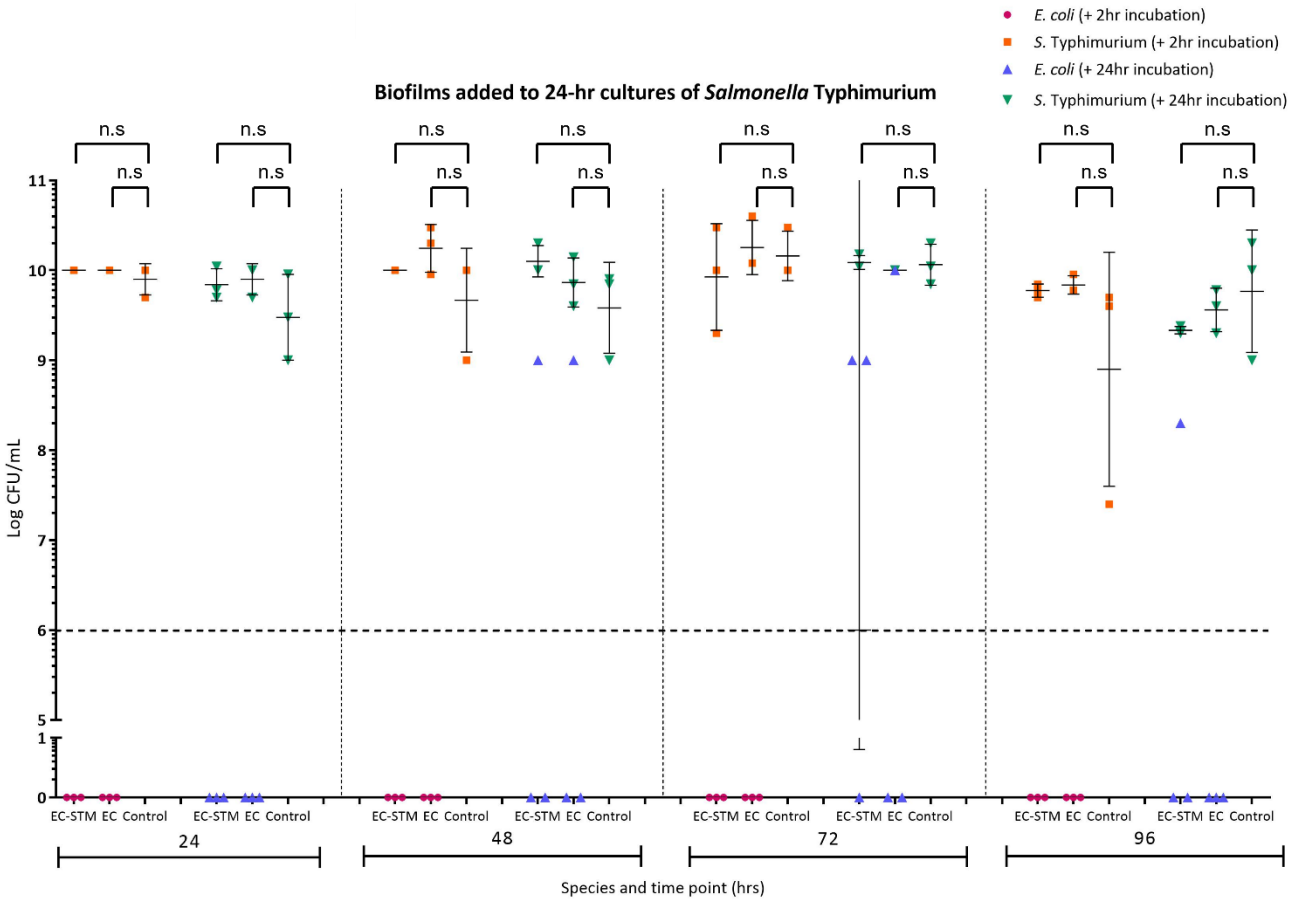

Figure 27. Adding biofilms to planktonic *S.* Typhimurium.

E. coli EC166, *S.* Typhimurium STM2 were grown on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in triplicate and cells were incubated at 30°C and passaged at 24 hours. Beads were inoculated with equal volumes of EC166 and Salmonella at 0 hours and at 24, 48, and 72 hours, five beads were taken and added to 3 hour or 24 hour planktonic cultures of *S.* Typhimurium. Planktonic *S.* Typhimurium + bead were incubated for 2 hours then 20 µL of planktonic cells were diluted and counted. Tubes were incubated for a further 22 hours and 20 µL of planktonic cells were removed, diluted and counted again. *E. coli* and *S.* Typhimurium cells were distinguished using blue-white screening and colony counts were used to calculate CFU/mL. The graph shows results from each replicate, the mean log CFU/mL (horizontal lines), and the error bars show +/- one standard deviation from the mean. Tukey's multiple comparisons test was used to identify significant differences between the mean CFU/mL of *S.* Typhimurium STM2 in the control compared with that in the two biofilm conditions, n.s = not significant. Horizontal line shows the detection threshold (lowest CFU/mL detectable for this experiment).

Pre-formed *E. coli* EC166 biofilms prevent the growth of Salmonella

To further investigate the effect *E. coli* EC166 biofilms on *Salmonella* growth, *E. coli* biofilms were grown in a 24-well plate and planktonic *Salmonella* was added. In each well of a 24-well plate, *E. coli* was added to LB without NaCl and incubated for 24 hours at 30^{o}C, shaking at 60 rpm to induce biofilm formation. Waste media was removed and replenished with fresh media before overnight cultures of planktonic *Salmonella* diluted 1:40 in the fresh broth was added to each well. *S. Typhimurium* STM2 and *S.* Typhimurium STM1 and *S.* Enteritidis STM14 were exposed to *E. coli* EC166 biofilms and *S.* Typhimurium STM51 was exposed to *E. coli* BW25113. *E. coli* BW25113 was included as a control as this strain did not inhibit the growth of *S.* Typhimurium STM51 in the biofilm model. Controls of each *Salmonella* strain alone were used to identify inhibitory activity of *E. coli.*

E. coli EC166 or *E. coli* BW25113 growth was present in each relevant multispecies condition and *Salmonella* growth was present in each control condition (Figure 28). However, in the multispecies conditions *Salmonella* growth was reduced: *S.* Typhimurium STM2 growth was absent in six of eight replicates; *S.* Typhimurium STM1 growth was absent in three of eight replicates; *S.* Enteritidis STM14 growth was absent in four of eight replicates; and *S.* Typhimurium STM51 growth was absent in two of eight replicates (Figure 28). Unpaired t-tests were used to identify whether there was a significant difference between the mean CFU/mL of *Salmonella* controls and *Salmonella* in the presence of *E. coli* biofilms. When *E. coli* EC166 was present, there was a significant difference between the CFU/mL of *S.* Typhimurium STM2 compared with that of the *S.* Typhimurium STM2 controls ($t=3.39$, $df=10$, $p=0.0069$). However, there was no significant difference between the CFU/mL of *S.* Typhimurium STM1 ($t=1.60$, $df=10$, $p=0.14$) or *S.* Typhimurium STM14 ($t=2.17$, $df=10$, $p=0.055$) with and without the presence of *E. coli* EC166 biofilms or between *S.* Typhimurium STM51 CFU/mL with and without the presence of *E. coli* BW25113 biofilms ($t=1.427$, $df=10$, $p=0.18$) (Figure 28).

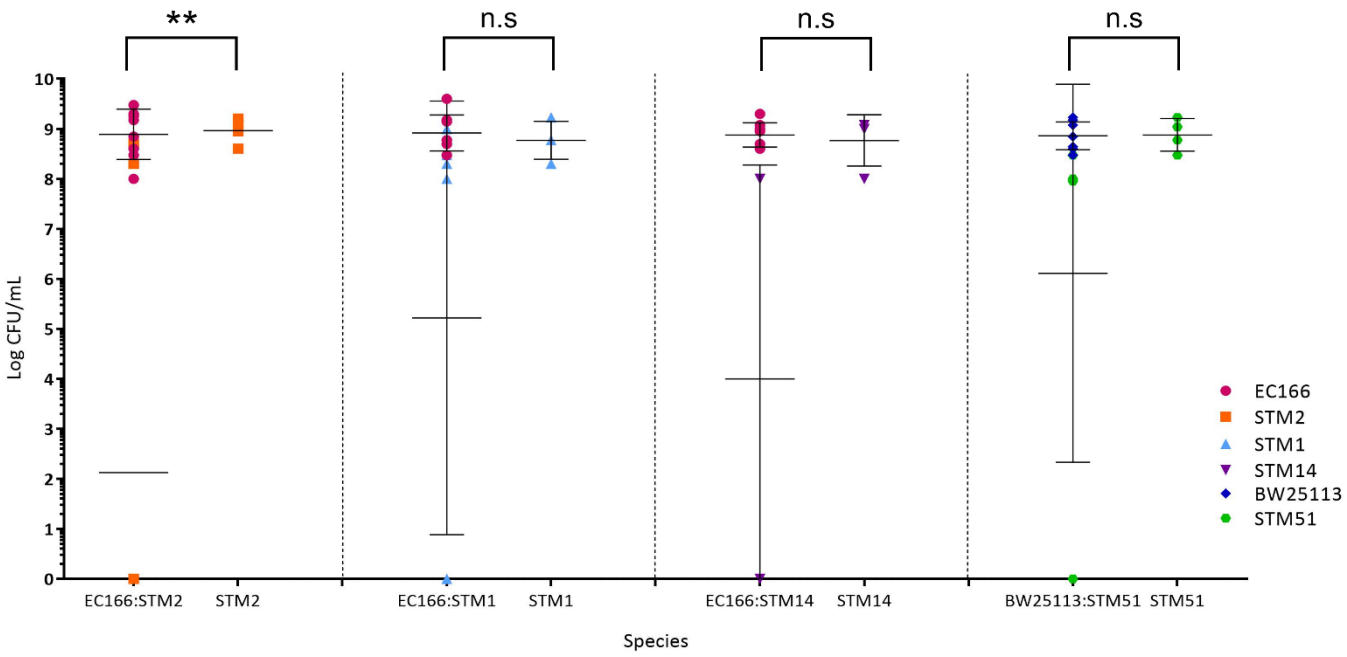

Figure 28. Effects of pre-formed biofilms on *Salmonella* growth

E. coli (EC166; BW25113) biofilms were grown in a 24-well plate and incubated for 24 hours. At 24 hours, waste media was removed from each well without disturbing the biofilms. Each well was replenished with 1 mL of LB broth without salt and *S.* Typhimurium (STM2; STM1; STM51) and *S.* Enteritidis (STM14) were added to the preformed *E. coli* biofilms, or to sterile media as a control. The plate was incubated for 24 hours then cells were diluted, plated and CFU/mL was calculated from colony counts. *E. coli* and *Salmonella* were distinguished using blue-white screening. The graph shows results from two experimental replicates each containing four technical replicates, the mean log CFU/mL (horizontal lines), and the error bars show +/- one standard deviation from the mean. Unpaired t-tests were carried out between *Salmonella* in multispecies and single species conditions (** = $p<0.01$).

Salmonella is lost from multispecies biofilms over time

The biofilm model only shows the abundance of *E. coli* and *Salmonella* at one time point per 24 hours. To observe how abundance changes over 24 hours, the biofilm model was carried out as previously described, however six beads were inoculated so that one bead could be removed to count biofilm cells every 2 hours. Multispecies conditions included *E. coli* EC166 grown with *S.* Typhimurium STM2, *S.* Typhimurium STM1, or *S.* Enteritidis STM14 and the experiment was passaged at 24 hours.

In all 3 cases, the CFU/bead of *Salmonella* was higher than that of *E. coli* EC166 at 2 hours post inoculation of the biofilm model (Figure 29). However, in all three conditions, at 4 hours post inoculation, the CFU/bead of *E. coli* EC166 increased above that of each *Salmonella* species and remained higher until 8 hours post inoculation (Figure 29). At the passage, the abundance of *E. coli* EC166 to *Salmonella* was different for each *Salmonella* species. The CFU/bead of *S.* Typhimurium STM2 was slightly higher than that of *E. coli* EC166 whereas the CFU/bead of *S.* Typhimurium STM1 was lower than that of *E. coli* EC166, finally, the CFU/bead of *S.* Enteritidis STM14 was the same as that of *E. coli* EC166 (Figure 29). At 2 hours after passaging one bead into fresh media, the CFU/bead of each *Salmonella* species was higher than *E. coli* EC166 in all three conditions. However as previously, the CFU/bead of *E. coli* EC266 was higher than Salmonella at 4 hours post passage (Figure 29).

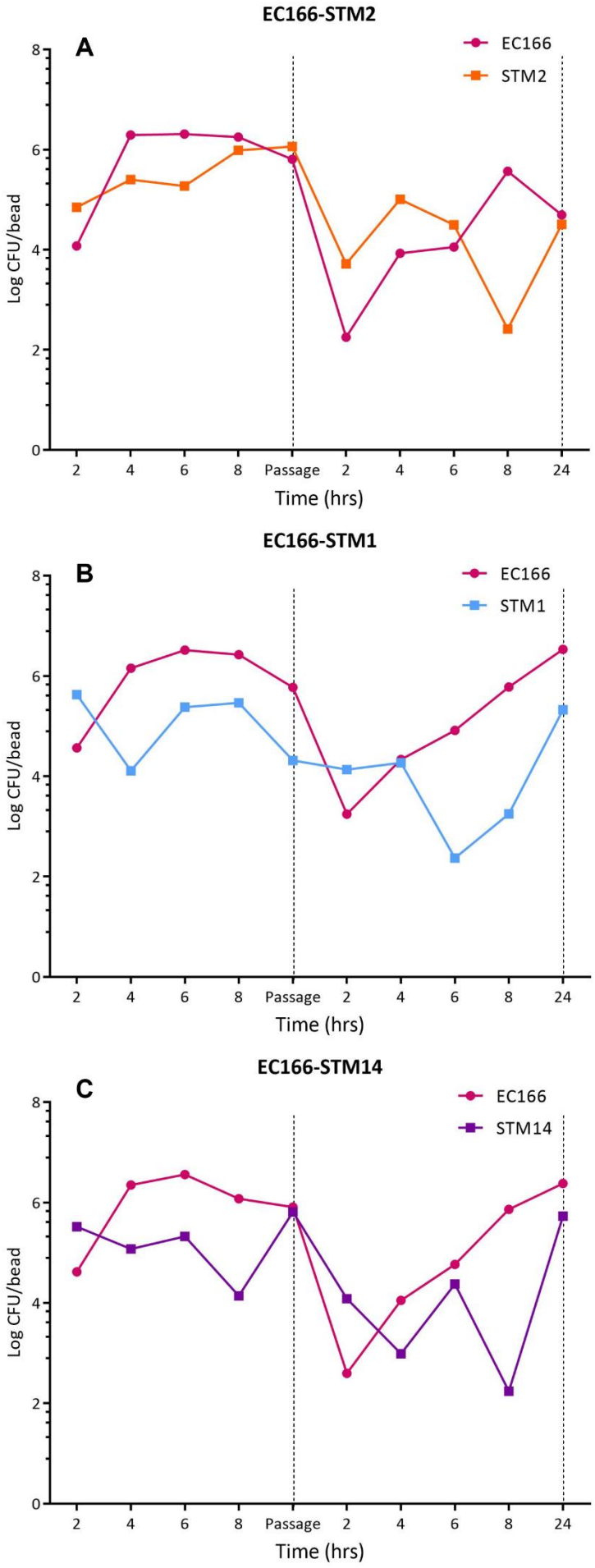

Figure 29. Abundance of *E. coli, S.* Typhimurium, and *S.* Enteritidis biofilm cells in a multispecies biofilm over time.

E. coli EC166, *S.* Typhimurium STM2 (A), *S.* Typhimurium STM1 (B), and *S.* Enteritidis (C) were grown on glass beads in LB broth without NaCl to form biofilms. Each condition was carried out in quadruplicate and cells were incubated at 30°C. Beads were inoculated with equal volumes of EC166 and *Salmonella* at 0 hrs and one bead was removed from the tube and washed for counting every 2 hours until 8 hours. At 24 hrs, one bead was removed and washed for counting (passage) and one bead was removed and added to fresh media to inoculate new sterile beads. Following the passage, one bead was taken and washed for counting every 2 hours until 8 hours post passage. At 24 hours post passage, one bead was removed and from the tube and washed for counting (24). *E. coli* and *Salmonella* cells were counted using blue-white screening and colony counts were used to calculate CFU/bead and CFU/mL which were then logged.

Salmonella survives in a multispecies biofilm with _E. coli_ EC166 in BioFlux conditions

The biofilm model shows that abundance of *E. coli* and *Salmonella* cells within a multispecies biofilm but does not show where each species is located within the biofilm. Using a BioFlux to grow biofilms allows visualisation of biofilms in real time and using fluorescently-tagged strains can allow us to see the location of two species. Unfortunately, due to time constraints it was not possible to make a fluorescently-tagged strain of *E. coli* EC166, however we already had an *mplum*-tagged *S.* Typhimurium STM2 (STM95). This meant we could visualise whether *S.* Typhimurium STM2 biofilms were present in mixed conditions and whether co-culture with *E. coli* EC166 impacted how the biofilms formed. To do this, a BioFlux plate was inoculated with different ratios of *E. coli* EC166 to *S.* Typhimurium STM95. LB without NaCl was run through the BioFlux plate for 24 hours then waste was removed, and fresh media replenished for another 24 hours to allow biofilms to grow.

Figure 30 shows fluorescent *S.* Typhimurium STM95 in the flow cell at different ratios to *E. coli* EC166; large red shapes are biofilms and small red dots are individual cells or small clusters of cells (Figure 30). *S.* Typhimurium STM95 biofilms were present when *E. coli* and *S.* Typhimurium STM95 were at a ratio of 1:1, 1:2, 1:3, 1:5 and 2:1, shown by the large sections of red. No biofilms were present in the 5:1 ratio or the *E. coli* EC166 control (Figure 30). In the *S.* Typhimurium STM95 control, most of the image is taken up by a large fluorescent biofilm with few single cells/small clusters. To quantify biofilms, Fiji (Image J) was used; red particles (fluorescent cells) were analysed and counted by the programme and the area covered by fluorescent cells per three repeats was plotted (Figure 31). The *S.* Typhimurium STM95 control had the largest area covered by fluorescent cells and the *E. coli* EC166 control showed no fluorescence (Figure 31). From a ratio of 1:2 to 1:5 (left to right) area covered by *S.* Typhimurium STM95 decreased (Figure 31). Fiji (Image J) was also used to count the number of cells per image (Figure 32). The number of *S.* Typhimurium STM95 cells was highest at a ratio of 1:1 and lowest at 5:1 (EC:STM), and no STM95 cells were seen in the *E. coli* EC166 control. From a ratio of 1:2 to 2:1 (left to right, Figure 32), the average number of cells increased.

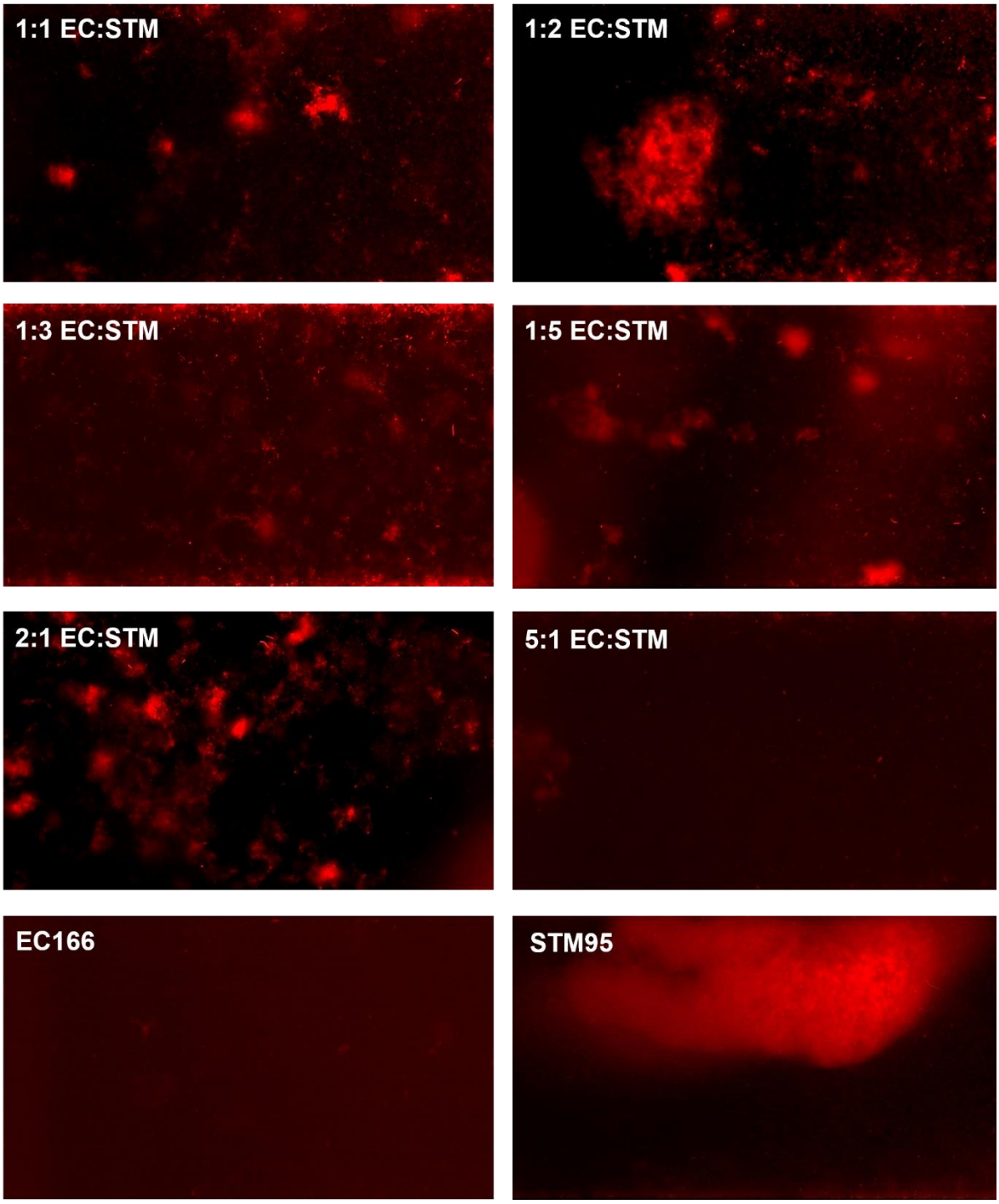

Figure 30. *E. coli* and *S.* Typhimurium mixed species biofilms

E. coli EC166 (not fluorescent) and *S.* Typhimurium STM95 (tagged with mplum) were added to inlet wells of a BioFlux plate at various ratios (EC:STM). LB broth without salt was added to the inlet wells of the plate which was run on a BioFlux at 0.3 dyne for 48 hours to induce biofilm formation. Waste media was removed, and fresh media was replenished at 24 hours. At 48 hours, the plate was removed, and the centre of each flow cell was imaged using a fluorescence microscope. Each image was taken at 20X magnification, using 590 nm wavelength. Each condition was carried out in triplicate and the images show one of three technical replicates.

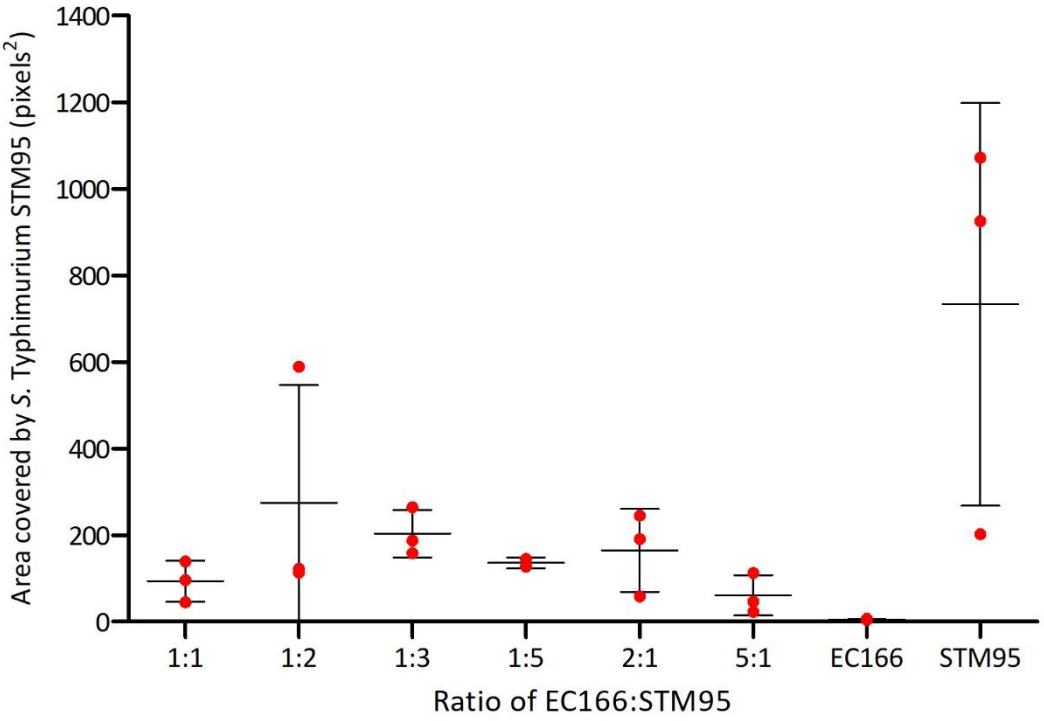

Figure 31. Area covered by *S.* Typhimurium in a multispecies biofilm with *E. coli.*

E. coli EC166 (not fluorescent) and *S.* Typhimurium STM95 (tagged with mplum) were added to inlet wells of a BioFlux plate at various ratios (EC:STM). LB broth without salt was added to the inlet wells of the plate which was run on a BioFlux at 0.3 dyne for 48 hours to induce biofilm formation. Waste media was removed and fresh media was replenished at 24 hours. At 48 hours, the plate was removed and the centre of each flow cell was imaged using a fluorescence microscope. Each image was taken at 20X magnification, using 590 nm wavelength. Images were analysed using Fiji (ImageJ) to count the total fluorescent area per image. The graph shows results from three technical replicates, the mean area covered by *S.* Typhimurium STM95 (horizontal line), and the error bars show +/- one standard deviation from the mean.

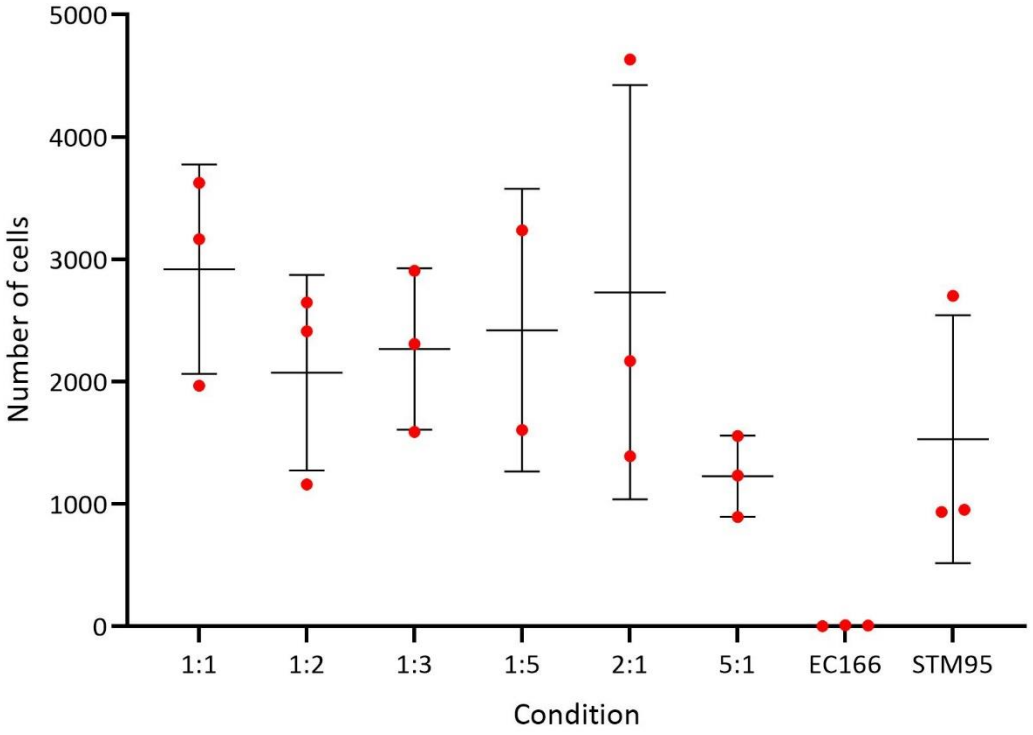

Figure 32. Abundance of *S.* Typhimurium in a multispecies biofilm with *E. coli.*

E. coli EC166 (not fluorescent) and *S.* Typhimurium STM95 (tagged with mplum) were added to inlet wells of a BioFlux plate at various ratios (EC:STM). LB broth without salt was added to the inlet wells of the plate which was run on a BioFlux at 0.3 dyne for 48 hours to induce biofilm formation. Waste media was removed and fresh media was replenished at 24 hours. At 48 hours, the plate was removed and the centre of each flow cell was imaged using a fluorescence microscope. Each image was taken at 20X magnification using 590 nm wavelength. Images were analysed using Fiji (ImageJ) to count the number of fluorescent particles per image. The graph shows results from three technical replicates, the mean number of cells (horizontal line), and the error bars show +/- one standard deviation from the mean.

Genomic analysis shows *E. coli* EC166 may contain a type VI secretion system

The *E. coli* EC166 genome was analysed to identify potential mechanisms involved in *E. coli* EC166 inhibition of *Salmonella*. The *E. coli* EC166 genome was assembled and analysed using Spades and Prokka using a Galaxy installation. To find potential genes involved in inhibition of *Salmonella,* Artemis was used to search the genome for genes involved in bacteriocin production and secretion systems.

Table 3 shows the gene hits within the *E. coli* EC166 genomes associated with a T6SS and bacteriocins. A potential T6SS was found between 3324200 and 3352800 base pairs (forward strand) encoding 19 genes (Figure 33; Table 3). A protein-protein BLAST search was carried out on the amino acid sequence of each gene to identify potential protein products. Of the 19 genes, 17 had an amino acid sequence associated with a T6SS including the VgrG tip protein and the Hcp tail protein (Table 3) and no stop codons were found within any gene sequences. The T6SS cluster contains a minimum of 13 essential genes required for assembly and function of the secretion system (139,154). All 13 essential genes were found in the T6SS within the *E. coli* EC166 genome.

A potential colicin, Microcin S (P_0481) was also identified by BLAST search as well as the colicin V production protein (*cvpA*) and a colicin V secretion protein (*cvaA*) (Table 3). However, genes surrounding P_0481, *cvpA*, and *cvaA* were not involved in bacteriocin production, regulation, or secretion (Figure 34).

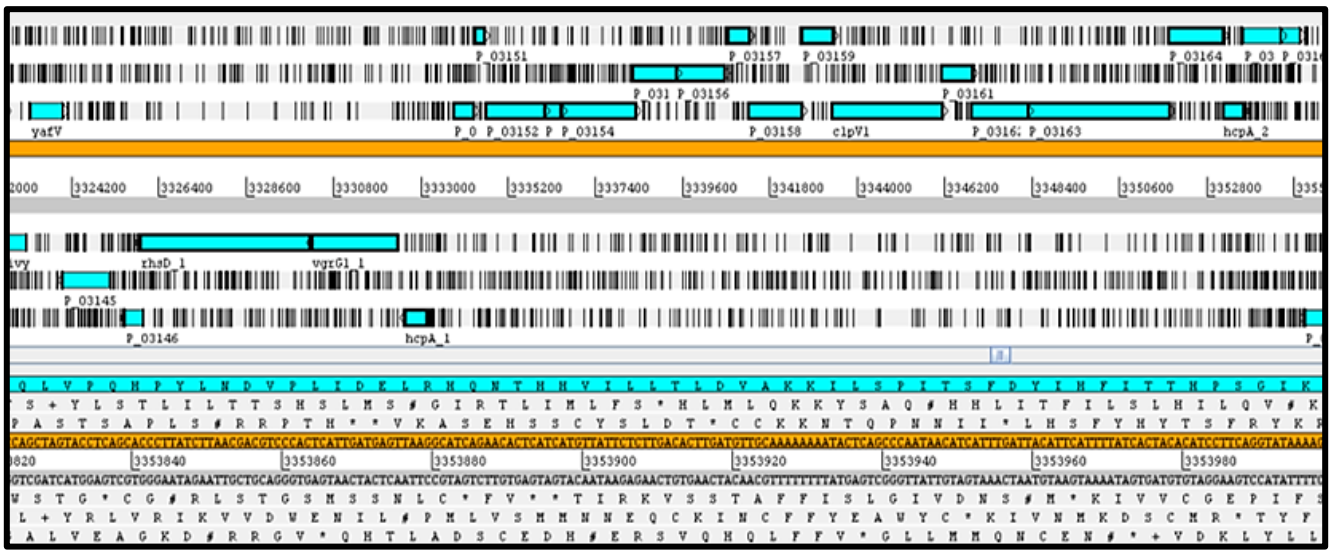

Figure 33. Type VI secretion system genes in *E. coli* EC166 genome

The *E. coli* EC166 genome was assembled and annotated in Galaxy then viewed in Artemis. The screenshot shows a section of the genome containing a potential type VI secretion system with 19 genes outlined in black.

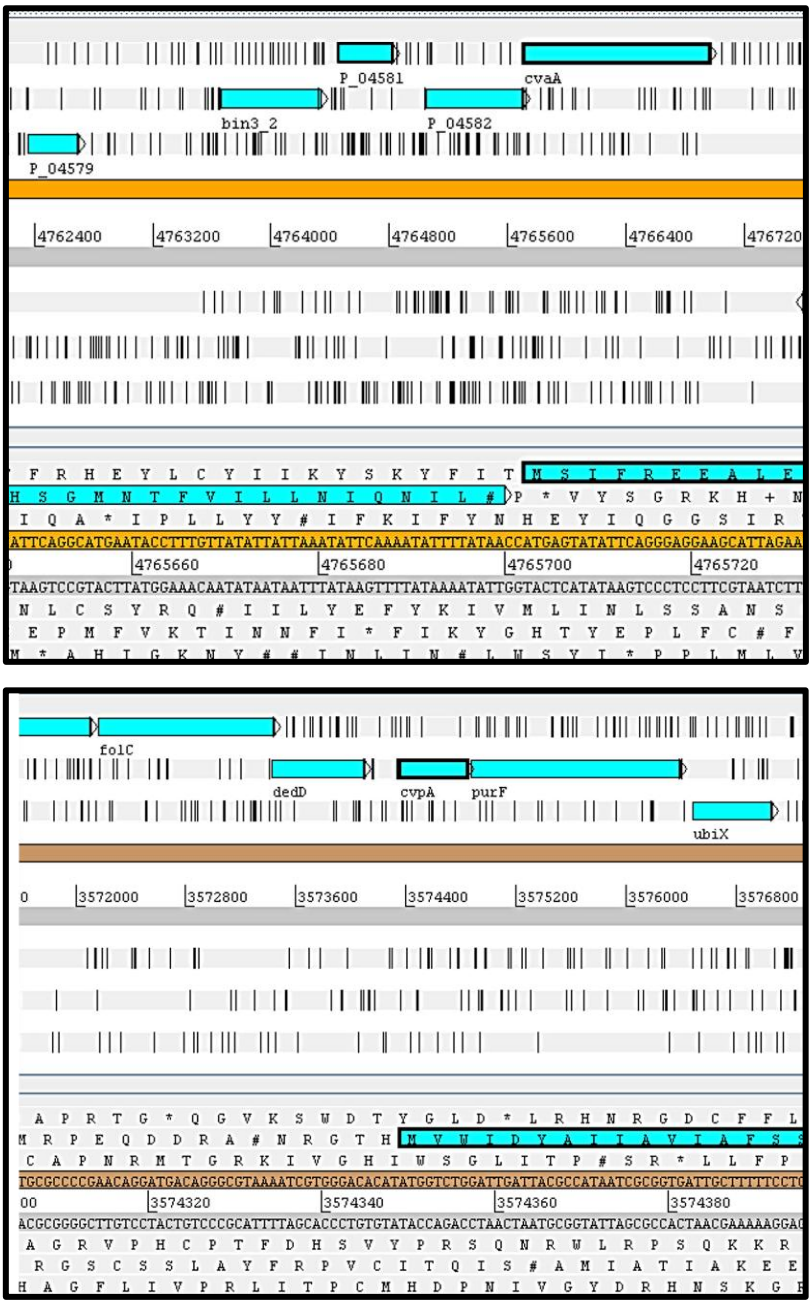

Figure 34. Bacteriocin-associated genes in *E. coli* EC166 genome

The *E. coli* EC166 genome was assembled and annotated in Galaxy then viewed in Artemis. The top screenshot shows a section of the genome containing a potential microcin gene (P_04581) and a colicin V transporter (*cvaA*). The bottom screenshot shows a potential colicin V production protein (*cvpA*).

Table 3. Protein hits from genomic analysis. T6SS = type VI secretion system.

Function	Gene	Strand	Potential protein	Expect value	Percent identity
Type VI Secretion System	*rhsD_1*	Reverse	RhsD effector protein	0	100.00
	vgrG1_1	Reverse	T6SS tip protein	0	99.86
	hcpA_1	Reverse	T6SS Hcp protein	4×10^{-127}	100.00
	P_03150	Forward	Contractile sheath small subunit	1×10^{-115}	100.00
	P_03151	Forward	No hits	N/A	N/A
	P_03152	Forward	Contractile sheath large subunit	0	100.00
	P_03153	Forward	Baseplate TssE	2×10^{-96}	100.00
	P_03154	Forward	Baseplate TssF	0	99.84
	P_03155	Forward	Baseplate TssG	0	100.00
	P_03156	Forward	T6SS protein ImpI	0	100.00
	P_03157	Forward	T6SS lipoprotein TssJ	5×10^{-125}	100.00
	P_03158	Forward	Baseplate TssK	0	99.55
	P_03159	Forward	T6SS protein IcmH/PtU	0	100.00
	clpV1	Forward	T6SS ATPase TssH	0	100.00
	P_03161	Forward	T6SS associated protein TagO	2×10^{-179}	100.00
	P_03162	Forward	T6SS TssA	0	100.00
	P_03163	Forward	T6SS TssM	0	99.49
	P_03164	Forward	T6SS-associated protein	0	100.00
	hcpA_2	Forward	T6SS Hcp protein	6×10^{-116}	100.00
Bacteriocins	P_0481	Forward	Microcin S	2×10^{-75}	100.00
	cvaA	Forward	Colicin V secretion protein	0	100.00
	cvpA	Forward	Colicin V production protein	4×10^{-109}	100.00

Discussion

Bacteria exist in biofilms which are less susceptible to antibiotics, making them harder to treat than their planktonic counterparts. Biofilms typically comprise more than one strain or species and interactions in biofilms are common due to the proximity of cells and the pressure from limited nutrients and space. These factors increase competition between bacteria and there are various ways that bacteria can inhibit or kill their competitors. We identified a food isolate of *E. coli* with the ability to reduce growth or inhibit multiple strains of *Salmonella* and carried out various experiments to uncover the mechanism behind this inhibition.

Initially, *E. coli* BW25113 was used for multispecies biofilm evolution experiments, however *E. coli* BW25113 is a K-12 lab strain and is generally a poor biofilm former which may be why this species was lost from the biofilm conditions at 15°C (155,156). When incubated at 15°C, *E. coli* BW25113 was lost from both single species and multispecies biofilm conditions, however this occurred more rapidly in the multispecies condition, thus *S.* Typhimurium STM51 may inhibit or slow *E. coli* BW25113 growth. However, *E. coli* BW25113 was not completely lost from the multispecies biofilm at 30°C, thus *S.* Typhimurium STM51 may have a growth advantage at 15°C, rather than actively killing or preventing *E. coli* BW25113 growth. Similar results were seen in the planktonic cells; *E. coli* BW25113 was lost from the 15°C multispecies condition by 144 hours and overall reached a lower CFU/mL in the presence of *S.* Typhimurium STM51 compared to in the single species planktonic condition. As the loss of *E. coli* BW25113 was more profound at 15°C than at 30°C, it is likely that growth of this species is more temperature dependent than *S.* Typhimurium STM51.

The biofilm evolution model was carried out again with an additional planktonic control containing no beads. A two-way ANOVA with Dunnett's multiple comparisons test identified a significant difference between CFU/mL of *E. coli* BW25113 in a multispecies compared with a single species biofilm at 24 hours, however all other time points showed no difference. No significant difference between CFU/mL of *S.* Typhimurium STM51 in a multispecies compared with a single species biofilm was seen at any time point. This shows that the presence of *S.* Typhimurium STM51 biofilms does not reduce overall growth of *E. coli* BW25113 biofilms. Both *E. coli* BW25113 and *S.* Typhimurium STM51 were maintained throughout the experiments in the 30°C multispecies biofilms however, a multispecies biofilm model ideally desires a stable community which was not achieved. In this case we aimed for both species to be present in equal numbers to test the effects of stress on the biofilm without either species impacting the growth of another. For this reason, *E. coli* BW25113 cannot be used as the *E. coli* strain within the biofilm model.

Under multispecies planktonic conditions, Dunnett's multiple comparison test showed a significant difference in mean CFU/mL at 72 and 96 hours for both *E. coli* BW25113 and *S.* Typhimurium STM51 CFU/mL. In the case of *S.* Typhimurium STM51, CFU/mL was higher in the presence of beads, compared to without beads at 72 and 96 hours, suggesting that the presence of biofilms in the multispecies planktonic conditions permits increased *S.* Typhimurium STM51 growth. However, for multispecies planktonic *E. coli* BW25113, CFU/mL was higher in the absence of beads at 72 hours. Together these results show that *S.* Typhimurium STM51 has an advantage over *E. coli* BW25113 in the presence of biofilms which is why the two could not be used in the biofilm model together.

To determine whether an environmental strain of *E. coli* could persist in a multispecies biofilm with *S.* Typhimurium, a food isolate of *E. coli* was used. When grown in single species biofilms, *E. coli* EC166 reached a higher CFU/bead than *S.* Typhimurium STM2. This may mean *E. coli* EC166 grows faster, allowing this species to take limited nutrients and space on the bead, causing a reduction in *S.*

Typhimurium STM2 growth when in a multispecies biofilm. However, as *S.* Typhimurium STM2 was also unable to persist planktonically in the presence of *E. coli* EC166 biofilms, *E. coli* EC166 may actively reduce or prevent *S.* Typhimurium STM2 growth. *E. coli* can produce bacteriocins, antimicrobial peptides that inhibit bacterial growth by acting on the cell envelope or inside the cell (157). A study by Zihler *et al.* (133) identified four commensal *E. coli* strains that produced bacteriocins with activity against 68 clinical isolates of *S.* Typhimurium and *S.* Enteritidis. Furthermore, Rendueles *et al*. identified an environmental *E. coli* isolate with ability to produce a bacteriocin only under biofilm conditions (158). It is therefore possible that *E. coli* EC166 produces a bacteriocin with activity against *S.* Typhimurium STM2.

The biofilm evolution experiment using *E. coli* EC166 and *S.* Typhimurium STM2 was also carried out using an additional planktonic control, containing no beads, to identify whether inhibition of *S.* Typhimurium STM2 growth was biofilm dependent. In this case, when the experiment was passaged every 24 hours, *S.* Typhimurium STM2 was not completely lost from the biofilm; therefore, a shorter passage window may reduce the impact of *E. coli* EC166 on *S.* Typhimurium STM2. It is possible that a longer period is required for *E. coli* EC166 to produce or accumulate a product that prevents growth of, or kills, *S.* Typhimurium STM2.

Two-way ANOVA simple main effects analysis showed that there was a significant difference between *S*. Typhimurium STM2 CFU/mL in a multispecies compared with single species biofilm; Dunnett's multiple comparisons test showed a significant difference at 72 and 96 hours. For *E. coli* EC166, a two-way ANOVA revealed that there was a significant interaction between multispecies vs. single species biofilms and time point on CFU/mL, however Dunnett's multiple comparisons test showed no significant difference between CFU/mL of *E. coli* EC166 in a multispecies compared with single species biofilm at any time point. This suggests that the presence of *E. coli* EC166 in a multispecies biofilm with *S.* Typhimurium STM2 reduces growth of the latter whilst CFU/mL of *E. coli* EC166 remains the same as when grown alone; these species therefore cannot be used in a multispecies biofilm model.

Under planktonic conditions, no significant difference was seen in the CFU/mL of *S.* Typhimurium STM2 or *E. coli* EC166 in the multispecies planktonic conditions with or without beads. In the multispecies planktonic conditions, both *S.* Typhimurium STM2 and *E. coli* EC166 growth was lower with beads compared to without beads. This may have been because biofilm cells must disperse from the bead and begin a planktonic lifestyle in the planktonic with beads control, however planktonic controls without beads were inoculated with an overnight culture. In the planktonic controls without beads, *S.* Typhimurium STM2 was maintained throughout the experiment, however in the presence of beads, three out of four replicates were lost by 96 hours, suggesting that the presence of *E. coli* EC166 as a biofilm reduces *S.* Typhimurium STM2 growth, however not at a statistically significant level. As discussed previously, this may be due to a bacteriocin produced by *E. coli* EC166 that kills or prevents growth of *S.* Typhimurium STM2, possibly only during biofilm mode of life. Due to the reduced growth of *S.* Typhimurium STM2 in a multispecies biofilm with *E. coli* EC166, it is not possible to use these strains to model a multispecies biofilm as this requires equal numbers of each species. However, we decided to further investigate the mechanism behind *E. coli* EC166 inhibition of S. Typhimurium STM2.

The biofilm model was carried out using *E. coli* EC166 with another *S.* Typhimurium strain and a strain of *S.* Enteritidis to identify whether inhibition by *E. coli* EC166 was strain or serovar specific. A two-way ANOVA was used to identify significant differences in the CFU/mL of *S.* Typhimurium STM1 and *E. coli* EC166 in multispecies compared with single species biofilms. Interactions between multispecies vs. single species biofilms and time point had a significant effect on the CFU/mL of *S.* Typhimurium STM1. Dunnett's multiple comparisons test revealed the CFU/mL of *S.* Typhimurium STM1 was significantly

different between multispecies and single species biofilms at 48, 72, and 96 hours. however, no significant interaction between multispecies vs. single species biofilm and time point was identified for CFU/mL of *E. coli* EC166. Furthermore, *E. coli* EC166 CFU/mL of multispecies compared with single species biofilms was not significantly different at any time point. *S.* Typhimurium STM1 was lost from the multispecies biofilm condition by 48 hours, however remained throughout the experiment as a single species biofilm. This suggests that the presence of *E. coli* EC166 biofilms results in reduced growth of *S.* Typhimurium STM1 biofilms. Growth inhibition or killing by *E. coli* EC166 is therefore not strain-specific. It is possible that *E. coli* EC166 uses the limited resources more quickly than *S.* Typhimurium STM1, allowing *E. coli* EC166 to outgrow *S.* Typhimurium STM1. However as with *S.* Typhimurium STM2, *S.* Typhimurium STM1 can only persist under planktonic conditions, when *E. coli* EC166 biofilms are not present, suggesting that *E. coli* EC166 in a biofilm actively reduces growth of *S.* Typhimurium STM1.

Planktonic controls were also analysed by two-way ANOVA which showed a significant interaction between *S.* Typhimurium STM1 with and without beads and time point in the multispecies planktonic condition however no significant interaction was seen for *E. coli* EC166. Dunnett's multiple comparisons test showed a significant difference between *S.* Typhimurium STM1 CFU/mL in the multispecies planktonic condition with compared to without beads at 48, 72, and 96 hours. All eight *S.* Typhimurium STM1 replicates were lost from the multispecies planktonic condition with beads at 72 hours and seven out of eight replicates were lost at 96 hours. Dunnett's multiple comparisons test showed a significant difference between multispecies planktonic *E. coli* EC166 CFU/mL with and without beads at 24 and 72 hours. Despite this, all *E. coli* EC166 replicates remained in the multispecies planktonic condition with beads throughout the experiment. In the multispecies planktonic condition without beads, *S.* Typhimurium STM1 had a lower mean CFU/mL than *E. coli* EC166 despite having a higher CFU/mL than *E. coli* EC166 when each were grown as a single species without beads. This suggests that *E. coli* EC166 may also reduce growth of *S.* Typhimurium STM1 when grown together planktonically in the absence of biofilms. This may be in the same way as in the multispecies biofilm condition or due to a higher growth rate of *E. coli* EC166 compared with *S.* Typhimurium STM1, however this effect is less pronounced in the absence of beads. Overall, these results suggest that the presence of *E. coli* EC166 biofilms can reduce the growth of biofilm and planktonic *S.* Typhimurium STM1.

Following this, the biofilm model was carried out using *E. coli* EC166 and *S.* Enteritidis STM14. Mixed effects analysis showed a significant interaction between *S.* Typhimurium STM14 in a multispecies vs. single species biofilm and time point on the CFU/mL of *S.* Typhimurium STM14. Dunnett's multiple comparisons test showed a significant difference between the CFU/mL of *S.* Typhimurium STM14 in a multispecies compared with single species biofilm at all time points. *S.* Typhimurium STM14 was lost from the multispecies biofilm condition by 72 hours but remained in a single species biofilm throughout the experiment. The reduction of *S.* Enteritidis STM14 in the presence of *E. coli* EC166 shows that the inhibitory effect of *E. coli* EC166 is not strain or serovar specific.

Two-way ANOVA showed no significant interaction between *E. coli* EC166 in a multispecies vs. single species biofilm and time point and Dunnett's multiple comparisons test showed no significant difference between the CFU/mL of *E. coli* EC166 in a multispecies vs. single species biofilm at any time point. This demonstrates the ability of *E. coli* EC166 to survive in the presence of *S.* Typhimurium STM14 at the same level as when grown alone. This may be due to the faster generation time of *E. coli* EC166 compared to *S.* Typhimurium STM14 or due to competitive action of *E. coli* EC166.

Under multispecies planktonic conditions, mixed effects analysis showed a significant interaction between *S.* Typhimurium STM14 with vs. without beads and time point, furthermore Dunnett's multiple comparisons test showed a significant difference between the CFU/mL of *S.* Typhimurium STM14 with and without the presence of beads at 48, 74, and 96 hours in the multispecies planktonic control. There was no significant interaction between *E. coli* EC166 with vs. without beads and time point, however Dunnett's multiple comparisons showed a significant difference between CFU/mL of *E. coli* EC166 with and without beads at 24, 72, and 96 hours. The mean CFU/mL of *E. coli* EC166 in the multispecies planktonic conditions with *S.* Typhimurium STM14 with beads was higher than that of *E. coli* EC166 without beads—this was significant in three out of four time points. This was also seen in the multispecies planktonic *E. coli* EC166 with *S.* Typhimurium STM1 with compared to without beads and was significant in two of three time points. This may be due to inhibition of *Salmonella* by *E. coli* EC166 biofilms which would result in more nutrients and space available for *E. coli* EC166 causing the higher CFU/mL and suggests that *E. coli* EC166 inhibition of *S.* Typhimurium STM14 is biofilm-dependent.

Overall, these results show that the reduction in *Salmonella* is not strain or serovar specific. The presence of *E. coli* EC166 in the multispecies biofilm condition resulted in a significantly lower CFU/mL of *Salmonella* strain by 72 hours for all three strains. Furthermore, there was a significant difference in CFU/mL of planktonic *Salmonella* in the presence of *E. coli* EC166 biofilms in all three strains. This may be due to a shorter generation time of *E. coli* EC166, however each *Salmonella* strain survived with *E. coli* EC166 under planktonic conditions in the absence of *E. coli* EC166 biofilms suggesting an active mechanism of *Salmonella* inhibition by *E. coli* EC166 that is biofilm dependent. It would be interesting to carry out the biofilm evolution experiment using more strains of *Salmonella*, particularly clinically-relevant strains such as isolates from the chicken gut or clinical samples to identify whether *E. coli* EC166 is protective against pathogens, not just lab strains.

Growth kinetics showed that *E. coli* EC166 had a significantly lower generation time compared with the *Salmonella* strains. This supports the possibility that in a multispecies biofilm, *E. coli* EC166 can take up space and nutrients more quickly. However, under planktonic control conditions, *E. coli* EC166 and *Salmonella* strains were able to persist together throughout the biofilm experiment, suggesting the difference in generation time is not enough for *E. coli* EC166 to outcompete the *Salmonella* strains. Biofilm growth on a bead is more competitive than planktonic conditions due to limited space and nutrients which could exaggerate the faster growth rate of *E. coli* EC166 compared to *Salmonella* in a multispecies biofilm. However, by looking at the abundance of each species in a multispecies biofilm over 24 hours, we know that *Salmonella* can colonise the bead and is the primary strain immediately after inoculation and passaging.

When supernatant from biofilm conditions was added to planktonic *E. coli* EC166, generation time was significantly increased compared to the control in all cases aside from the *E. coli* EC166 planktonic supernatant. However, if this effect was due to an inhibitory factor produced by *E. coli* EC166, it should not have inhibited its own growth. It is possible that this increase in generation time was because addition of supernatant can reduce growth of cells as it contains waste from the culture. Additionally, a drop in pH caused by waste in the supernatant could have resulted in an increased generation time. A study by Chorianopoulos *et al.* (159) added cell-free filtered supernatant to cultures of *S.* Enteritidis to investigate the impact of *Hafnia alvei* supernatant on biofilm formation. The group used a bead model supplemented with 20% or 50% supernatant and investigated biofilm development between 12 and 72 hours and found that 50% supernatant reduced biofilm cells after 24 hours of incubation. This method could be used to identify whether presence of each *Salmonella* strain is required for *E. coli*

EC166 to inhibit or kill the *Salmonella*. To do this a biofilm model using each strain of *Salmonella* should be supplemented with cell-free, filtered supernatant from pre-grown biofilms of *E. coli* EC166 as a single species or multispecies with each *Salmonella* strain. As all three *Salmonella* strains were not lost from the multispecies biofilm until at least 48 hours in the experiment, it is possible that reduced *Salmonella* growth is dependent on time. Observing growth rate over a 24-hour period did not allow for this to be investigated, however supplementing the biofilm model with supernatant would allow us to see the impact of the supernatant on the development of *Salmonella* biofilms.

When supernatant from biofilm conditions was added to planktonic *S.* Typhimurium STM2 biofilms, generation time was significantly higher compared with the control in all cases. Addition of supernatant from planktonic cultures containing no beads increased generation time further than with addition of cultures containing beads. This may have been because less cells were present within the tubes containing beads, therefore produced less waste to prevent growth when the supernatant was added. Overall, a significant difference was seen between the controls in the case of *E. coli* EC166 and *S.* Typhimurium STM2, however if this was due to inhibitory activity of *E. coli* EC166 then it should not have reduced its own growth.

When the mixed planktonic supernatant was added to *E. coli* EC166, a 12% increase (27.9 min to 31.3 min) was seen, however a 25% increase (39.3 min to 49.5 min) increase was seen when the same supernatant was added to *S.* Typhimurium STM2. Additionally, when the *E. coli* planktonic supernatant was added to *E. coli* EC166, a 0% increase (27.9 min to 27.9 min) was seen, and a 16% increase (39.3 min to 45.9 min) was seen when this supernatant was added to *S.* Typhimurium STM2. However, when the supernatant from the biofilm conditions was added to *E. coli* EC166 and *S.* Typhimurium STM2, a larger increase in generation time was seen in *E. coli* EC166 A 24% increase (27.9 min to 34.8 min) was seen when mixed biofilm and *E. coli* EC166 were added to *E. coli* EC166 compared to a 16% (39.3 min to 45.8 min) increase when the same supernatant was added to *S.* Typhimurium STM2. This suggests that the biofilm conditions had more impact on *E. coli* EC166 and that the planktonic conditions had more impact on *S.* Typhimurium STM2. However, as mentioned previously, adding supernatant to *E. coli* EC166 was a control as an inhibitory product produced by *E. coli* EC166 should not inhibit its own growth, therefore we cannot be certain that the significant increase in generation time seen when supernatant was added to *S.* Typhimurium STM2 is due to competitive inhibition by *E. coli* EC166.

The biofilm model was adapted to determine whether pre-formed *E. coli* EC166 biofilms grown on beads could prevent or reduce growth of *S.* Typhimurium STM2. This was done by adding one bead or five beads (containing either *E. coli* EC166 and *S.* Typhimurium STM2 in a biofilm or containing only *E. coli* EC166 biofilms) to 3-hour and 24-hour planktonic *S.* Typhimurium STM2. No significant difference was seen in *S.* Typhimurium STM2 CFU/mL in control condition compared to when grown with biofilms. It is possible that the activity of *E. coli* EC166 occurs very early therefore even after only 3 hours of growth, the planktonic *S.* Typhimurium STM2 was sufficiently established so *E. coli* EC166 could not reduce its growth. This experiment used colonies from agar plates to inoculate the planktonic *S.* Typhimurium STM2, however the experiment should be repeated using overnight cultures as this is what the biofilm model uses. Additionally, more time points should be carried out; biofilms should be added to cultures of *S.* Typhimurium STM2 at inoculation and 1 and 2 hours post-inoculation to identify whether activity of *E. coli* EC166 occurs early in *S.* Typhimurium STM2 planktonic growth.

When *Salmonella* strains were exposed to pre-formed *E. coli* EC166 biofilms grown in a 24-well cell culture plate, CFU/mL of *S.* Typhimurium STM2 was significantly lower than *S.* Typhimurium STM2 grown alone. This experiment appears to contradict the previous experiment in which *S.* Typhimurium STM2 was not inhibited when incubated with pre-formed biofilm beads. Biofilms formed around the

wall of each well within the 24-well plate meaning planktonic *Salmonella* in the plate may have been physically exposed to biofilms more often than in the biofilm model in which the beads roll on the bottom of the universal tube. Furthermore, surface properties have an influence on biofilm initiation and attachment which may account for the differences seen between this and the previous experiment (160,161). The smaller volume of the wells, compared to the tubes may have resulted in a higher biomass of biofilm per well compared to per tube. Interestingly *E. coli* BW25113 formed a very thick biofilm on the well but was unable to significantly reduce the growth of *S.* Typhimurium STM51. This suggests that biofilm presence alone is not enough to inhibit *Salmonella* growth. It is therefore likely *E. coli* EC166 can actively inhibit the growth of *Salmonella. E. coli* EC166 biofilms were unable to significantly reduce the growth *S.* Typhimurium STM1 and *S.* Typhimurium STM14 however three of eight (STM1) and six of eight (STM14) replicates showed no growth in the presence of biofilms. This suggests inhibitory activity of *E. coli* EC166 biofilms however 24 hours may not have been a long enough incubation period to see a significant effect. I would repeat this experiment using different concentrations of planktonic *Salmonella* to identify whether there is a minimum amount of *Salmonella* required to prevent the inhibition by *E. coli* EC166.

To observe any changes in the abundance of *E. coli* EC166 and *Salmonella* in a multispecies biofilm, the biofilm model was used. However, in this case, six beads were inoculated, and one bead was removed for counting every 2 hours (up to 8 hours post inoculation) rather than once every 24 hours. At 2 hours post-inoculation, the CFU/bead of each *Salmonella* species was around 1 log higher than the CFU/bead of *E. coli* EC166. This was surprising as growth kinetics showed the generation time of *E. coli* EC166 was significantly shorter than the generation time of each *Salmonella* species. It was therefore expected that *E. coli* EC166 would colonise the bead more quickly and use available resources before *Salmonella*, causing the loss of *Salmonella* from the multispecies biofilm. At 4 hours post inoculation, CFU/bead of *E. coli* EC166 was higher than that of each *Salmonella* species, suggesting *E. coli* EC166 is able to actively kill or inhibit *Salmonella* at this point. However, at 2 hours post passage, CFU/bead of each *Salmonella* species was higher than *E. coli* EC166 again, demonstrating that *Salmonella* was present in the biofilm and able to colonise beads before inhibition by *E. coli* EC166.

Commensal microbes can prevent colonisation of the gut by pathogens (162). A study by Litvak *et al*. showed commensal *E. coli* isolates protect germ-free mice from colonisation by *S.* Enteritidis by competing for oxygen (163). Another group demonstrated that *E. coli* strain Nissle 1917 prevents colonisation of *S.* Typhimurium in mouse models by sequestering available iron (164). It is possible that in the bead model, *Salmonella* can colonise the bead at the beginning of the experiment when resources are high, however as competition for resources increases, *E. coli* EC166 has an advantage and outcompeted *Salmonella,* causing it to be lost from the multispecies biofilm. When the experiment is passaged to fresh media, competition for resources is lower again, potentially allowing *Salmonella* to colonise the new bead, however over time, as competition for resources increases, *E. coli* EC166 outcompetes *Salmonella*. This experiment should be repeated with additional passages to identify whether *Salmonella* can continue to colonise the bead after the second or third passage. *E. coli* EC166 may also inhibit *Salmonella* growth by producing a bacteriocin or using a T6SS that is not activate immediately. If the mechanism is activated via quorum sensing, *E. coli* EC166 would have to reach a high cell density before activation, potentially allowing *Salmonella* to colonise the bead before being inhibited by *E. coli* EC166. When repeating this experiment, a single species control of *E. coli* EC166 and each *Salmonella* species should be carried out to determine how quickly each species colonises a bead without the presence of the other.

To visualise the multispecies biofilm, fluorescently-tagged *S.* Typhimurium STM2 (STM95) was grown with *E. coli* EC166 in a BioFlux system. As we did not have a fluorescently-tagged *E. coli* EC166, it was only possible to view the *S.* Typhimurium STM95 cells and thus the data outputted is crude. In future the experiment should be repeated with a *gfp*-tagged *E. coli* EC166 to allow distinction between each cell. This would allow us to determine where each species is located with the multispecies biofilm; whether the cells are mixed within a biofilm or if microcolonies of each species form. *S.* Typhimurium STM95 was able to form biofilms after 48 hours when grown as a multispecies biofilm with *E. coli* EC166. It is possible that the BioFlux system increases the ability of *S.* Typhimurium STM95 to form biofilms in the presence of *E. coli* EC166 compared to on beads. The BioFlux system has a constant flow of fresh media and removal of waste which may reduce competition between the two species, allowing *S.* Typhimurium STM95 to form biofilms under this condition. Image J was used to determine the area covered by fluorescent *S.* Typhimurium STM95 and to count the number of cells present. The *S.* Typhimurium STM95 control had the highest area covered by fluorescence (733 pixels^2) but the second lowest number of cells (1530 cells) (excluding *E. coli* EC166 control) suggesting the *S.* Typhimurium STM95 control formed large biofilms and had few single cells or microcolonies. In contrast, at a ratio of 1:1 (EC:STM), number of *S.* Typhimurium STM95 cells was highest (2919 cells), but area covered (93 pixels^2) was second lowest (excluding *E. coli* EC166 control). This suggests that when grown with *E. coli* EC166 at a ratio of 1:1, *S.* Typhimurium STM95 may form smaller biofilms, or exist as microcolonies and single cells compared to when grown as a single species.

However, the number of *E. coli* EC166 cells and area covered by *E. coli* EC166 were not quantified therefore we cannot say how the number of *E. coli* EC166 cells/biofilms influenced the number of cells/biofilms of *S.* Typhimurium STM95. Furthermore, Image J was not able to count individual cells within a biofilm therefore number of cells per well is not accurate. To count individual cells, a *gfp*-tagged *E. coli* EC166 should be used in the BioFlux system and flow cytometry should be used to count cells. I would do this by removing cells from the BioFlux system every 24 hours and using flow cytometry to quantify the abundance of red vs. green cells in the sample to observe how abundance changes over time.

Genomic analysis was used to search for genes responsible for *E. coli* EC166 inhibition of *Salmonella*. Overall, no biofilm-specific mechanisms were identified, however a potential T6SS and three bacteriocin genes were identified. We identified a potential T6SS containing 18 genes encoding T6SS proteins including *vgrG* and *hcp*. T6SSs are involved in contact-dependent killing and can be found in *E. coli* (129,143,144). Within a biofilm cells live in proximity which would allow contact-dependent killing by a T6SS. Using the BioFlux system to visualise where *E. coli* EC166 and the *Salmonella* species exist within a biofilm would allow us to observe whether the two species mix within a multispecies biofilm or form distinct microcolonies and thus whether they are close enough to interact. However, a T6SS would not explain why planktonic *Salmonella* cannot survive in the presence of *E. coli* EC166 biofilms in the biofilm evolution model or when exposed to pre-formed *E. coli* EC166 biofilms. It is possible that there is an additional mechanism resulting in inhibition of planktonic *Salmonella* that results in secretion of an inhibitory product into the media.

To identify whether this T6SS is responsible for the inhibition of *Salmonella,* then next step would be to knockout the T6SS from *E. coli* EC166 and repeat the biofilm evolution experiment with this strain compared to a wild-type control. If no difference is seen in *Salmonella* growth between the knockout and wild-type, then it is unlikely that the T6SS is responsible for inhibition of *Salmonella*. Additionally, I would knock-in the T6SS into *E. coli* BW25113 and carry out the biofilm evolution experiment with

Salmonella; if the T6SS is responsible for inhibition of *Salmonella*, the *E. coli* BW25113 should gain the ability to inhibit *Salmonella*.

BLAST analysis of the *rhsD*_1 amino acid sequence was a match for the protein RhsD; Rhs proteins are a class of T6SS effector proteins of various species including *E. coli* (165–167). Rhs proteins contain a C-terminal domain with endonuclease activity that results in chromosomal and plasmid DNA degradation and subsequently growth inhibition and cell death (139,168). To confirm that RhsD is responsible for inhibition of *Salmonella*, the protein could be purified and added to *Salmonella* cultures to identify whether *Salmonella* can grow in its presence.

Genomic analysis also identified potential bacteriocin genes. The amino acid sequence of P_0481 was a 100% match to that of MccS, a microcin produced by a probiotic strain of *E. coli* (169). Zschüttig *et al*. (169) characterised the microcin S gene cluster which was located on a plasmid and had toxic activity against susceptible *E. coli* strains—strains absent of the *mcsI* gene which confers immunity (169). However, other members of the microcin S gene cluster were not present in the *E. coli* EC166 genome, therefore it is unlikely that this strain can produce microcin S to inhibit the growth of *Salmonella*. Two genes encoding colicin V proteins were also identified via BLAST analysis. Colicin V is a bacteriocin produced by *E. coli* that inserts into the inner membrane of a target cell to disrupt the membrane potential and cause lysis (170). Genome analysis identified *cvaA*, the gene encoding the colicin V secretion protein and *cvpA*, the gene encoding the colicin V production protein (170). However, analysis of surrounding genes showed no hits for any members of the colicin V gene cluster, thus it is unlikely that colicin V is produced by *E. coli* EC166 to inhibit the growth of *Salmonella.*

Conclusion

We have identified a food isolate of *E. coli* with the ability to inhibit *S.* Typhimurium and *S.* Enteritidis. Inhibitory activity of *E. coli* EC166 is biofilm dependent; *Salmonella* was lost from multispecies conditions but only when an *E. coli* EC166 biofilm was present in the culture. The generation time of *E. coli* EC166 was significantly shorter than that of each *Salmonella* strain however it is unlikely that this is the sole reason for reduced growth of *Salmonella* in the presence of *E. coli* EC166. Adding planktonic *Salmonella* to pre-formed *E. coli* EC166 biofilms resulted in reduced *Salmonella* growth compared with controls, indicating that an active mechanism is involved in inhibition, rather than simply a difference in growth rate. Furthermore, immediately after inoculation and passaging, *Salmonella* was more abundant than *E. coli* EC166 in multispecies biofilms, suggesting *E. coli* EC166 may employ an active mechanism of growth inhibition later in biofilm maturation. A T6SS was identified within the *E. coli* EC166 genome which may be responsible for growth inhibition of *Salmonella*, however further work is required to confirm whether the T6SS is essential for this inhibition. Commensal strains of *E. coli* can protect the gut microbiota from invasion by pathogens and commensal bacteriocins can be used as probiotics to prevent infections. Uncovering the mechanism of inhibition by *E. coli* EC166 will allow us to understand interactions between commensal and pathogenic bacteria and could identify new probiotics for the prevention or treatment of infection.

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