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Indirect resistance to antibiotics between bacteria of different species

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Abstract

Indirect resistance (IR) is a well documented polymicrobial interaction, defined as the protection of an antibiotic-sensitive strain by a neighboring resistant strain through the detoxification of the surrounding environment. It is typically observed as a consequence of antibiotic-based treatments, with a notable proclivity for β-lactam antibiotics. The large majority of IR occurrences involve cohabiting strains of diverging species. By comparison, examples of intra-species IR remain scarce. To explain this discrepancy, we propose that resource competition can counteract IR by rendering intra-species strain coexistence untenable. Likewise, inter-species IR can occur unimpeded as different strains can coexist by occuppying different metabolic niches, or even share metabolic by-products through cross-feeding. To test this hypothesis, we performed intra- and inter-species co-cultures of resistant and sensitive strains, in ampicillin-supplemented minimal media with competing and non-competing carbon sources. Also, cross-feeding was evaluated by performing co-cultures in media supplemented with carbon sources only consumable by one of the strains. While intra-species competition was invariable, inter-species competition was conditional of the carbon source. We found that sensitive strains exhibited significant growth reduction in intra-species co-culturing compared to when in mono-culture. By contrast, when paired with a resistant strain belonging to a different species, sensitive strains were able to match the growth observed in mono-culture. Co-cultures also showed potential cross-feeding interactions allowing the growth of both resistant and sensitive strains in the absence of their specific carbon source. Our results show that IR may be optimized in the absence of strain competition, which could potentially explain the lack of observable intra-species IR when compared to inter-species. Furthermore, we identified that cross-feeding mechanisms between strains might strenghten the cooperative nature of IR by permiting the growth of the resistant cells. Conversely, it may also extend the exploitation of the resistant strain to include metabolic nutrients in addition to antibiotic-disabling enzymes.

Keywords: indirect resistance, cross-feeding, bacteria, coexistence, competitive exclusion.

Resumo

A resistência indireta (RI), também conhecida como patogenicidade indireta, define-se como a proteção de uma estirpe sensível por uma estirpe resistente vizinha contra determinado antibiótico através da desintoxicação do ambiente circundante. Tem sido observada na clínica hospitalar tipicamente como consequência de tratamentos com antibióticos, revelando uma tendência notável para antibióticos β-lactâmicos. Para tal, contribui o facto de que as enzimas β-lactamases (principal defesa contra β-lactâmicos) são normalmente alvo de transporte para o meio extracelular. Este mecanismo de acção favorece fortemente a RI, pois permite que estas enzimas circulem livremente desintoxicando o meio, em igual benefício tanto para as estirpes produtoras de β-lactamases (resistentes) como para as estirpes não-produtoras (sensíveis). De facto, qualquer molécula que seja transportada para o espaço extracelular, poderá em teoria contribuir para uma interação deste género. Um caso semelhante será o efeito de *cross-feeding*, outra interação polimicrobiana que se define como a partilha (passiva ou activa) de produtos metabólicos entre estirpes, tipicamente pertencentes a espécies diferentes. Neste caso, uma espécie poderá produzir um metabolito secundário, posteriormente transportado para o espaço extracelular, que irá ser consumido por espécies vizinhas não-produtoras. Esta partilha poderá também ser recíproca, sendo que duas ou mais estirpes diferentes poderão sintetizar diferentes produtos metabólicos e partilhá-los entre si, estabelecendo-se assim um equílibrio entre as várias populações e promovendo-se a biodiversidade.

Apesar da importância deste tipo de interações para o estudo de comunidades bacterianas, a RI permanece um tópico largamente subvalorizado e pouco compreendido. Uma das questões mais notáveis sobre a ocorrência de RI na Natureza será a diferença observada entre a frequência de RI em situações de colonização inter-espécies e situações intra-espécies. A grande maioria das ocorrências de RI normalmente envolvem estirpes bacterianas co-habitantes de espécies diferentes. No entanto, casos de RI entre estirpes pertencentes à mesma espécie estão raramente documentados na literatura. Ao longo dos anos, pouco tem sido feito para elucidar a(s) causas(s) desta discrepância.

No âmbito deste projecto, propomos que a competição por fontes de carbono poderá desfavorecer a RI, tornando a co-existência de estirpes da mesma espécie insustentável. Pela mesma lógica, a RI inter-espécies pode ocorrer com maior propensão, pois diferentes estirpes podem co-existir ocupando diferentes nichos metabólicos ou mesmo compartilhando subprodutos metabólicos por *cross-feeding*.

Para testar esta hipótese, realizámos co-culturas de estirpes resistentes e sensíveis, sendo elas da mesma espécie ou não, em meio mínimo suplementado com o antibiótico ampicilina (um β-lactâmico) e com fontes de carbono comuns ou exclusivas a cada estirpe. Para tal, construíram-se quatro estirpes, duas estirpes *E. coli* resistente e sensível, e duas estirpes *S. enterica* resistente e sensível. Como fontes de carbono, utilizou-se glucose, lactose e citrato. Aqui, a glucose funciona como fonte consumível por ambas as espécies, enquanto que a lactose e o citrato são exclusivas às estirpes *E. coli* e *S. enterica*, respectivamente. A lactose e o citrato, foram utilizados em combinação para gerar co-culturas com estirpes pertencentes a espécies diferentes em ausência de competição. Em co-culturas em que a fonte de carbono seja consumível pelas duas estirpes, espera-se que a subsequente competição pelas mesmas dificulte o crescimento das estirpes sensíveis, mesmo após a inactivação do antibiótico no meio. Especificamente, será de esperar que enquanto o meio não esteja suficientemente desintoxicado, a estirpe resistente tenha acesso exclusivo à fonte de carbono, deixando pouco da mesma disponível para sustentar o posterior crescimento da sensível. Deste modo, prevê-se que a estirpe sensível não tenha

carbono suficiente para atingir a sua taxa de crescimento e concentração máximas. Por outro lado, em meios suplementadas com fontes de carbono exclusivas às estirpes sensível e resistente, espera-se que a resistente consuma exclusivamente a sua fonte enquanto inactiva a ampicilina circundante. Após a desintoxicação do meio, a estirpe sensível ainda terá acesso à sua fonte de carbono e conseguirá atingir a sua taxa de crescimento e concentração final máximas. Adicionalmente, procurámos avaliar qualitativamente a ocorrência de *cross-feeding* realizando co-culturas em meios suplementados com fontes de carbono apenas consumíveis pelas estirpes sensíveis. O crescimento bem-sucedido das sensíveis implica a ocorrência de interações de *cross-feeding* que favoreçam a proliferação limitada da estirpe resistente e a subsequente desintoxicação do meio.

Inicialmente, realizámos mono-culturas das estirpes resistentes de *E. coli* e *S. enterica* em lactose e citrato. Enquanto que a estirpe *E. coli* mostrou o comportamento previsto, crescendo em lactose e permanecendo estacionária em citrato, *S. enterica* revelou resultados mais inesperados. De facto, a estirpe *S. enterica* mostrou um crescimento limitado na presença de lactose, para além do crescimento previsto em citrato. Como a metabolização de lactose não é uma característica típica da *S. enterica*, inoculámos a mesma em meio M9 sem qualquer fonte de carbono para confirmar se de facto a sua proliferação estaria a ser suportada pela lactose. Verificou-se que, mesmo na ausência total de fonte de carbono, a estirpe *S. enterica* resistente continuou a revelar crescimento. Propomos que esta proliferação seja devida a potenciais mecanismos de armazenamento de carbono (por exemplo, síntese de glicogénio), que permitam produzir e armazenar moléculas de carbono durante a sua cultura *overnight*. Esta capacidade foi tomada em conta durante a análise das várias co-culturas inter- e intra-espécies efectuadas ao longo do trabalho. Enquanto que competição intra-espécies se verificou em todas as fontes de carbono testadas, a ocorrência de competição inter-espécies era totalmente dependente da fonte de carbono utilizada no meio. Nomeadamente, estirpes sensíveis exibiram reduções significativas nas suas concentrações finais (em comparação com mono-culturas) quando cultivadas em co-cultura com estirpes resistentes da mesma espécie. Isto verificou-se tanto para estirpes *E. coli* em meio M9 com lactose, como para estirpes *S. enterica* em M9 com citrato. De modo a comprovar que esta redução no crescimento das estirpes sensíveis ocorreu devido à competição metabólica com as resistentes, fizeram-se também co-culturas com estirpes de espécies diferentes em meio M9 suplementado com glucose. Observámos que enquanto que a estirpe *E. coli* sensível sofreu uma redução no seu crescimento quando posta em co-cultura com *S. enterica* resistente, o mesmo não aconteceu com as estirpes *S. enterica* sensível e *E. coli* resistente. Uma possível explicação será a capacidade de armazenamento de carbono por parte da *S. enterica*, previamente observada quando a mesma foi posta em meio M9 sem fonte de carbono. Este mecanismo poderá ter permitido às estirpes *S. enterica* suportar o seu crescimento face à exaustão da glucose no meio. Outra razão poderá também ter sido o facto da *E. coli* ter tendência a diminuir acentuadamente em concentração no início de cada cultura. Possivelmente esta redução terá anulado a vantagem em concentração inicialmente usufruída pela *E. coli* resistente, adiando a exaustão da glucose em relação à desintoxicação do meio. Por outro lado, quando estirpes pertencentes a espécies diferentes foram postas em co-cultura em meio M9 suplementado por ambos lactose e citrato, as estirpes sensíveis foram sempre capazes de alcançar a concentração máxima observada em monocultura. Isto fortalece a hipótese de que na ausência de competição nutricional, as estirpes sensíveis podem beneficiar da RI sem qualquer decréscimo no seu fitness.

Potenciais interações de *cross-feeding* foram também observadas ao longo das co-culturas inter-espécies, permitindo o crescimento de estirpes resistentes e sensíveis na ausência da sua fonte de carbono específica. Quando a estirpe *S. enterica* sensível foi cultivada em co-cultura com a estirpe *E. coli* resistente em meio M9 com citrato, observámos que *E. coli* conseguiu proliferar o suficiente para desintoxicar o meio e permitir o crescimento da *S. enterica*. Em conformidade, quando *E. coli* era a estirpe sensível, esta também revelou proliferação limitada mesmo quando em co-cultura em M9

complementado com citrato. É de notar que quando a estirpe *E. coli* resistente foi cultivada em monocultura em meio M9 com citrato, esta não revelou qualquer proliferação, e o mesmo aconteceu quando duas estirpes *E. coli* (resistente e sensível) foram co-cultivadas em citrato. Dado que o crescimento da *E. coli* em citrato só foi observado quando em co-cultura com *S. enterica*, podemos inferir que o *cross-feeding* pela *S. enterica* na ausência de uma fonte de carbono metabolizável pela *E. coli* terá suportado o crescimento da mesma. No entanto, não foi possível observar *cross-feeding* entre as estirpes *E. coli* resistente e *S. enterica* sensível, uma vez que devido aos mecanismos de armazenamento de carbono previamente mencionados, o crescimento da *S. enterica* em M9 com lactose foi sempre independente de estar em mono- ou co-cultura.

Os nossos resultados mostram que a RI pode ser otimizada na ausência de competição por fonte de carbono, o que ajuda a explicar a escassez de casos de RI intra-espécies observados em comparação com inter-espécies. Além disso, identificámos que mecanismos de *cross-feeding* entre estirpes podem fortalecer a natureza cooperativa da RI, sustentando o crescimento das células resistentes. Por outro lado, pode também proporcionar uma relação parasítica entre estirpes, sendo que estirpes sensíveis poderão tirar proveito não só de enzimas inactivadoras de antibióticos produzidas pelas resistentes, como também de quaisquer nutrientes metabólicos libertados pelas mesmas.

Palavras-chave: resistência indirecta, cross-feeding, bactéria, co-existência, exclusão competitiva.

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1. Introduction

1.1 Indirect Resistance

In bacteriology, the phenomenon of indirect resistance (IR), a form of indirect pathogenicity, occurs when an antibiotic-sensitive strain is protected by a resistant strain from the action of surrounding antibiotic molecules (Brook, 1989; Nicoloff H. and Andersson, 2016a; Domingues *et al.*, 2017). Considering that one of the possible antibiotic resistance mechanisms consists in the production of antibiotic-specific inactivating enzymes, it stands to reason that a non-resistant strain of bacteria could be protected by enzymes produced by another local resistant strain (Brook, 1989; Brook, 2004; Wright, 2005; Nicoloff H. and Andersson, 2016a). Let's suppose that there is an enclosed environment embedded with a given antibiotic and containing two strains of bacteria, one susceptible and one resistant. Through the production of resistance enzymes, the local medium will eventually be deprived of active antibiotic molecules, thus allowing for the unimpeded growth of both the resistant as well as any neighboring non-resistant strains.

It becomes clear that an antibiotic susceptible strain can be shielded by a nearby resistant strain without enduring the fitness limiting costs of maintaining one or more functional resistance genes [Vogwill and Maclean, 2015; Dugatkin et al., 2005]. In practice, IR has only been observed in certain cases, largely between strains belonging to different species. For instance, in the context of cystic fibrosis, the patients' lungs are colonized by a multi-species community of bacteria, thus constituting an ideal setting for inter-species IR to occur [Vandeplassche et al., 2019]. Similarly, chronic bronchial infections can also present cases of inter-species IR. Here, enterobacteria have been shown to protect *Haemophilus influenzae* from clinical treatment through the production of penicillinase [Maddocks and May, 1969]**.** As a final example, various *in-vivo* and *in-vitro* studies highlight β-lactamase producing bacteria as protectors of group A beta-hemolytic *streptococci* (GABHS) in tonsillar infections [Brook, 1984]**.** The scarcity of intra-species IR suggests that there are other factors at play which may diminish the benefits of IR and neutralize the potential growth of the sensitive strains. These include a wide array of interactions such as ion sequestration, carbon utilization, byproduct exchange as well as the overall spatial structuring and organization of both strains [Braga et al., 2016]. Indeed, IR presents itself as an intriguing component in the study of microbial ecological communities, serving as a valuable contributor for the foundation of inter- and intra-species interactions. Nevertheless, IR cannot be considered an isolated phenomenon, but otherwise the result of multifactorial driving forces that constrains the relationships between neighboring bacterial populations [Weiland-Bräuer, 2021; Coyte et al., 2015; Antoniewicz, 2020; Stubbendieck et al., 2016].

1.2 Competitive exclusion theory

Metabolism is paramount when considering the dynamics of bacterial communities as it can be a powerful driver of ecological competition between populations [Stubbendieck et al., 2016; Bauer et al., 2018]. This is known as the principle of competitive exclusion [den Boer, 1986; Macarthur and Levins, 2015]. Under this principle, it is unlikely that two strains can successfully co-infect a host, or cohabitate an ecological domain, while also occupying the same metabolic niches [Watkins et al., 2016; Bauer et al., 2018; Li et al., 2018] (Fig. 1.1). In a pre-established bacterial community, an invading species will either fail to colonize the new microbiota or displace competing populations, resulting in their decline and a reduction in the local biodiversity [Stubbendieck et al., 2016; Bauer et al., 2018]. This resource competition works as a negative driving force that will most likely interfere with other inter-populational interactions, including IR, which requires a stable inter-strain relationship in a community with sustainable biodiversity. An invading resistant species may not be capable of successfully colonizing a new habitat if its metabolic requirements are in direct conflict with the native sensitive strain(s), thus rendering IR unfeasible regardless of whether it would have benefited the sensitive strains or not. As such, differences in nutrient sources, rate of consumption or macromolecule binding receptors are imperative in order to boost coexistence and maintain species' diversity in the community [Watkins et al., 2016].

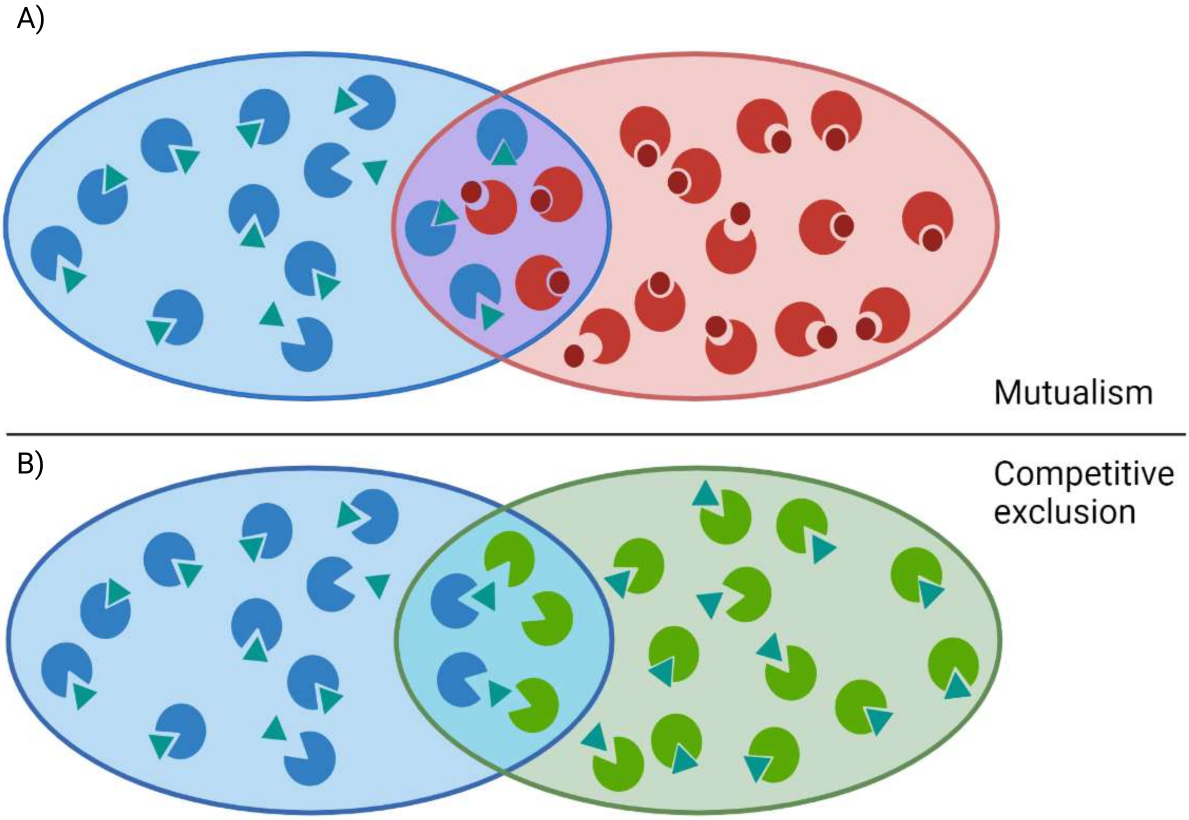

Figure 1.1 Comparison between competitive exclusion and mutualism. Populations and niches are distinguishable by color (blue, red and green). Within each population, individuals are represented as indented concentric circles, the shape of their grooves correspond to their preferred nutrient source. Food sources are represented either as triangules (shared between blue and green individuals) or small red circles (exclusive to red individuals). (A) Mutualism: If two niches overlap but both populations possess their own exclusive food source, the two populations will successfully coexist as nutrient competition will not occur. (B) Competitive exclusion: If two niches overlap and both populations use the same nutrients, competition will be unavoidable and one population will eventually displace the other by consuming the bulk of the available nutrients.

1.3 Cross-feeding

Natural environments are often less than ideal for the growth of bacteria. As such, it is of great importance for bacterial species to display flexibility in the acquisition and consumption of metabolites (Brückner and Titgemeyer, 2002; Stubbendieck, Vargas-Bautista and Straight, 2016). This is the case for carbon source macromolecules, which play a vital role in cellular ATP production and biosynthesis [Waschina et al., 2016; Peter Jurtshuk, 1996]. Bacterial species are typically capable of consuming multiple types of carbon sources, often presenting different preferences for each in a hierarchical fashion (Brückner and Titgemeyer, 2002; Aidelberg *et al.*, 2014; Kremling *et al.*, 2015; Wang *et al.*, 2019). For the vast majority of these species, glucose is the optimal carbon source for growth (Brückner and Titgemeyer, 2002; Kenyon *et al.*, 2005; Aidelberg *et al.*, 2014). However, glucose is in very short supply in many environments, so other potential sugars must be utilized instead. Furthermore, when consuming carbon macromolecules, it is common for bacteria to generate other less-optimal sugars as by-products, like lactate or TCA cycle intermediates. Once the original carbon source is depleted, the strain can then switch to the less-optimal by-product to maintain its growth, albeit at a slower rate (Brückner and Titgemeyer, 2002; Aidelberg *et al.*, 2014; Kremling *et al.*, 2015).

In accordance with the competitive exclusion principle, the degree of diversity in carbon source metabolization is important within the context of multi-species bacterial communities. Here, a greater variance in carbon utilization ensures that competition between strains is minimized, allowing for a higher level of coexistence and cooperation [Stubbendieck et al., 2016; Bauer et al., 2018; Watkins et al., 2016]. This flexibility can serve as an important pillar for cooperation through cross-feeding mechanisms. Cross-feeding can best be defined as the uni- or bi-directional exchange of metabolites, between two or more bacterial strains, conducive to enhanced growth [Braga et al., 2016; D'Souza et al., 2018]. It can be reciprocal or not, as the metabolites traded may simply be by-products of essential chemical processes or actively generated in order to sustain symbiotically dependent neighboring strains [D'Souza et al., 2018]. It is both an inter- and intra-species phenomenon, as a single strain can diverge into multiple strains with differing metabolic needs, forming chains of consumers and producers with each linking strain consuming the by-product(s) of the previous while supplying its own by-product(s) to the next [Waschina et al., 2016; D'Souza et al., 2018]. Cross-feeding may enrich IR, resulting in either greater benefits for the sensitive strain or in a mutualistic relationship where antimicrobial inactivating enzymes are exchanged for essential by-products. Indeed, cross-feeding is ubiquitous in both ecological and clinical settings having been found in a diverse range of microbiota, including the human gut [D'Souza et al., 2018]. In the human gut, several strains of bifidobacteria have been found to sustain eachother through the metabolization of glycans and the subsequent cross-feeding of the resulting subproducts [Turroni et al., 2016]. Similarly, many sulfate-reducing gut bacteria, such as *Desulfovibrio piger*, can obtain sulfate through cross-feeding with sulfatase-producing species like *Bacteriodes* [Rey et al., 2013].

Whether or not the metabolic interplay between neighboring bacterial populations plays a direct role in IR is still not fully determined. Its potential for such, however, should not be ignored.

1.4 Hypothesis

With IR involving two strains of the same species, sensitive strains have an overall competitive advantage over resistant ones by avoiding the need to maintain relevant resistance genes operational [Vogwill and Maclean, 2015; Dugatkin et al., 2005]. Owing to this, we might be tempted to evaluate the relationship between the two strains as invariably commensal, with the sensitive strain as the ultimate beneficiary. This, however, may not always be the case. Assuming that the resources essential to the growth of both sensitive and resistant strains are finite, it is likely that, by the time the surrounding medium has been sufficiently detoxified, these resources will have already been depleted by the resistant strain. In this case, we see that the relationship has evolved from commensal to competitive, with the resistant strain as the only victor. Therefore, it is likely that resource competition is at the heart of the discrepancy between the incidence of inter-species and intra-species IR.

Being the primary limiting nutrient for bacterial growth, we expect that carbon source competition is at the core of IR development. Specifically, if the resistant and sensitive strains belong to the same species, carbon source competition and subsequent inhibition of IR may occur. Alternatively, different preferences in carbon utilization may significantly potentiate the occurrence of IR between two or more bacteria strains. Following this rationale, in situations where the sensitive and resistant strains belong to the same species (intra-species), we predict that competition for the carbon source will arise, thereby impeding the growth of the sensitive strain (Fig. 1.2A). When the strains belong to different species (inter-species), it is expected that competition will be absent when each strain has their own exclusive carbon source available (Fig. 1.2B). As a consequence, both strains will coexist, with the resistant strain successfully protecting the sensitive one. However, even if the strains belong to different species, competition may still occur if the only source of carbon available is common to both (as is the case with glucose). Therefore, in this case, IR will be significantly hindered. In this project, we set out to demonstrate if the carbon source can be a powerful moderator of IR, acting as both inhibitor and enabler, depending on whether resource competition occurs.

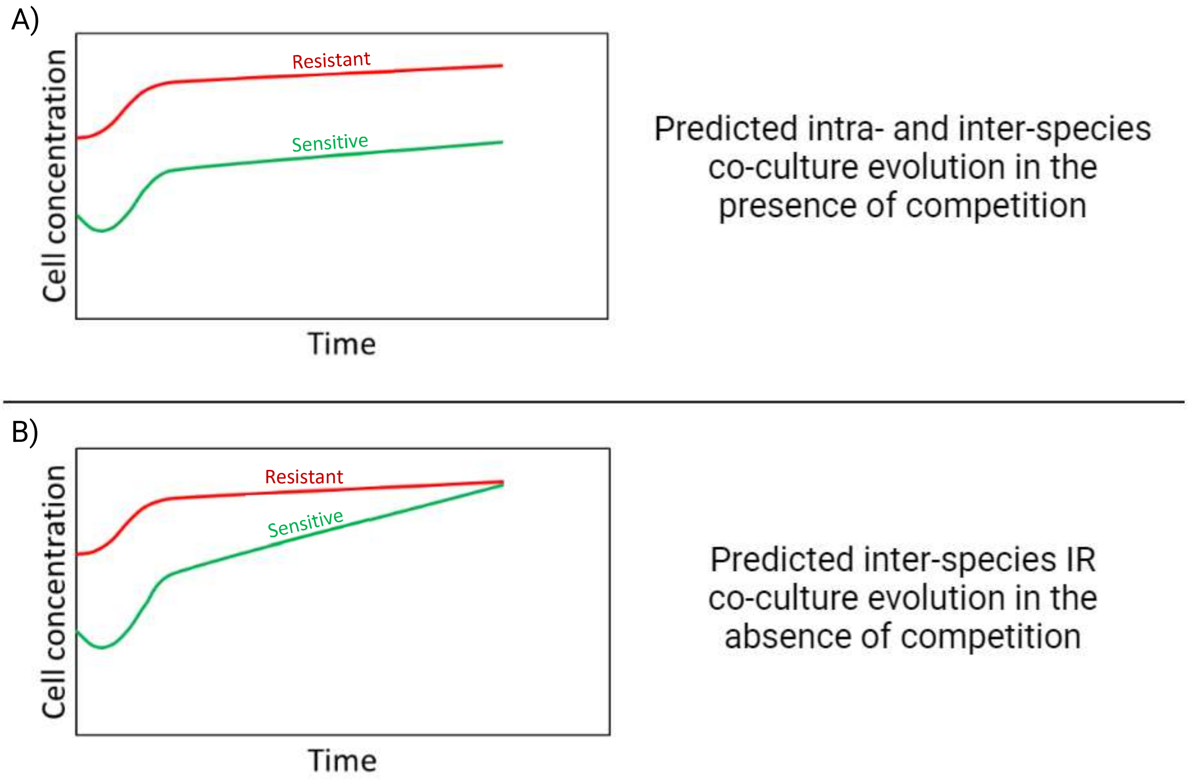

Figure 1.2 Models for co-culture progression in the context of metabolic competition. (A) When strains are in direct resource competition, resistant strains will have a temporal advantage over the sensitive strains. As the media is slowly detoxified, resistant strains will have exclusive access to the available resources, diminishing the amount left available for the sensitive strains once the surrounding media has been sufficiently detoxified. (B) When there is no resource competition between strains, resistant strains will consume their respective nutrient source while detoxifying the media. Once enough antibiotic molecules have been nullified, sensitive strains will still have a considerable resource reservoir available to them, allowing them to proliferate and achieve a final cell concentration comparable to that of the resistant strains. The red and green curves denote the resistant and sensitive strains, respectively, as indicated in the figure. Note: Co-culture progression estimations were made along five time-points (0h, 2.5h, 5h, 7h, and 24h).

1.5 The project

Given the possibility of IR to be hindered by carbon source competition, we sought to juxtapose how intra and inter-species IR develops in defined minimal media supplemented with “competing” and “non-competing” carbon sources. For that, we performed competition assays involving two different species: *Escherichia coli*, a common human gut commensal bacterium, and *Salmonella enterica* a pathogenic bacterium that can colonize the human gut, causing gastroenteritis [Blount, 2015; Cherubin et al., 1969; Ahmer and Gunn, 2011]. For each species, we had a resistant and an ampicillin-sensitive strain, yielding a total of four strains. Resistance was conferred by the natural RP4 plasmid that codes for the ampicillin (Amp) detoxifying enzyme β-lactamase [Wright, 2005]. With these four strains, we performed multiple co-cultures using lactose, citrate, glucose or a combination of lactose and citrate as the carbon sources. Whilst glucose can be used by both species, only *E. coli* is presumed to be able to metabolize lactose, and the same applies to *S. enterica* and citrate [Aidelberg et al., 2014; Kenyon et al., 2005; Reynolds and Silver, 1983; Loomis and Magasanik, 1967; Leonard et al., 2015; Santillán and Mackey, 2008; Brocker et al., 2009]. Eventual transconjugants that may have arisen during the co-cultures were also considered. Our results show that carbon competition is a powerful modulator of IR, although other factors such as glycogen reserves, iron availability and/or by-product formation, may be at stake.

2. Materials and methods

2.1 Strains

This study used two bacterial species, the *Escherichia coli* K12 MG1655 and the *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028. This thesis's fundamental experiments consist of co-culturing a β-lactamase-producing strain with a non-producer strain. However, to facilitate the manipulation of these strains in mixed cultures, we started by isolating two spontaneous mutants for each species, one resistant to the antibiotic nalidixic acid (henceforth denoted as NalR) and another to the antibiotic rifampicin (RifR). We then introduced the RP4 plasmid (that encodes for a β-lactamase, conferring ampicillin-resistance, henceforth denoted as AmpR) into the NalR strains. Ultimately, we obtained four strains: *E. coli* NalR(RP4). *E. coli* RifR, *S. enterica* NalR(RP4) and *S. enterica* RifR. The strains and their respective characteristics can be reviewed in Table 2.1.

Briefly, the isolation of spontaneous NalR and RifR mutants was performed by plating 100 µL of an overnight (ON) culture of *E. coli* or *S. enterica* in LA plates, either supplemented with nalidixic acid (Nal) or rifampicin (Rif), and incubated for 24 h at 37ºC - the colonies grown in the presence of Nal and Rif resulted from resistant clones. These clones were isolated by streaking one colony in a new LA plate supplemented with the respective antibiotic, and incubated at 37ºC for 24 h. We repeated this last procedure. The isolated NalR and RifR clones were then stored in 50% glycerol stock at -20º.

As mentioned above, the Amp-resistant strains were obtained by transforming NalR clones with the RP4 plasmid. Briefly, a conjugation protocol was performed between a lab's Nal-sensitive RP4 donor strain and the NalR clones (see protocol details below). The isolated NalR AmpR clones were stored in 50% glycerol stock at -20ºC.

Table 2.1 All four strains utilized throughout the study, their preferred carbon sources and the presence/absence of resistance to ampicillin.

Strains	**Glucose**	**Lactose**	**Citrate**	**Ampicillin resistance**
E. coli RifR	+	+	−	−
E. coli NalR RP4	+	+	−	+
S. enterica RifR	+	−	+	−
S. enterica NalR RP4	+	−	+	+

2.2 Plasmid

The natural plasmid RP4 (also known as RK2) was chosen since its a a well-studied plasmid in the context of gene transfer and IR to Amp [Adamczyk and Jagura-Burdzy, 2003]. Particularly, the RP4 plasmid belongs to the IncP-116 incompatibility group and has the genes for resistance to kanamycin (km^{r}), tetracycline (tc^{r}) and ampicillin (*ampC),* which encodes for a β-lactamase [Adamczyk and Jagura-Burdzy, 2003; Soda et al., 2008]. The latter is responsible for the resistance to β-lactams, such as Amp. Briefly, the β-lactamase produced accumulates in the periplasmic space and is released in the environment [Georgiou et al., 1988]. If Amp is present, the β-lactamase will promote the nucleophilic attack of the β-lactam ring, leading to the hydrolysis of Amp and, consequently, detoxification of the surrounding environment/media [Wright, 2005].

2.3 Plasmid transfer

The *E. coli* and *S. enterica* NalR strains resistant to Amp were obtained through conjugation with an in-house *E. coli* Ara^{-} ValR RP4 donor strain. Briefly, the conjugation was performed by incubating 100 µL of the donor and recipient (*E. coli* or *S. enterica* NalR) strains from an ON culture, in 10 mL of Luria broth media (LB) at 37ºC without agitation in a 50 mL Falcon tube. The conjugation was allowed to occur during 5 hours, after which the Falcon tubes were vigorously vortexed for at least 2-3 minutes, to break the conjugation bridges. The conjugation mix was diluted 100x in LB, plated in LA supplemented with Nal and Amp and incubated for 24 hours at 37ºC to select for transconjugants. The colonies obtained were isolated in new plates of LA with Nal and Amp, and stored at -20ºC in 50% glycerol.

2.4 Media and carbon sources

LB media was prepared according to the manufacturer's instructions (Invitrogen, Waltham, USA). LA media was obtained by adding 15 mg of agar (Merck, Darmstadt, Germany) for 1 mL of LB media. The M9 minimal media was prepared by mixing 100 mL of 5x base salts (BD, Eisyns, Switzerland); 5 mL of trace elements (consisting in 0.63 mM of $ZnSO_4$ (Merck), 0.7 mM of $CuCl_2$ (Merck), 0.71 mM of $MnSO_4$ (Merck) and 0.76 mM of $CoCl_2$ (Merck)); 0.5 mL of 0.1 M $CaCl_2$ (Merck); 0.5 mL of 1 M $MgSO_4$ (Merck); 0.3 mL of 0.1 M $FeCl_3$ hexahydrate (Merck); 1 mL of 1.4 mM Thiamine-HCl (Merck); 50 mL of carbon source solution and the addition of milliQ H2O for a final volume of 500 mL. A total of three sugars were used as carbon sources, namely lactose (50 gL^{-1}) (Merck), sodium citrate (20 gL^{-1}) (PanReac, Spain), and glucose (50 gL^{-1}) (Merck), alone or in different combinations: lactose; citrate, glucose and lactose + citrate. All the solutions were sterile-filtered or autoclaved before mixing. When needed, the media was supplemented with the respective antibiotics at defined concentrations, namely: 100 µg/ml of ampicillin (Merck); 100 µg/mL of rifampicin (Merck) and/or 40 µg/mL of nalidixic acid.

2.5 Antibiotics

The antibiotics used throughout this work were prepared at defined concentrations, filtered with 0.22 µm filters (except rifampicin) and stored in stock solutions at -20ºC. Namely, Rifampicin (Rif) was prepared in methanol 100% at a concentration of 20 mg/mL; Nalidixic acid (Nal) was prepared in 1 M NaOH at 100 mg/mL; Kanamicin (Kan) and Amp were prepared in milliQ water at 100 mg/mL, respectively.

2.6 Competition assays

Prior to the competitions, it was necessary to determine the adequate inoculation/plating dilutions for each strain. For this, all four strains were grown in overnight (ON) cultures on M9 + glucose at 37º C and 220 rpm. Afterwards, strains were plated into the appropriate media with several dilutions. We concluded that all strains showed a maximum ON growth of $1x10^9$ CFUs/mL. Thus, a preceding $1x10^{-2}$ dilution of the sensitive strains was deemed sufficient to ensure a desired ratio of 1:99 when co-cultured with the resistant strains. Throughout the project, all necessary dilutions were made in eppendorfs containing 0.01 M $MgSO_4$ (Merck).

For the competition assays, the Amp-resistant and Amp-sensitive strains, previously grown ON in M9 with glucose, were inoculated in 6-well plates containing 5.7 mL of M9 supplemented with the competitive or non-competitive carbon sources, and in the presence of Amp. Specifically, 57 µL of the resistant strain and 57 µL of a previously 100x diluted sensitive strain, were inoculated to a final concentration of $1x10^7$ CFUs/mL and $1x10^5$ CFUs/mL, respectively. Cultures were incubated at 37ºC at 130 rpm during 24 hours. At defined time points (0 h, 2.5 h, 5 h, 7 h and 24 h) co-culture samples were withdrawn and, after properly diluted, plated in LA with rifampicin, to select for the Amp-sensitive strain, LA with Amp and Nal to select for the Amp-resistant strain and with Rif and Amp to account for any transconjugants arisen during the competition. A schematic representation of the competition assay protocol is depicted in Figure 2.1.

2.7 Statistical analysis

Competition assays were made in replicates of three. In sections 3.4 and 3.5 of the Results section, final co-culture curves were constructed using the means of the values obtained in each replicate at the various time-points. When applicable, concentration values are given as the mean ± the standard deviation. To calculate the amplification factors used in Figs. 3.8 and 3.9, the formula shown in equation 2.1 was used. The final and initial concentrations correspond to the values of CFUs/mL registered at the 24h and 0h time-points, respectively. Mean differences were performed with the Student t-test in Microsoft Excel. Differences were considered significant when $p < 0.05$.

Equation 2.1:

$$Amplification\ factor = Log_{10}(\frac{Final\ concentration}{Initial\ concentration})$$

2.8 Figures

All illustrations displayed in this work are original creations made with Biorender (biorender.com).

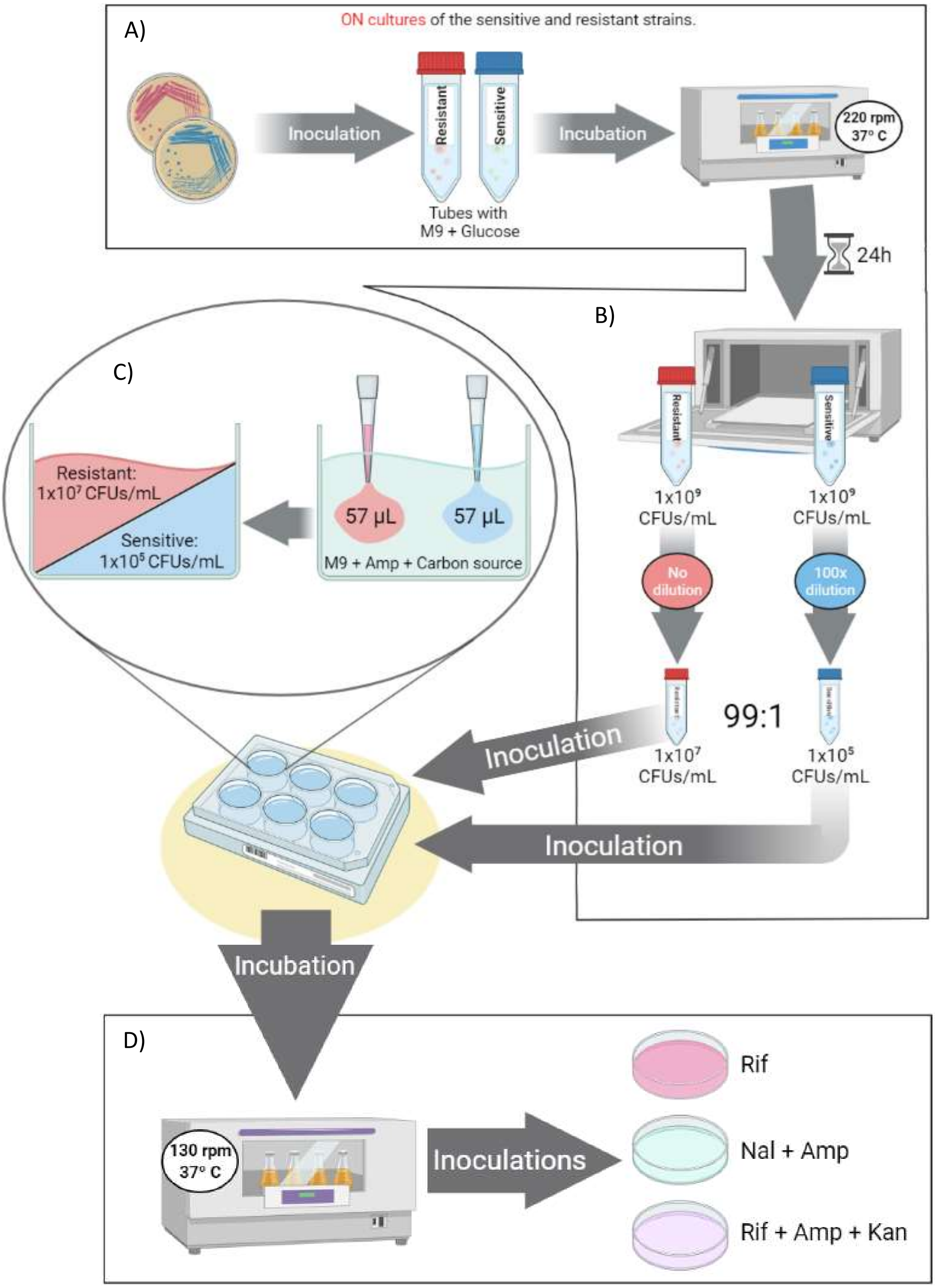

Figure 2.1 General project outline. (A) Strains are inoculated from stored plates into falcons containing M9 minimal media supplemented with glucose. These are then placed in an orbital incubator at 220 rpm and 37ºC for overnight culturing. (B) After 24 hours, sensitive ON cultures will be diluted by a factor of 100. This is done in order to assure that resistant strains will begin each co-culture with a 99:1 proportion relative to the sensitive strains. (C) Strains are then inoculated into a six-well plate. Each well will contain 5.7 mL of M9 minimal media complemented with a predetermined carbon source or combination of carbon sources. 57 µL of a resistant and a sensitive strain will be inoculated into every well, leaving each with an initial concentration of 1x10^7 CFUs/mL for the resistant strain and 1x10^5 CFUs/mL for the sensitive. (D) The six-well plate is then placed in an orbital incubator at 130 rpm and 37ºC. From then on, the plate will be removed periodically at specific time-points (0h, 2.5h, 5h, 7h and 24h) to make the necessary petri dish inoculations and assess co-culture progression.

3. Results

3.1 Mono-culture growth controls of *E. coli* and *S. enterica* resistant strains in non-competitive carbon sources

To evaluate the growth of ampicillin-resistant *E. coli* and *S. enterica* strains, we performed monocultures in non-competitive carbon sources (Fig. 3.1). Namely, *E. coli* was grown in the presence of lactose and *S.enterica* in citrate. As a negative control, we inoculated *E. coli* in M9 supplemented with citrate and *S. enterica* in M9 supplemented with lactose. We observed that both *E. coli* and *S. enterica* respectively grew from 9.45x10^5 and 5.3x10^5 CFUs/mL to their final 1.14x10^9 and 7.33x10^8 CFUs/mL in their optimal non-competitive carbon sources, lactose and citrate. (Fig. 3.1B and C). In any case, both strains achieved approximately 10 generations. Of notice, *S. enterica* was able to grow in the presence of lactose, however only reaching only 6.7x10^7 CFUs/mL (6 generations) (Fig. 3.1D). Nevertheless, *E. coli* was unable to grow in its non-metabolizable carbon source, citrate, remaining viable in the 24 hours culture time (Fig. 3.1A). Since lactose should not be metabolized by *S. enterica*, we performed further analysis to address what could be supporting its growth.

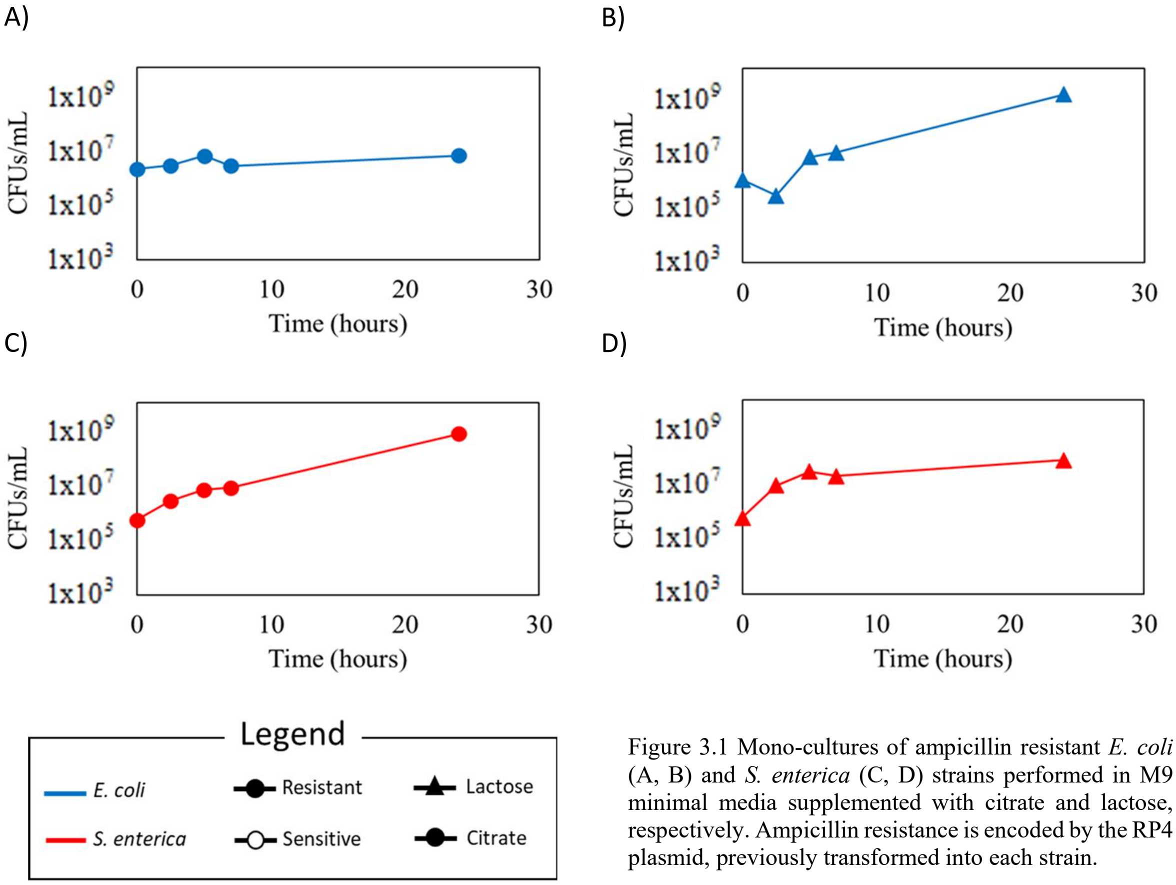

Figure 3.1 Mono-cultures of ampicillin resistant *E. coli* (A, B) and *S. enterica* (C, D) strains performed in M9 minimal media supplemented with citrate and lactose, respectively. Ampicillin resistance is encoded by the RP4 plasmid, previously transformed into each strain.

3.2 Co-cultures of *S. enterica* and *E. coli* show potential cross-feeding interactions

To further address if *S. enterica*'s growth in lactose is supported by metabolic by-products of coexisting bacteria (i.e. cross-feeding), resistant *E. coli* and *S. enterica* were co-cultured with sensitive strains of the opposing species in M9 medium, supplemented with citrate for the resistant *E. coli,* and lactose for the resistant *S. enterica* (Fig. 3.2). Similar to what we observed in mono-cultures, *S. enterica* was able to grow in the presence of lactose when in co-culture with sensitive *E. coli* (compare Fig. 3.2A with Fig. 3.1D). Specifically, both in monoculture and co-culture, *S. enterica* completed more than six generations (6.4 generations in monoculture and 6.9 in co-culture). As expected, when in co-culture, *E. coli* completed many more generations, well over 14 (Fig. 3.2A).

Surprisingly, when co-cultured with the sensitive *S. enterica*, the resistant *E. coli* performed 2.8 generations on citrate. This growth contrasts with the results obtained in mono-culture, where *E. coli* was unable to grow (Fig. 3.2B and Fig. 3.1A). We can postulate that this unexpected growth is not due to citrate metabolization but cross-feeding interactions with the sensitive *S. enterica*. These interactions, however, appear not to be reciprocal, as *S. enterica*'s growth on lactose shows little divergence between mono- and co-cultures (Fig. 3.2B and Fig 3.1D). As expected, the sensitive *S. enterica* completed approximately nine generations in citrate, one of its consumable carbon sources.

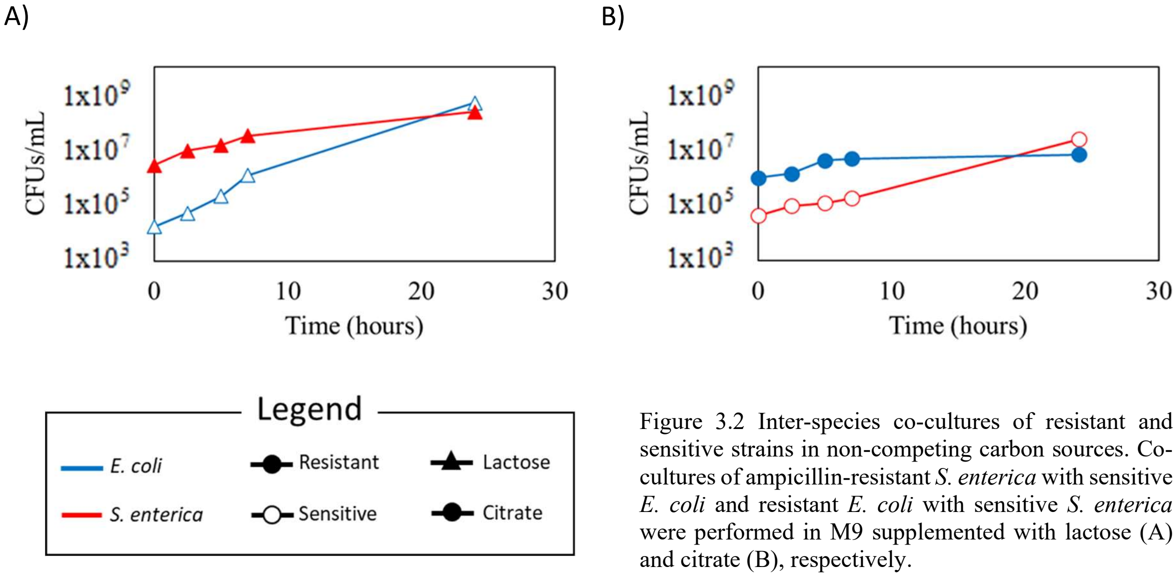

Figure 3.2 Inter-species co-cultures of resistant and sensitive strains in non-competing carbon sources. Co-cultures of ampicillin-resistant *S. enterica* with sensitive *E. coli* and resistant *E. coli* with sensitive *S. enterica* were performed in M9 supplemented with lactose (A) and citrate (B), respectively.

3.3 *S. enterica*'s growth on media without carbon source reveals potential carbon storage mechanisms

The expectation was that the *S. enterica* strain should not be able to metabolize lactose. Therefore, we evaluated if M9 media components could be supporting its growth. With this aim, we performed mono- and co-cultures with the resistant *S. enterica* and sensitive *E. coli* strains in M9 without any carbon source. *S. enterica* growth was observed in both mono- and co-cultures. Specifically, when in mono-culture, *S. enterica*'s CFU concentration increased from 4.96×10^6 to 4.19×10^7 CFUs/mL, achieving three generations in the process (Fig. 3.3A). When in co-culture, *S. enterica* and *E. coli* exhibited non-negligible growth, with both strains reaching approximately three generations (Fig. 3.3B).

Contrary to standard practice, our M9 medium was complemented with iron. According to the literature, iron has been pointed as a potentiator of the proliferation and virulence of various pathogenic enteric bacteria, such as *S. enterica* [Kortman et al., 2012]. In order to measure the potential effect of iron in its lactose-independent proliferation, we performed mono- and co-cultures involving resistant *S. enterica* and sensitive *E. coli* in M9 media deprived of both iron and carbon source. *S. enterica* was once again able to complete roughly three generations, thereby, showing nearly identical results as when grown in the presence of iron (Fig. 3.3C). The co-cultures showed small differences, namely, both strains performed slightly less generations when compared to the iron supplemented media. Here, *S. enterica* proliferated through only one complete generation while *E. coli* underwent two generations. As such, *S. enterica* and *E. coli* experienced a respective growth reduction of 74% and 59% in the absence of iron (compare Fig. 3.3B and D).

It is now apparent that *S. enterica*'s previous proliferation on lactose was not due to the sugar itself but *S. enterica*'s own intrinsic properties (compare Fig. 3.3A and C with Fig. 3.1D). Indeed, despite not as elevated as in the presence of citrate (10 generations), *S. enterica* is capable of growing in the total absence of both carbon source and iron (approximately three generations in both cases).

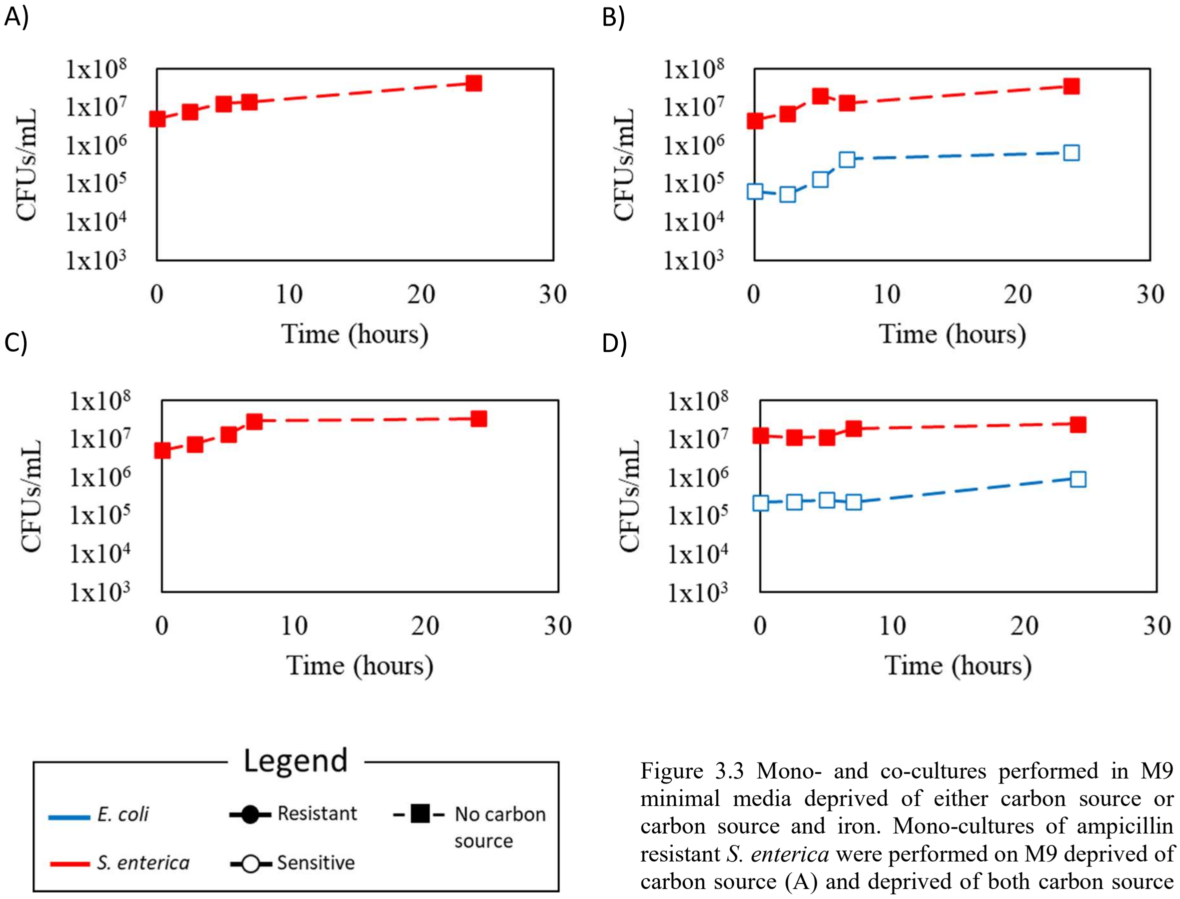

Figure 3.3 Mono- and co-cultures performed in M9 minimal media deprived of either carbon source or carbon source and iron. Mono-cultures of ampicillin resistant *S. enterica* were performed on M9 deprived of carbon source (A) and deprived of both carbon source and iron (C). Co-cultures with the sensitive *E. coli* were also made in carbon source deprived M9 (B) and M9 deprived of both carbon source and iron (D).

3.4 Intra-species competitions on competitive and non-competitive carbon sources

Intra-species competitions were performed in M9 media supplemented with either lactose or citrate. Resistant and sensitive *E. coli* were co-cultured with their competitive carbon source, lactose, as well as with citrate. Similarly, *S. enterica* strains were co-cultured in either citrate, their competitive carbon source, or in lactose, which they cannot metabolize.

3.4.1 Intra-species co-cultures on competitive carbon sources

On lactose, the resistant *E. coli* population completed nine generations, having reached a final concentration of (9.64 ± 6.8)x10^8 CFUs/mL, while the sensitive *E. coli* grew by ten. However the sensitive strain only reached (5.02 ± 1.71)x10^7 CFUs/mL, presumably because the resistant strain had already consumed most of the lactose (Fig. 3.4A). In the case of the intra-species competition involving *S. enterica* (Fig. 3.4B), the final density of the resistant strain is akin to the one reached when grown alone (Fig. 3.1C), while the final concentration of the sensitive strain was nearly ten-fold lower. Presumably, this difference was caused by the weak growth of the sensitive strain in the first hours while the medium is still toxic, allowing the resistant cells to monopolize the available citrate.

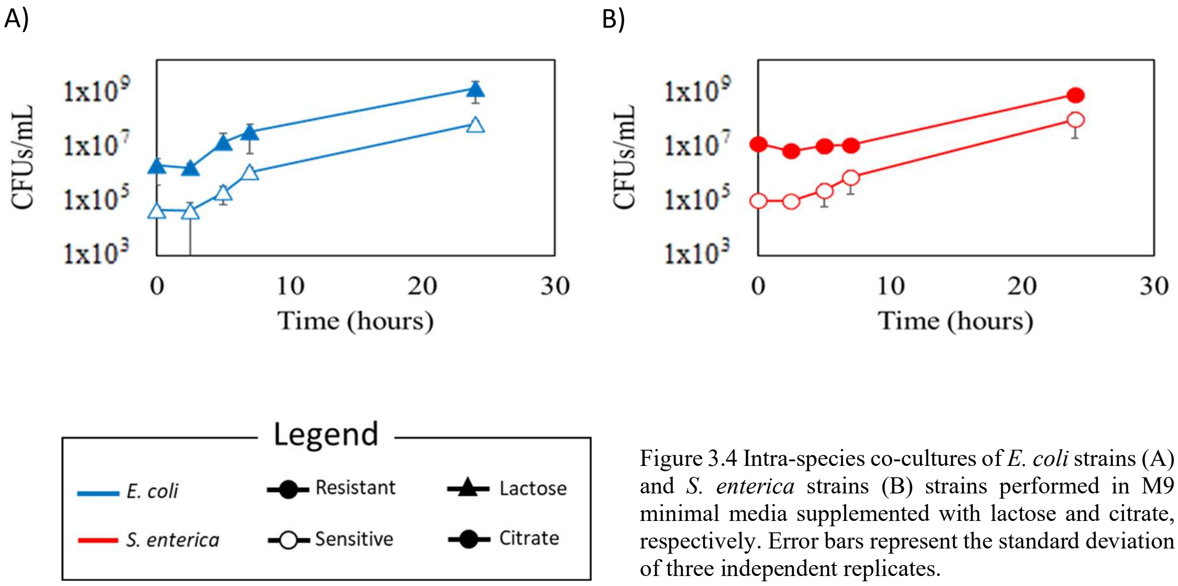

Figure 3.4 Intra-species co-cultures of *E. coli* strains (A) and *S. enterica* strains (B) strains performed in M9 minimal media supplemented with lactose and citrate, respectively. Error bars represent the standard deviation of three independent replicates.

3.4.2 Intra-species co-cultures on non-competitive non-optimal carbon sources

As seen in the previous subsections, *E. coli* strains showed no measurable proliferation on citrate (Figure 3.5A). On the other hand, both *S. enterica* strains showed some increase on lactose, with both the resistant and sensitive strains attaining approximately 3 generations. Nevertheless, their final densities in lactose were much lower than the ones observed when grown in citrate (compare Fig. 3.5B with Fig. 3.1C).

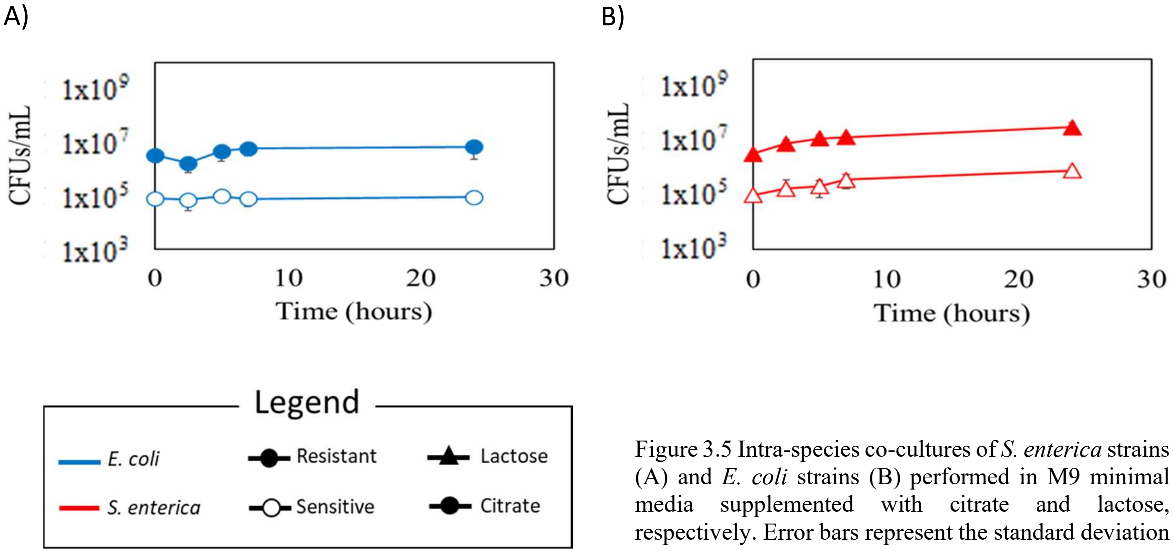

Figure 3.5 Intra-species co-cultures of *S. enterica* strains (A) and *E. coli* strains (B) performed in M9 minimal media supplemented with citrate and lactose, respectively. Error bars represent the standard deviation of three independent replicates.

3.5 Inter-species competitions on competitive and non-competitive carbon sources

Co-cultures were made with resistant and sensitive strains belonging to two different species (*E. coli* and *S. enterica*, respectively). The two strains compete for glucose. Individually, lactose and citrate are non-consumable sugars for the *S. enterica* and *E. coli* strains, respectively. Therefore, there is no competition for carbon sources if the medium contains both lactose and citrate (and no other sugar).

3.5.1 Inter-species competitions on competitive carbon sources

E. coli and *S. enterica* compete for carbon sources in media supplemented with glucose and no other carbon source. In the case of the resistant *E. coli* competing with the sensitive *S. enterica* (Fig. 3.5A), the density of the *E. coli* population unexpectedly decreased in the first time interval. We do not know why such a decrease occurred; however, because of this decrease, the cell densities of *E. coli* and *S. enterica* became fairly similar at the second time point. In the following hours, cell densities were practically identical at all time points, presumably because the medium was already non-toxic. When *S. enterica* was the resistant strain (Fig. 3.5B), both strains completed close to 8 generations, with *S. enterica* reaching $(1.06 \pm 0.72)\text{x}10^9$ CFUs/mL and the sensitive *E. coli* only reaching $(1.19 \pm 0.87)\text{x}10^7$ CFUs/mL. In this experiment, competition for glucose was more evident. While *S. enterica*'s final concentration was equitable to when mono-cultured in citrate, the sensitive *E. coli* ceased its growth after reaching a concentration of only $(1.19 \pm 0.87)\text{x}10^7$ CFUs/mL, almost a hundred times lower than the one achieved in lactose mono-culture (Fig. 3.1B).

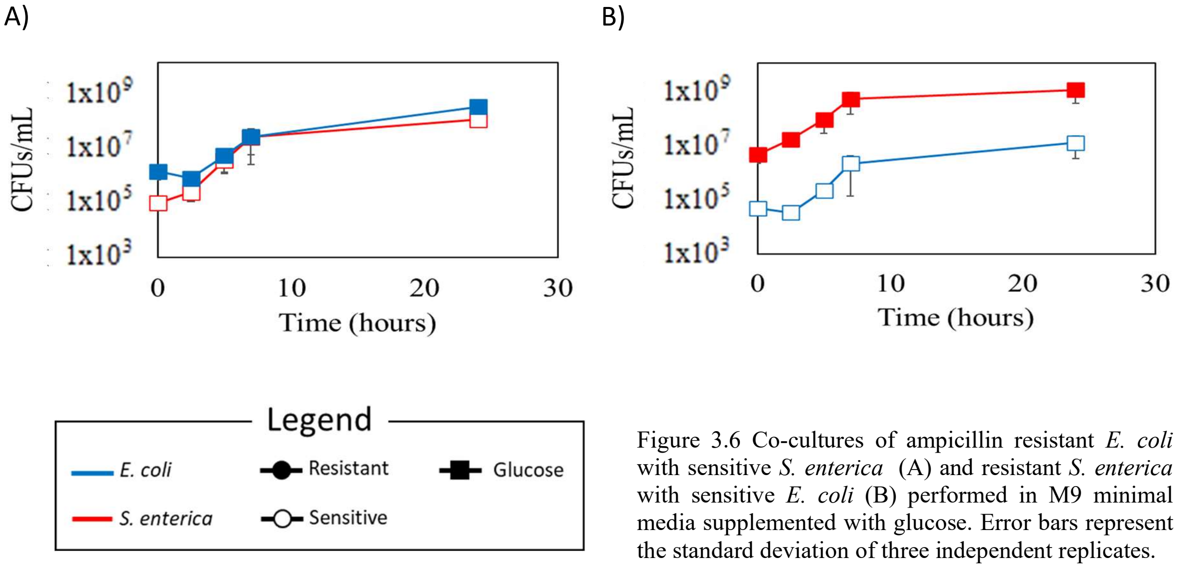

Figure 3.6 Co-cultures of ampicillin resistant *E. coli* with sensitive *S. enterica* (A) and resistant *S. enterica* with sensitive *E. coli* (B) performed in M9 minimal media supplemented with glucose. Error bars represent the standard deviation of three independent replicates.

3.5.2 Inter-species competitions on non-competitive carbon sources

The selected non-competitive carbon sources were lactose (in monocultures of *E. coli*) and citrate (in monocultures of *S. enterica*) as well as the lactose + citrate combination in co-cultures. On lactose + citrate, both resistant *E. coli* and sensitive *S. enterica* showed considerable growth, having each completed 9 and 10 generations, respectively (Fig. 3.6A). When the resistant *S. enterica* was co-cultured with the sensitive *E. coli*, similar results were obtained as *S. enterica* achieved 8 generations while *E. coli* reached 13 (Fig. 3.6B). Of note, while initially, the resistant strain began 93x more abundant than the sensitive, by the end this difference had decreased by 98%. This result supports our assumption that competition for carbon sources between the two species growing in lactose + citrate is non-existant.

In lactose supplemented media, both the resistant *E. coli* and the sensitive *S. enterica* experienced considerable growth (Fig. 3.6C). Here, *E. coli* grew to $(9.62 \pm 9.07)\times10^{8}$ CFUs/mL and *S. enterica* to $(1.71 \pm 0.47)\times10^{8}$ CFUs/mL. When the resistant *S. enterica* was co-cultured with the sensitive *E. coli* (Fig. 3.6D), we found similar results. Both strains exhibited significant proliferation and obtained near identical final concentrations, with *S. enterica* completing 6 generations and *E. coli* almost 13.

In citrate, however, co-culture growth showed remarkable differences, with the resistant *S. enterica* reaching $(5.5 \pm 0.58)\times10^{8}$ CFUs/mL (6 generations) and the sensitive *E. coli* only reaching $(7.9 \pm 3)\times10^{6}$ CFUs/mL (7 generations). Thus, *S. enterica* was able to maintain an advantage over *E. coli* throughout the co-culture (Fig. 3.6F). When the resistant strain was *E. coli*, *S. enterica* showed remarkable growth reaching $(2.78 \pm 1.09)\times10^{8}$ CFUs/mL after completing 11 generations. By contrast, *E. coli* did not display any measurable increment at its final concentration, although proliferation most likely took place at several points, as proven by the media's resulting detoxification (Fig. 3.6 E). This limited proliferation would have potentially been the product of cross-feeding with the sensitive *S. enterica*.

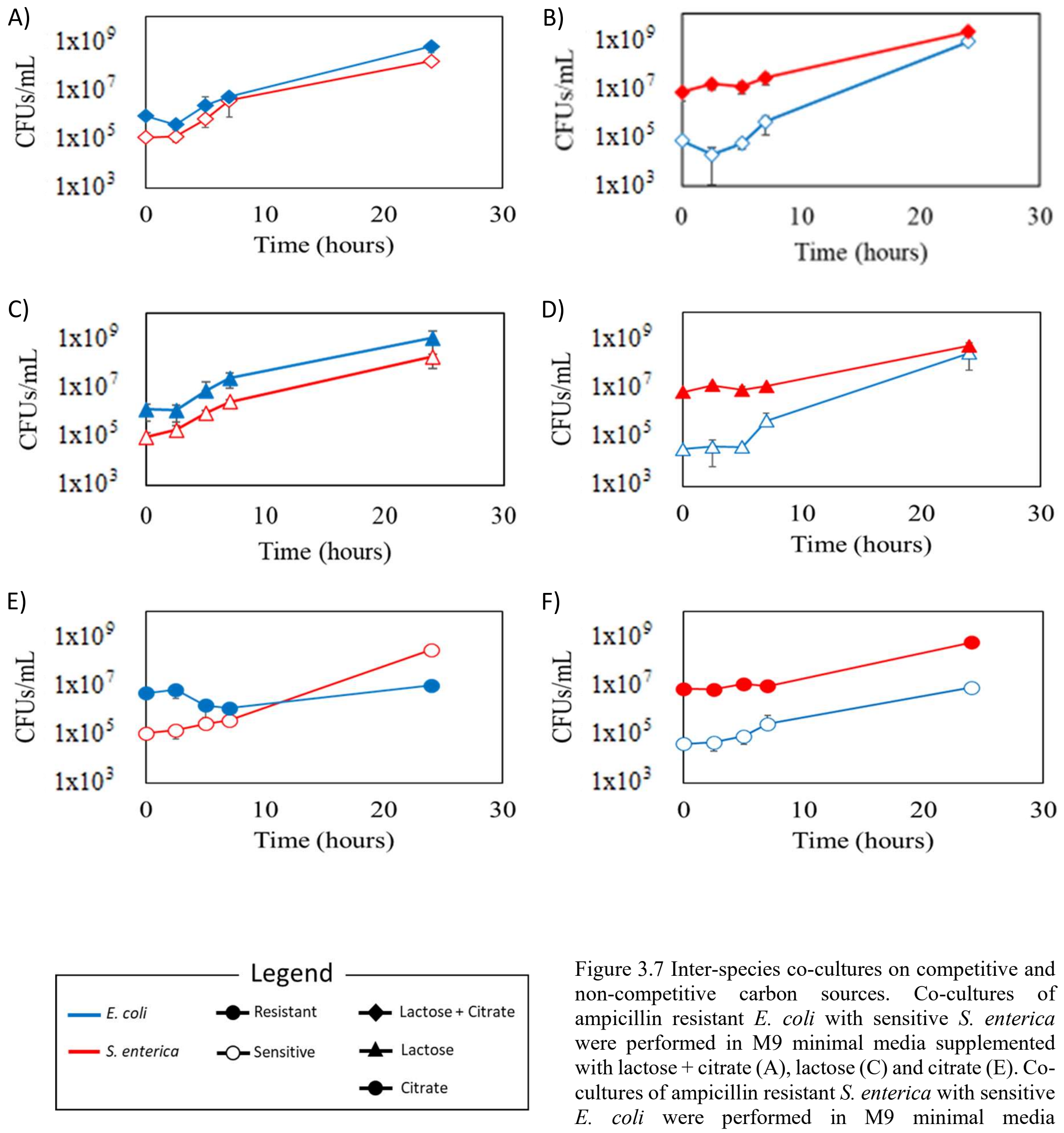

Figure 3.7 Inter-species co-cultures on competitive and non-competitive carbon sources. Co-cultures of ampicillin resistant *E. coli* with sensitive *S. enterica* were performed in M9 minimal media supplemented with lactose + citrate (A), lactose (C) and citrate (E). Co-cultures of ampicillin resistant *S. enterica* with sensitive *E. coli* were performed in M9 minimal media supplemented with lactose + citrate (B), lactose (D) and citrate (F). Error bars represent the standard deviation of three independent replicates.

3.6 Inter- and intra-species competition data analysis

For each strain, amplification factors were calculated using the formula displayed in equation 2.1 (See Methods section). Amplification factors were then compared between different co-cultures in order to quantify the degree to which competition, or lack thereof, affected co-culture progression. Statistical significance was assessed through Student’s t-tests.

3.6.1 Inter-species: competitive vs non-competitive

Inter-species co-cultures were compared between competitive (glucose) and non-competitive (lactose + citrate) carbon sources (Fig. 3.8). While resistant strains displayed fairly similar ranges of amplification factors, the sensitive strains showed more varied results. Namely, sensitive *S. enterica* revealed little difference in proliferative success when co-cultured with *E. coli*, regardless of whether competition occurred or not. In contrast, sensitive *E. coli* had a significantly higher amplification factor when not in direct competition with the resistant *S. enterica*, compared to when competition took place. As previously noted, the fact that the resistant *E. coli* had a sharp decrease in cell concentration in the beginning of several co-cultures with *S. enterica* may explain why it was unable to exhaust the carbon source prior to media detoxification.

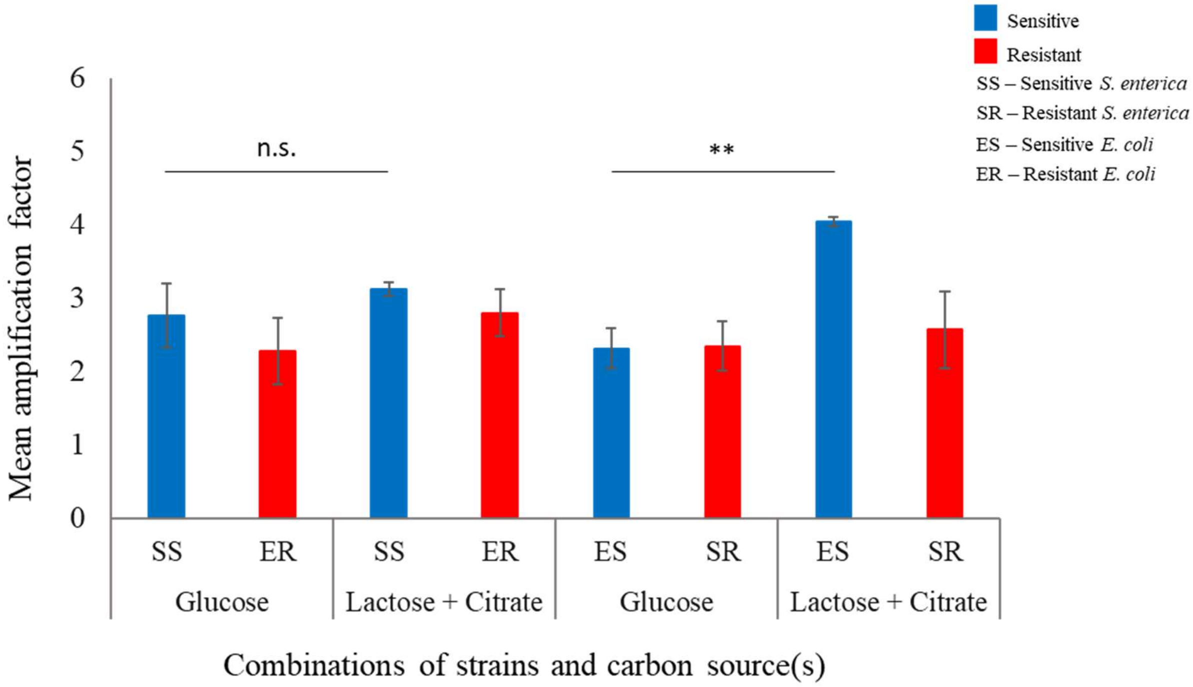

Figure. 3.8 Comparison of the amplification factors of strains grown in inter-species co-cultures in M9 media supplemented with competing and non-competing carbon sources. Statistical analysis was made with Student's t-tests with a 95% confidence interval. A significant difference (p-value = 0.009) was found between the sensitive *E. coli* grown in glucose and the sensitive *E. coli* grown in lactose + citrate. Error bars represent the standard deviation of three independent replicates.

3.6.2 Competitive intra-species vs non-competitive inter-species

As previously mentioned, intra-species co-cultures were expected to show competition between the sensitive and resistant strains. As such, comparisons were drawn between the sensitive strains' amplification factors in intra-species co-cultures and the amplification factors obtained in non-competitive inter-species (Fig. 3.9). Here, we identified that sensitive *E. coli* had significantly less proliferation when in competition with the resistant (lactose) than when growing in a non-competitive media (lactose + citrate). Unlike *E. coli*'s case, the difference of the growth of sensitive *S. enterica* between the occurrence (citrate) and the non-occurrence (lactose + citrate) of competition was not statiscally significant (p>0.05). In part this is due to the sheer variance associated with *S. enterica*'s amplification factors in intra-species co-culture. Potentially, this could have been also been the result of anticipated media detoxification, although reasons for this are yet to be determined.

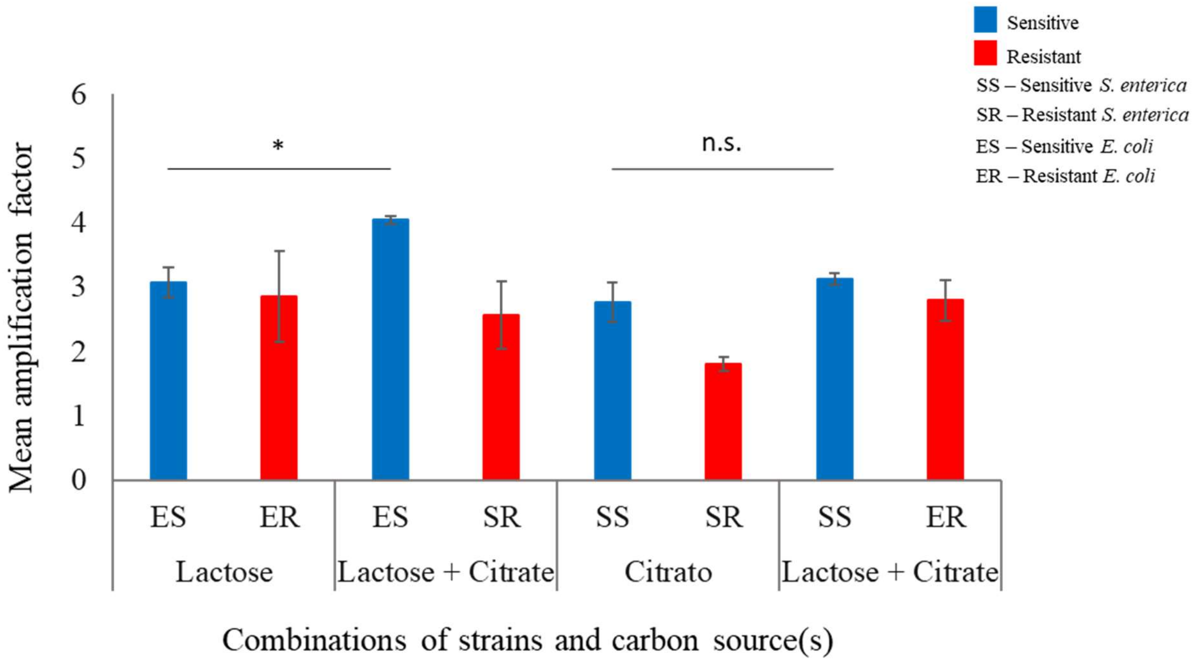

Figure. 3.9 Comparison of the amplification factors of strains grown in intra- and inter-species co-cultures in M9 media supplemented with competing and non-competing carbon sources. Statistical analysis was made with Student's t-tests with a 95% confidence interval. A significant difference (p-value = 0.02) was found between the sensitive *E. coli* grown in lactose and the sensitive *E. coli* grown in lactose + citrate. Error bars represent the standard deviation of three independent replicates.

4. Discussion

This work aims at disclosing if resource competition modulates indirect resistance (IR). Specifically, we hypothised that if carbon source competition occurs, intra-species IR is unlikely to take place. In a nutrient-limited environment, a sensitive strain would struggle to compete with the resistant as the latter would have early access to nearby carbon prior to the complete detoxification of the media. Thus, resistant strains, despite enduring fitness costs to maintain resistance genes functional [Vogwill and Maclean, 2015; Dugatkin et al., 2005], would still have an evolutionary advantage over a sensitive strain occupying the same metabolic niche. On the other hand, if coexisting strains metabolize different carbon sources, bacteria can successfully coexist and partake in the sharing of "public goods" such as antibiotic-inactivating enzymes [Nicoloff H. and Andersson, 2016b; Özkaya et al., 2017]. In this regard, inter-species IR is possible, and can even be facilitated if cross-feeding mechanisms exist. Indeed, under the scope of indirect pathogenicity, both cross-feeding and IR may serve as critical mechanisms in the development of complex inter-populational relationships between pathogenic and non-pathogenic strains.

To address the impact of carbon source competition in IR, we performed several co-cultures with resistant and sensitive strains belonging to *Escherichia coli* or *Salmonella enterica* species. Lactose and citrate were the carbon sources selected for *E. coli*'s and *S. enterica*'s growth, respectively. Citrate is readily metabolized by *S. enterica* as it possesses the citrate TctABC inducible transporter system. Of note, *E. coli* is also capable of citrate incorporation through its CitT transporter, however, only in strictly anaerobic conditionsis is this transporter active. Therefore, since we performed aerobic cultures, *E. coli* cannot metabolize citrate [Brocker et al., 2009]. On the other hand, *E. coli* metabolizes lactose as it possesses a lac operon, contrasting with *S. enterica* which does not [Loomis and Magasanik, 1967; Santillán and Mackey, 2008]. Despite this, both *E. coli* and *S. enterica* were found to grow in the presence of lactose when each were in mono-culture (Fig. 3.1B and D). *S. enterica*'s proliferation, while not as high as *E. coli*'s, was still non negligible. Although *S. enterica* isolates possessing functional chromosomal lac operons have been detected in the past [Leonard et al., 2015], we ruled this possibility out as inoculation on lactose-supplemented petri plates resulted in no growth (data not shown). Subsequent culturing proved that *S. enterica*'s growth was not due to lactose metabolization, as further culturing on sugar-deprived media yelded the same amplification factor as observed in lactose. Ferric citrate has been found to strongly potentiate the proliferation and virulence of enteric pathogens such as *S. enterica* during intestinal infections [Kortman et al., 2012]. In addition, iron has also been identified as a critical factor in the survival and growth of *S. enterica* in tomato fruits [Nugent et al., 2015]. As our M9 medium was supplemented with iron, we next sought to identify if extracellular iron could have been the cause of *S. enterica*'s sugar-independent growth. Like before, *S. enterica* continued to display an increase in cell concentration even in the combined absence of both sugar and iron (Fig. 3.3A and C). We hypothesized that *S. enterica* may hold internal carbon storing mechanisms that allow it to grow without any available carbon sources. One of the main forms of carbon storage in *Enterobacteriaceae* is glycogen [McMeechan et al., 2005]. In accordance, the literature shows that glycogen production has been identified in most *S. enterica* serotypes, including our isolate, *S. enterica typhimurium* [McMeechan et al., 2005]. Specifically, *S. enterica typhimirum LT- 2* has been found to possess all three enzymes necessary for glycogen synthesis, namely ADPglucose pyrophosphorylase, glycogen synthase, and branching enzyme [Steiner et al., 1977]. Before each competition, ON cultures of selected strains are made in glucose-supplemented M9 media. It is possible that *S. enterica*'s intracellular carbon storage may be composed primarily of glycogen, and that the production of this macromolecule took place during ON culturing. By acting as a carbon reserve molecule, glycogen can support *S. enterica*'s growth in the absence of a metabolizable carbon source.

Another previously unexplored aspect of IR is its potential interplay with cross-feeding mechanisms. In a microbial community, cross-feeding is defined as the uni- or bi-directional exchange of metabolites between two or more bacterial strains [Braga et al., 2016; D'Souza et al., 2018]. Carbon metabolic by-products are an examble of such metabolites. It is possible that the underlying mechanism enabling *E. coli*'s apparent growth on citrate may have been cross-feeding between *E. coli* and *S. enterica*. As previously stated, *E. coli* is not expected to proliferate in citrate-supplemented media [Reynolds and Silver, 1983]. While *E. coli* mono-cultures did in fact not show growth on citrate (Fig. 3.1A), *E. coli* consistently reported growth in citrate-supplemented media when co-cultured with *S. enterica* (Fig. 3.2B and 3.7F). Indeed, *E. coli* proliferation continued to be observed when co-cultured with *S. enterica* even in the total absence of carbon source (Fig. 3.3B and D), further reinforcing that *E. coli*'s growth was being supported through cross-feeding with *S. enterica*. In fact, cross-feeding between *E. coli* and *S. enterica* has been documented in the past, where the same strains were found to partake in metabolic exchanges involving methionine, among other byproducts [Harcombe, 2010]. By contrast, support of *S. enterica*'s growth by *E. coli* could not be conclusively ascertained, owing to *S. enterica*'s aforementioned capability to proliferate in lactose-supplemented media.

Cross-feeding has been pointed as a potentiator of IR in humans tissues, as commensal bacteria may provide invading pathogens with the necessary nutrients for colonization. *P. aureginosa*, for example, has been found to benefit from cross-feeding with resident anaerobic bacteria in the cystic fribrosis lung [Flynn et al., 2020]. Through the production of L-lactate, commensal *S. gordonii* was also observed to assist the pathogenic *A. actinomycetemcomitans*' colonization of the oral cavity [Ramsey et al., 2011], thereby facilitating the occurrence of periodontitis infections [Slots et al., 1980]. Cross-feeding can involve a number of different externalized molecules, from enzymes, to metabolic by-products, to biofilm components and even molecules that serve to enhance cross-feeding itself [Braga et al., 2016; D'Souza et al., 2018; Fritts et al., 2021]. It is extremely common within biofilms, where multi-species communities are often found [Germerodt et al., 2016]. These interactions often implicate a number of changes in the genetic expression in individual cells, such so that a predicted 17% to 42% of a bacteria's open reading frames may be involved in microbial interactions [Phelan et al., 2012; Hansen et al., 2016]. Several genes related to protein expression, cellular receptor production, metabolic pathways and many others are subject to alterations which, in the long run, will increase the cooperation and cohesion of multi-populational bacterial communities [Fritts et al., 2021; Hansen et al., 2016; Davey and O'toole, 2000]. Molecules involved in antibiotic resistance may also be exchanged within bacterial communities, composing one dimension of the large network of molecules and signals shared among multiple strains and species [Fritts et al., 2021; Narisawa et al., 2008; Adamowicz and Harcombe, 2020; Adamowicz et al., 2018].

Cross-feeding and how it operates in the context of bacterial resistance has been an incredibly understudied and underappreciated phenomenon. From a mutualistic point of view, a sensitive strain may provide nutrients to the resistant strain in exchange for the subsequent detoxification of the surrounding media, thereby fostering a symbiotic-type relationship. Such relationship was at one point observed when our sensitive *S. enterica* was co-cultured with a resistant *E. coli*, whereby *E. coli* was able to grow and detoxify the media through cross-feeding with the sensitive *S. enterica* in citrate-supplemented M9 media (Fig. 3.2B). On the other hand, sensitive strains could also exploit the resistant cells by profiting from not only antibiotic-inactivating enzymes (in our case β-lactamase) but metabolic nutrients as well. In this aspect, cross-feeding may function as yet another exploitation mechanism in a parasitic relationship between resistant and sensitive strains. This kind of parasitic behaviour was observed when our sensitive *E. coli* was co-cultured with the resistant *S. enterica* on citrate (Fig. 3.7F). Here, *S. enterica* provided both β-lactamase and the carbon necessary to *E. coli*'s growth, while apparently receiving nothing in return.

Competition between resistant and sensitive strains was observed when they belonged to the same species regardless of the carbon source used (Fig. 3.5), and when the strains belonged to different species when cultured in glucose-supplemented media (Fig. 3.6). Altogether, our results indicate that inter-strain competition heavily disfavors IR. In accordance with our initial hypothesis, sensitive strains grew less when in competition with the resistant strain for the same carbon source, compared to when both strain's nutritional needs were met (see the case when two non-competing carbon sources were used, Fig. 3.7A and B). The introduction of antibiotics in a given system will differentiate resistant and sensitive populations. Excluding the resistance genes, these populations will be genetically akin and therefore, retain identical nutritional requirements. Under these conditions, intra-species metabolic competition is to be expected. Therefore, it is likely that in an antibiotic-rich environment, intra-species IR is recurrently selected against on an evolutionary scale. This may explain the discrepancy observed in the literature between the abundance of inter-species IR relative to the scarcity of intra-species.

To assess inter-species co-culture progression in the presence of IR without competition, co-cultures were grown in media supplemented with two non-competing carbon sources, lactose and citrate (Fig. 3.7A and B). In this context, lactose and citrate were carbon sources exclusive to *E. coli* and *S. enterica*, respectively. This ensured that there was no metabolic overlapping between the two species' nutritional needs. As expected, inter-species IR in the absence of carbon source competition resulted in proliferative success for the sensitive strains. Although starting from a lower concentration (10x to 100x times lower than the resistant), the sensitive strains were always able to grow to match the resistant's final concentration. As such, in the absence of competition, sensitive strains appear to benefit from IR without the negative fitness repercussions associated with antibiotic resistance [Vogwill and Maclean, 2015; Dugatkin et al., 2005]. Provided there is negligible metabolic overlapping, we postulate that inter-species IR is evolutionarily favored within bacterial communities, benefitting either sensitive or both sensitive and resistant strains, depending on the various inter-populational interactions at play.

IR depends on the initial acquisition of antibiotic resistance genes by a bacterial population in a given microbial community [Domingues et al., 2017; Nicoloff H. and Andersson, 2016b; Baquero et al., 2009]. As such, the exchange of resistance genes between populations is an extremely important factor in the establishment of IR [Harrison and Brockhurst, 2012; Huddleston, 2014]. The transfer of plasmids (often containing resistance genes) between bacteria, i.e. horizontal gene transfer [Burmeister, 2015], may not only condition the development of IR, but may itself also be regulated by it. In inter-species IR, sensitive strains could face fitness penalties by incorporating resistance plasmids from the resistant population [Vogwill and Maclean, 2015; Dugatkin et al., 2005; Hall et al., 2021; Domingues et al., 2022]. In addition, resistant strains would benefit from transferring their plasmids to any neighboring sensitive population of differing species [Hall et al., 2021; Prensky et al., 2021; Domingues et al., 2022]. Meanwhile in intra-species IR, where sensitive strains are at a clear disadvantage, plasmid incorporation might be more disearable. Likewise, resistant strains would gain by staying in lower frequencies and reducing the amount of plasmid transfer. By doing so, the resistant strain would lower the amount of "public goods" in the population and secure exclusive access to the media's carbon source(s) [Amanatidou et al., 2019]. Thus, in the context of IR, horizontal gene transfer might be locked in a evolutionary tug-of-war between resistant and sensitive strains. In this regard, sensitive strains would benifit if horizontal gene transfer was promoted in intra- and suppressed in inter-species IR, while resistant strains would benefit from the inverse, with suppression in intra- and promotion in inter-species.

Overall, our results contribute to elucidate the impact of carbon competition on IR and offer new insights into how it may interplay with other secondary inter-species interactions (i.e. crossfeeding). Our findings show that sensitive strains only benefit from IR when they are not in direct competition with the local resistant strains. Indeed, when inter-species IR occurred in media supplemented with both lactose and citrate, sensitive strains were always able to reach their maximum concentration, eventually matching the resistant's. When in competition, nearby metabolic nutrients will be exhausted by the resistant before the media can be fully detoxified, limiting the potential growth of the sensitive strains. This was observed whenever same-species IR occurred in either lactose or citrate. When inter-species IR occurred in glucose, we expected the sensitive strains' growth to be constricted, regardless of strain combination. In actuality, only when *S. enterica* was the resistant strain was this observed (Fig. 3.6B). When *E. coli* was the resistant strain, sensitive *S. enterica* was still capable of achieving its maximum final concentration (Fig. 3.6A). This was most likely due to the fact that *E. coli* had a tendency to drop sharply in concentration at the beginning of each competition. Thus the resistant population no longer possessed an initial concentration advantage over the sensitive strain, delaying the exhaustion of glucose in relation to the media's detoxification. Regardless, these results strongly support our initial hypothesis, that only when the resistant and sensitive strains possess distinct nutritional needs can both strains coexist and achieve their respective maximum concentrations and growth rates. Additionally, other interactions such as cross-feeding may influence the relationship between resistant and sensitive strains, as it may further strengthen cooperation or act as yet another mechanism of exploitation. Co-culturing of resistant *E. coli* and sensitive *S. enterica* on citrate reinforces this notion (Fig. 3.2B and 3.7E). Here, potential cross-feeding interactions between *S. enterica* and *E. coli* might have permitted the limited proliferation of *E. coli*, allowing for the media to be sufficiently detoxified for the sensitive *S. enterica* to grow freely on citrate. Hypothetically, horizontal gene transferring may also interplay with IR, conditioning the plasmid transfer flow within the larger community and the effective ratio equilibrium of antibiotic-sensitive to antibiotic-resistant cells.

5. Conclusion

According to our initial hypothesis, concerning the impact that resource competition can have over IR, sensitive strain growth should be hindered when in direct nutritional competition with the neighboring resistant strain. That being the case, we showed that sensitive strains were unable to reach their maximum final concentrations when co-cultures were made in glucose-supplemented media, most likely due to the sugar's exhaustion preceding the media's detoxification. Similarly, intra-species IR yelded identical results. Indeed, sensitive *E. coli* was also unable to reach its maximum concentration when co-cultured with the resistant *E. coli* in lactose. The same occurred with *S. enterica* when both strains were grown in citrate. This makes sense as metabolic competition is nearly always predicted to occur between strains belonging to the same species. These results suggest that metabolic competition may limit IR, which could explain why reports involving intra-species IR appear to be scarce. On the other hand, inter-species IR, when occurring in media supplemented with carbon sources exclusive to each strain, resulted in the maximum growth rate of the sensitive strain. The absence of metabolic competition maximizes the growth potential of the sensitive strains and, by extension, the cooperative nature of IR.

Throughout our competitions, *S. enterica* was observed to grow in lactose-supplemented media. Given that lactose metabolization is not a trait typical of *S. enterica*, it is likely that its proliferation was supported through the storage and consumption of glycogen. This capability acted as a confounding factor and minimizing its effects should be a priority for any future studies. As such, overnight cultures should be prepared in minimal media complemented with a carbon source other than glucose (such as lactose or citrate) to safeguard against the occurence of gluconeogenesis and its influence in co-culture progression.

Several inter-population interactions such as cross-feeding may also play a significant role, allowing for more complex interactions to occur. In a multi-populational microbial community, IR can interact with cross-feeding mechanisms to generate a multi-level network of bacterial associations resulting in a higher degree of biodiversity and an increment in the microbiota's stability. In order to combat indirect pathogenicity in the field of human health, it is paramount to dissect how cooperation between co-infecting pathogens and/or commensal bacteria may be disrupted. Likewise, the discrepancy between intra- and inter-species IR can lead to intriguing dynamics regarding the transfer of genetic material between strains. Depending on the circumstance, resistant strains may be more or less prone to transfer their resistant plasmid and sensitive strains more or less prone to receive them. As either strain could stand to benefit more from the advent of IR in any given context, horizontal gene transferring can be a relevant and crucial component to understanding IR establishment and its impact on the evolution of a given bacterial microbiota as a whole. In order to better understand role of genetics in the evolution and genetic makeup of bacterial communities, the role of IR cannot be underestimated. In the clinical use of antibiotics, IR remains a crucial obstacle to disease treatment and its study, a fundamental necessity.

6. References

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