University of Cyprus
Department of Biological Sciences

Bacterial Species Antagonizing *Pseudomonas aeruginosa* Infection

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Master's Thesis
in Biomedical Sciences

Supervisor: Associate Professor Yiorgos Apidianakis

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Abstract

Microbial species rarely exist in isolation in nature. Epithelial interfaces, such as the bilaterian intestine or the mammalian lung, harbour polymicrobial communities increasing infection complexity at these sites. We recently found that the Gram-negative bacterium *Pseudomonas aeruginosa* interacts antagonistically, additively, or neutrally with other Gram-negative bacterial species that colonize the human intestine, in the process of establishing a virulent infection in flies. More specifically, *P. aeruginosa* strain PA14 is inhibited upon interaction with *Serratia marcescens*, *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, and *Providentia* spp. These 6 bacterial strains exert a strong antagonistic effect to *P. aeruginosa* extending *Drosophila* survival upon co-infection.

The main purpose of this follow-up study was to investigate whether the same antagonistic interactions occur in isolation in culture under both normal and hypoxic (5% O_2) conditions mimicking the environment of the mammalian epithelia. In addition, all strains were tested in bacterial media containing or lacking glucose to also evaluate the impact of sugar fermentation on *P. aeruginosa* growth and pyocyanin production, a key virulence factor produced by this species. Under normoxia, *P. aeruginosa* growth remained unaffected upon interaction in culture with any of the six bacterial strains in the absence of glucose. On the other hand, when glucose was added, *Enterobacter aerogenes, Klebsiella spp., Acinetobacter baumannii* and *Provientia spp.,* inhibited *P. aeruginosa* growth and pyocyanin production. Under hypoxia, *P. aeruginosa* growth was compromised upon interaction with *Lactobacillus plantarum, Bifidobacterium infantis* and *E. coli* strain MGH only in the presence of glucose, while 3 more strains *Enterobacter aerogenes, Klebsiella spp.* and *Acinetobacter baumannii*, did not affect *P. aeruginosa* growth.

Co-infection of the hypoxic mouse lung with the *E. coli* strain MGH reduces virulence by the highly virulent *P. aeruginosa* strain B136-33. Moreover, *Drosophila melanogaster* under hypoxic (5% O_2) conditions revealed that fly mortality can be significantly delayed upon co-infections of P. aeruginosa with *Klebsiella* spp., *Enterobacter aerogenes, Acinetobacter baumannii, Salmonella paratyphi*, or *Providentia* spp.

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I particularly would like to thank my supervisor, associate professor Yiorgos Apidianakis for trusting and giving me this opportunity to work in his 'Host-Microbe-Diet Interactions and Carcinogenesis' Laboratory.
Working here, truly increased my scientific knowledge and research experience. Working with bacteria, flies and also mice is a valuable asset for and my future career as a scientist, improving my laboratory skills and knowledge of new techniques. Prof. Apidianakis has offered me valuable support, help, guidance, time, and patience in order to successfully complete both the experimental and the writing level of this work and I am grateful to him.

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Finally, I am grateful to my parents for their unconditional love, financial and emotional support, effort and advise for all these years allowing me to pursue my dream as a scientist.

Composition of the Examination Committee

Thesis Supervisor (Examination Committee coordinator): associate professor Yiorgos Apidianakis, Department of Biological Sciences, University of Cyprus

Committee Member: assistant professor Chrysoula Pitsouli, Department of Biological Sciences, University of Cyprus

Committee Member: assistant professor Katerina Strati, Department of Biological Sciences, University of Cyprus

Seminar Announcement

University of Cyprus
Department of Biological Sciences

Master Research Dissertation in Biomedical Sciences (BIO 830/600)

Student Presentation

Thursday, 27 May 2021 at 11:30

This seminar is open to the public via Zoom at the following link:

https://ucy.zoom.us/j/95067251241?pwd=Z1I3c1JHdnMzWVpQL2hwWExCUTFoZz09

Evangelos Grivogiannis

Thesis Supervisor: Assoc. Prof. Yiorgos Apidianakis

"Bacterial Species Antagonizing *Pseudomonas aeruginosa* Infection"

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Microbial species rarely exist in isolation in nature. Epithelial interfaces, such as the bilaterian intestine or the mammalian lung, harbour polymicrobial communities increasing infection complexity at these sites. We recently found that the Gram-negative bacterium *Pseudomonas aeruginosa* interacts antagonistically, additively, or neutrally with other Gram-negative bacterial species that colonize the human intestine, in the process of establishing a virulent infection in flies. More specifically, *P. aeruginosa* strain PA14 is inhibited upon interaction with *Serratia marcescens*, *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, and *Providentia* spp. These 6 bacterial strains exert a strong antagonistic effect to *P. aeruginosa* extending *Drosophila* survival upon co-infection.

The main purpose of this follow-up study was to investigate whether the same antagonistic interactions occur in isolation in culture under both normal and hypoxic (5% O_2) conditions mimicking the environment of the mammalian epithelia. In addition, all strains were tested in bacterial media containing or lacking glucose to also evaluate the impact of sugar fermentation on *P. aeruginosa* growth and pyocyanin production, a key virulence factor produced by this species.

Under normoxia, *P. aeruginosa* growth remained unaffected upon interaction in culture with any of the six bacterial strains in the absence of glucose. On the other hand, when glucose was added, *Enterobacter aerogenes, Klebsiella spp., Acinetobacter baumannii* and *Provientia spp.,* inhibited *P. aeruginosa* growth and pyocyanin production. Under hypoxia, *P. aeruginosa* growth was compromised upon interaction with *Lactobacillus plantarum*, *Bifidobacterium infantis* and *E. coli* strain MGH only in the presence of glucose, while 3 more strains *Enterobacter aerogenes, Klebsiella spp.* and *Acinetobacter baumannii,* did not affect *P. aeruginosa* growth.

Co-infection of the hypoxic mouse lung with the *E. coli* strain MGH reduces virulence by the highly virulent *P. aeruginosa* strain B136-33. Moreover, *Drosophila melanogaster* under hypoxic (5% O_2) conditions revealed that fly mortality can be significantly delayed upon co-infections of P. aeruginosa with *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, or *Providentia* spp.

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1. Introduction

Microbial species may interact with a host in a parasitic (leading to infection or even death), mutualistic, or commensal manner, but rarely in isolation from one another on barrier epithelia. In fact, recent studies have shown that bacterial species inhibit polymicrobial communities in both nature and in humans. Within those 'multicultural' communities, microbes interact with each other in various ways (Figure 1). (Sibley et al., 2008a; Vial and Deziel, 2008). The effect of those microbe-microbe interactions not only does it have a great impact on how a particular infection (in this case defined as a 'polymicrobial' infection) can be manifested (Short et al., 2014; Sibley et al., 2008b), but most importantly, on how host's health can also be affected directly accordingly, in a negative, neutral, or even positive fashion. Thus, studying polymicrobial-hosts interactions as well, is also imperative.

In a way to study both microbe-microbe interactions and polymicrobial-host interactions in a specifically designed polymicrobial setting, Sibley and colleagues (2004a) developed 'a novel *Drosophila melanogaster* infection model'. Flies were fed with 40 different oropharyngeal isolates (OF) taken from cystic fibrosis (CF) patients, chronically colonized with the bacterium **P. aeruginosa* strain PA01. Furthermore, all OF isolates were also fed to flies in combination with PA01 and survival rates were monitored every day.

The results they found were the following: 1) When PA01 was fed alone to *Drosophila*, survival rates immediately dropped to 50% (Figure 1e). As expected, PA01 killed the flies in about 6 days. 2) On the other hand, when OF isolates were fed alone, they exhibited that survival rates either remained unaffected (i.e., avirulent strains) or decreased to about 80% (i.e., virulent strains) (Both data is not shown in figure 1). 3) After flies were fed the OF isolates in combination of PA01 they revealed 4 different outcomes: A) When avirulent strains, unable to kill flies by themselves, were combined with PA01, the killing was not enhanced, as survival rates remained about 50%, being the same ones found when only PA01 was fed (Figure 1b). This means that the bacteria do not interact (neutral interactions) with each other. B) As expected, combination of virulent OF strains, which are able to kill flies alone, along with PA01 kills flies faster and survival rates decreased to ~30% (Figure 1c). Bacteria interacted in a so-called additive way, meaning that the result of the

bacteria combination equals the sum of the action of the two bacteria separately. C) Unexpectedly, they also demonstrated that certain OF avirulent strains or even strains beneficial for the human itself when combined with PA01 had the ability to dramatically reduce fly survival (Figure 1d). Those bacteria portrayed synergistic relationships with each other. (Korgaonkar et al., 2013) Most crucially, synergistic interaction between bacteria results to more dead flies (survival less than 20 %) compared to additive interactions between virulent strains. Surprisingly, antagonistic interactions, were not observed, despite the fact it is pretty likely that the co-feeding assay developed in this study could ultimately reveal such kind of interactions.

**Pseudomonas aeruginosa* is a rod-shaped, Gram-negative, commensal, and opportunistic human pathogen (Lyczak et al., 2000) that is widely found in soil and water. It infects a great variety of different hosts, including vertebrates, insects, nematodes, amoebae, and plants. (Apidianakis and Rahme 2009; Bayes et al., 2016; Mahajan-Miklos et al., 1999; Rahme et al., 1995). Generally, it affects immunocompromised individuals (those who suffer from chronic burns, or HIV for example) and is a major source of nosocomial infections. Most importantly, it is responsible for most of the morbidity and mortality associated with chronic lung infections of cystic fibrosis (CF) patients (Lutter et al., 2008). In addition, it is extremely resistant to a wide range of antibiotics, thus, making it difficult to treat any infections that is involved. (Lutter et al., 2008, Markou and Apidianakis, 2014, Mulcahy et al., 2011). During the last 25 years it has been shown that *P. aeruginosa* (as well as many pathogenic bacteria both Gram-negative and Gram-positive) has the ability to 'communicate' with other bacteria (being the same or even different species), 'exchange' information about their population density, and most essentially coordinate their gene expression. This process is termed Quorum sensing (QS). During this process bacteria seem to act in a pseudo-multicellular way, acting as a 'team', in order to regulate different processes, such as production of multiple virulence factors, biofilm formation, bioluminescence, production of secondary metabolites (antibiotics) and swarming motility (Bassler and Losick, 2006; Gonzalez and Keshavan, 2006; Jimenez et al., 2012; Rutherford and Bassler, 2012; Castillo-Juarez et al., 2015). QS has been suggested to be a promising therapeutic target against bacterial infections. (Hentzer et al., 2003; Ho Sui et al., 2012). One of the major virulence factors of *P. aeruginosa*, produced during the QS process is the blue-green pigment phenazine Pyocyanin (5-N-methyl-1-hydroxyphenazine), which contributes to both acute and chronic infections. It is a secondary

metabolite that can act as electron transfer facilitator in redox reactions. Furthermore, it is very toxic, as it reacts and produces oxygen free radicals, causing oxidative stress to organisms. (Rada and Leto, 2013). Finally, it has been found that pyocyanin is also a terminal signalling factor in the QS mechanism of *P. aeruginosa.* (Dietrich et al., 2006).

Among the several different projects that are carried out in our 'Host-Microbe-Diet Interactions and Carcinogenesis' Laboratory in University of Cyprus, a great effort has been also put in examining microbe-microbe interactions, both *in vitro* and *in vivo*, by the use of *Drosophila melanogaster* and *Mus musculus* as model organisms. Thus, following a similar approach of Sibley and colleagues that described earlier, as a first step, we examined whether particular intestinal human colonizers can also infect *Drosophila melanogaster.* Among 34 different bacterial strains (containing both Gram-negative and Gram-positive strains) tested for the experiment it was revealed that *P. aeruginosa* strain PA14 was the most pathogenic, as it killed flies in 5 to 7 days post-infection (about the same amount of time took for PA01 to kill the flies), while the least pathogenic was *Bacteroides thetaiotaomicron*. PA14 is one of the highly pathogenic strains of *P. aeruginosa* when infecting the various hosts, as it can cause high mortality rates, due to its ability to easily suppress their defences. (Apidianakis et al., 2005).
All the 34 bacteria were listed in a table (Figure 2) according to their degree of pathogenicity as we called it (i.e., time it took for the flies to be killed).

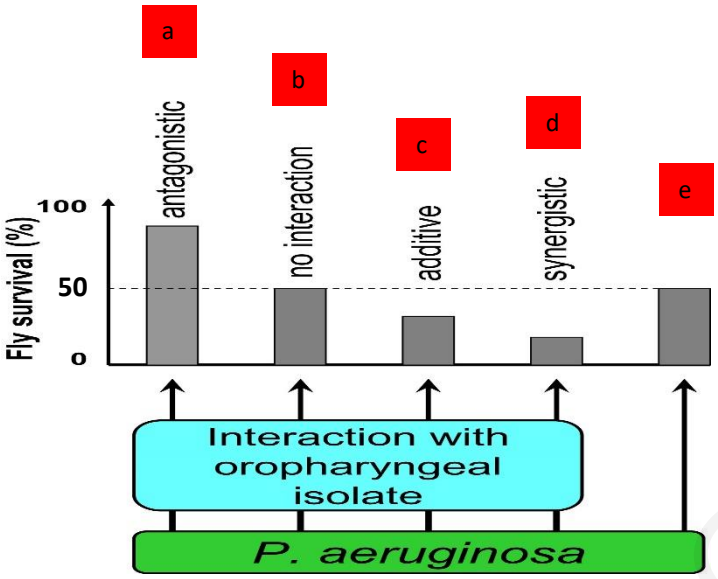


Figure 1. A *Drosophila melanogaster* Infection Model of Interactions Between *Pseudomonas aeruginosa* and Oropharyngeal Microflora from Cystic Fibrosis Patients. a-d: (potential) antagonistic, neutral, additive, and synergistic relationships respectively). e) Effect of *P. aeruginosa* on flies when fed alone. Oropharyngeal isolates were also fed to flies alone (data not shown). (Sibley et al., 2008a; Vial and Deziel, 2008)

(1) *P. aeruginosa* (PA14)	**(13)** *Enterobacter cloacae*	**(24)** *Vibrio Cholerae*
(2) *Yersinia pseudotuberculosis*	**(14)** *EPEC*	**(25)** *Yersinia Enterocolitica*
(3) *Serratia marcescenes*	***(15)*** *Streptococcus agalactiae*	**(26)** *Listeria monocytogenes*
(4) *P. multocida*	**(16)** *Salmonella cholerasuis*	**(27)** *Escherichea coli (DH5a)*
(5) *Proteus* spp.	**(17)** *Lactobacilus paraalimentarius*	**(28)** *P. aeruginosa (CF5)*
(6) *P.mirabilis*	**(18)** *Bifidobacterium infantis*	**(29)** *Lactobacillus acidophilus*
(7) *Morganella* spp.	**(19)** *Enterococcus faecalis*	**(30)** *Lactobacillus plantarum*
(8) *S. Dupentery*	**(20)** *Klebsiella pneumonia*	**(31)** *Lactobacillus brevis*
(9) *E. Aerogenes*	**(21)** *Salmonella paratyphi*	**(32)** *Providentia* spp.
(10) *Klebsiela spp.*	**(22)** *Salmonella typhi*	**(33)** *Bacteroidetes fragilis*
(11) *A.baumannii*	**(23)** *Enterococcus sp.*	**(34)** *Bacteroides thetaiotaomicron*
(12) *Citrobacter spp.*		
gram -	*gram +*	

Figure 2. Human Intestinal Bacteria Tested for Their Ability to Infect *Drosophila melanogaster*. All bacteria are listed according to their pathogenicity degree. Gram-negative and Gram-positive bacteria were listed in green and yellow colour, respectively. (Dieronitou Eirini, Master Thesis, 2016, University of Cyprus)

After defining which of the human intestinal bacterial strains can also infect flies and which ones are the most and least pathogenic, we wanted to explore the possibilities of microbe-microbe interactions, by implementing a co-feeding approach similar to Sibley and colleagues described. For our main bacteria we used PA14, since we find that it can dramatically kill flies in less than a week. Thus, we tested the potential interactions between the most pathogenic PA14 and all the other less pathogenic bacterial species. *E. coli* MGH strain was also included in the experiment. 4 different conditions were prepared. Flies were fed alone with sucrose as a vehicle (used as a negative control), PA14 (used as positive control) and each of the other 34 bacteria. Finally, a combination of PA14 and the 34 bacterial strains was also fed to *Drosophila.* Our results (Figure 3) bear some similarities with the previous study, as they exhibited 2 additive interactions between PA14 and *Enterococcus faecalis* and *Enterococcus* spp., which resulted in enhancing fly killing (Figure 4) as flies were killed much sooner (4 days) compared to PA14 alone (7 to 8 days). 17 neutral interactions were also found where no interactions occur (flies were dead at approximately at the same time as when fed with PA14 alone). Most essentially and in contrast to Sibley and colleagues, who did not observe any antagonist interactions earlier, we managed to show 15 antagonistic relationships, resulting in extending the life expectancy of the fly. In contrast to Sibley and colleagues who focused more on the synergistic and additive microbe interactions, we wanted to focus on the antagonistic interactions, which are paramount for host's health. We tried to 'quantify' the negative effect of the least pathogen bacteria to PA14 when fed in combination to flies. Thus, antagonistic interactions (Figure 3) were also given a measurement of negative effect, namely medium and strong. (Figure 3). The less pathogenic strain exerts a medium negative effect on the most pathogenic strain, when survival rates of the bacteria combination are higher than those of the more pathogenic bacterium (PA14) but lower than those of the less pathogenic strain (e.g., *Proteus* spp.) (Figure 5). On the contrary, when survival rates of the bacteria combination are much higher than those of the more pathogenic bacterium (PA14), or pretty close to those of the less pathogenic one (e.g., *Klebsiella* spp.), then the less pathogenic strain exerts a strong negative effect to PA14. From the 15 antagonistic interactions, only 6 bacteria exerted a strong negative effect upon PA14, namely *Serratia marcescens, Klebsiella* spp., *Enterobacter aerogenes, Acinetobacter baumannii, Salmonella paratyphi* and *Providentia* spp. (Figures 6-11)

	Pseudomonas aeruginosa (PA14)
E. coli (MGH)	Antagonistic Interactions (Strong Negative Effect)
Yersinia pseudotuberculosis	No Interactions (No Effect)
Serratia marcescens	Antagonistic Interactions (Strong Negative Effect)
P.multocida	No Interactions (No Effect)
Proteus spp.	Antagonistic Interactions (Medium Negative Effect)
P. mirabilis	No interactions (No Negative Effect)
Morganella spp.	No interactions (No Negative Effect)
S. dupentery	No interactions (No Negative Effect)
Enterobacter aerogenes	Antagonistic Interactions (Strong Negative Effect)
Klebsiella spp.	Antagonistic Interactions (Strong Negative Effect)
Acinetobacter baumannii	Antagonistic Interactions (Strong Negative Effect)
Citrobacter spp.	Antagonistic Interactions (Medium Negative Effect)
Enterobacter clocae	Antagonistic Interactions (Medium Negative Effect)
EPEC	Antagonistic Interactions (Medium Negative Effect)
Streptococcus agalactiae	No Interactions (No Effect)
Salmonella cholerasuis	Antagonistic Interactions (Medium Negative Effect)
Lactobacillus paraalimentarius	No Interactions (No Effect)
Bifidobacterium infantis	No Interactions (No Effect)
Enterococcus faecalis	Additive Interactions (Additive Effect)
Klebsiella pneumonia	Antagonistic Interactions (Medium Negative Effect)
Salmonella paratyphi	Antagonistic Interactions (Strong Negative Effect)
Salmonella typhi	Antagonistic Interactions (Medium Negative Effect)
Enterococcus spp.	Additive Interactions (Additive Effect)
Vibrio cholerae	No Interactions (No Effect)
Yersinia enterolytica	Antagonistic Interactions (Medium Negative Effect)
Listeria monocytogenes	No Interactions (No Effect)
E. coli (DH5a)	No Interactions (No Effect)
P. aeruginosa (CF5)	No Interactions (No Effect)
Lactobacillus acidophilus	No Interactions (No Effect)
Lactobacillus plantarum	No Interactions (No Effect)
Lactobacillus brevis	No Interactions (No Effect)
Providentia spp.	Antagonistic Interactions (Strong Negative Effect)
Bacteroidetes fragilis	No Interactions (No Effect)
Bacteroides thetaiotaomicron	No Interactions (No Effect)

Figure 3. Bacterial interactions between *P. aeruginosa* and 34 different human intestinal bacterial strains. Microbe-microbe interactions revealed 2 additive, 17 neutral, and 15 antagonistic (7 strong, 8 medium) relationships. (Dieronitou Eirini, Master Thesis, 2016, University of Cyprus / Michael Christina, 2019 University of Cyprus

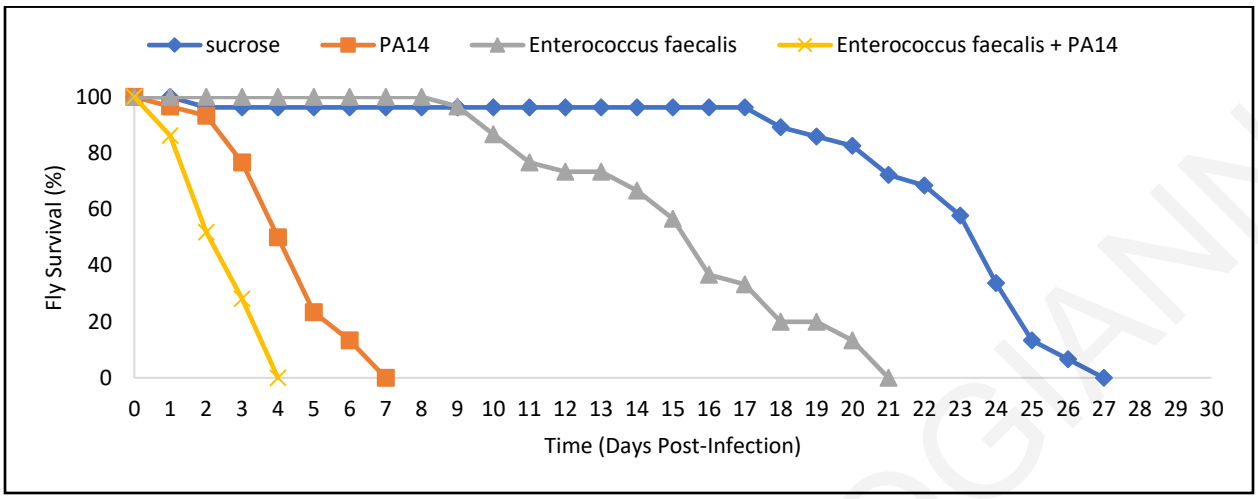


Figure 4. Interaction between PA14 and Enterococcus faecalis. Combination of E. faecalis and PA14 (yellow line marked with X) shows additive relationships. Orange line represents infection of PA14 alone. Blue and grey lines represent sucrose and the effect that E. faecalis exhibits to flies when fed alone, respectively. (Michael Christina, 2019, University of Cyprus)

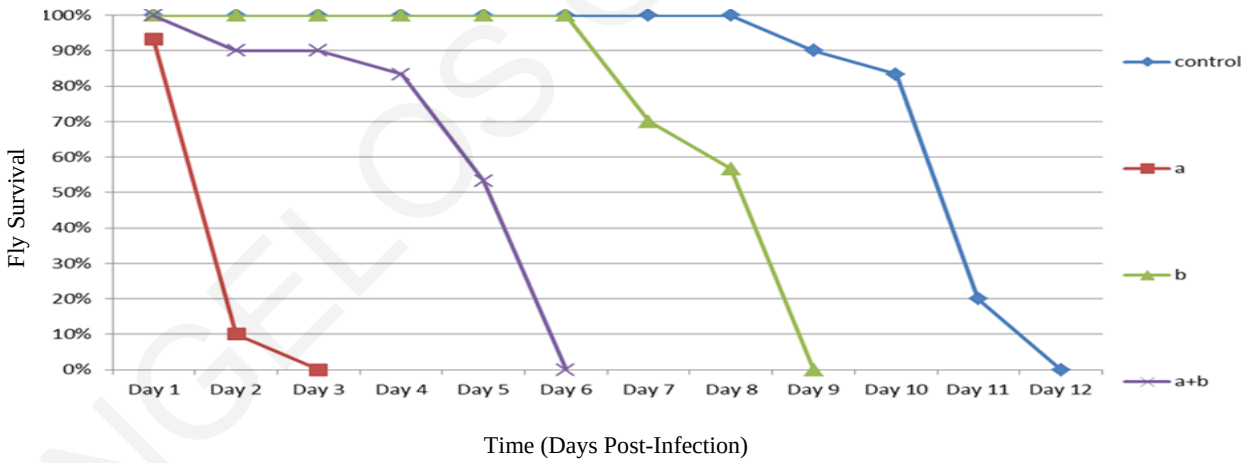


Figure 5. Antagonistic bacterial interaction with medium negative effect. 1Red line marked with boxes represents PA14 (most pathogenic strain), green line with triangles represents the less pathogenic strain and magenta line marked with X represents combination of PA14 and less pathogenic strain. When survival rates of the bacteria combination are higher than those of the more pathogenic bacterium but lower than those of the less pathogenic strain, the less pathogenic exerts a medium negative effect on PA14. (Michael Christina, 2019, University of Cyprus)

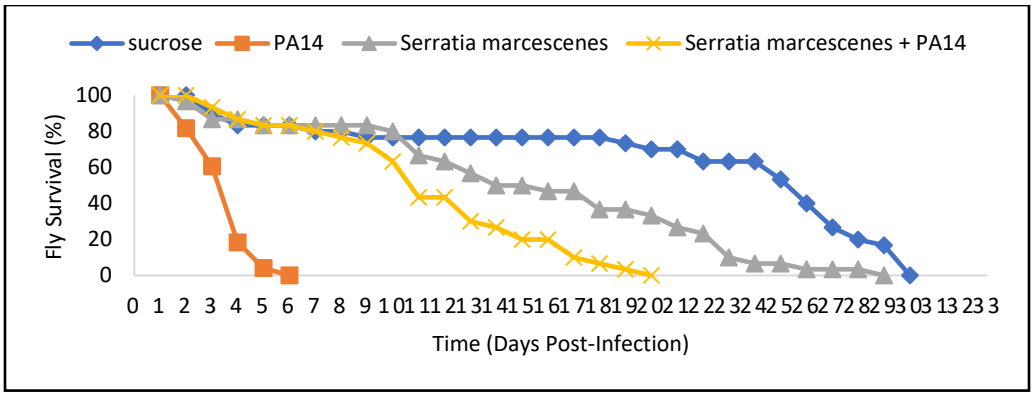
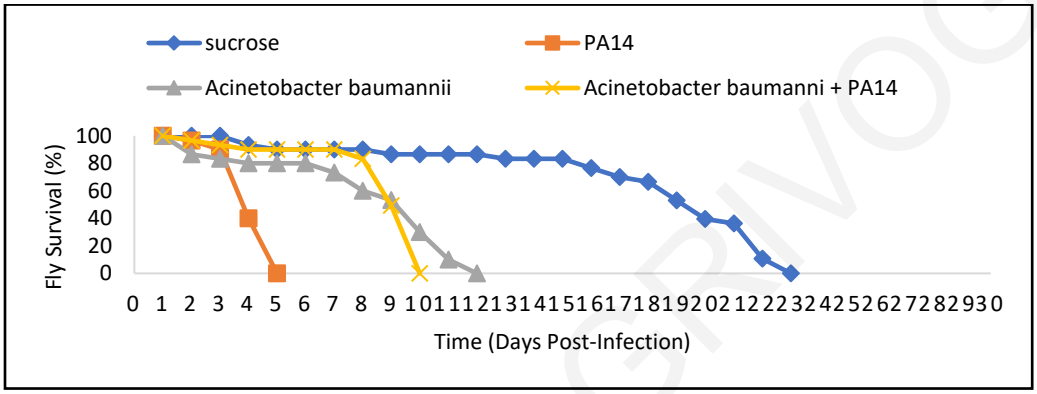


Figure 6.



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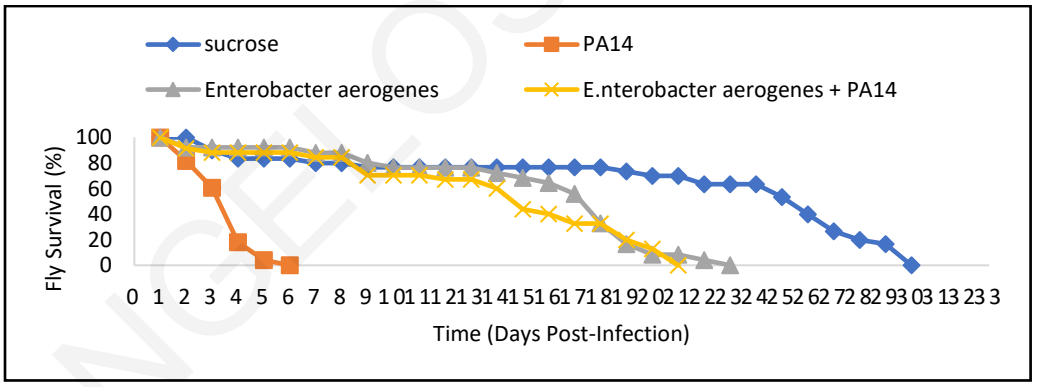

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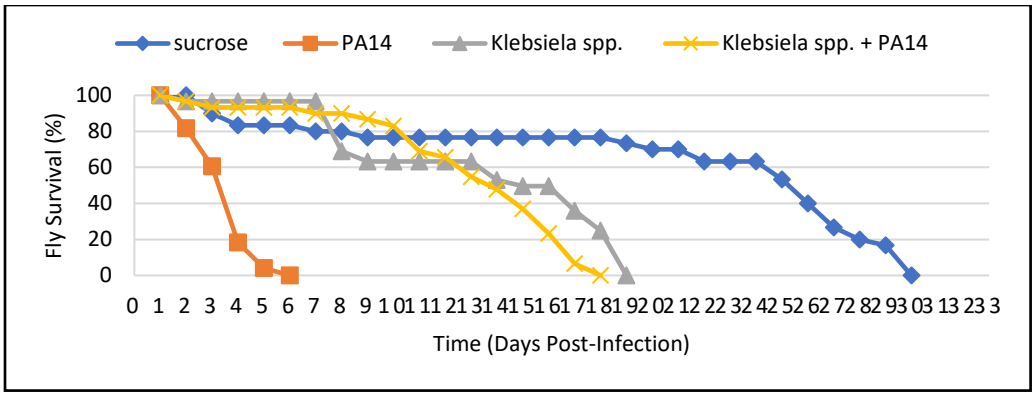

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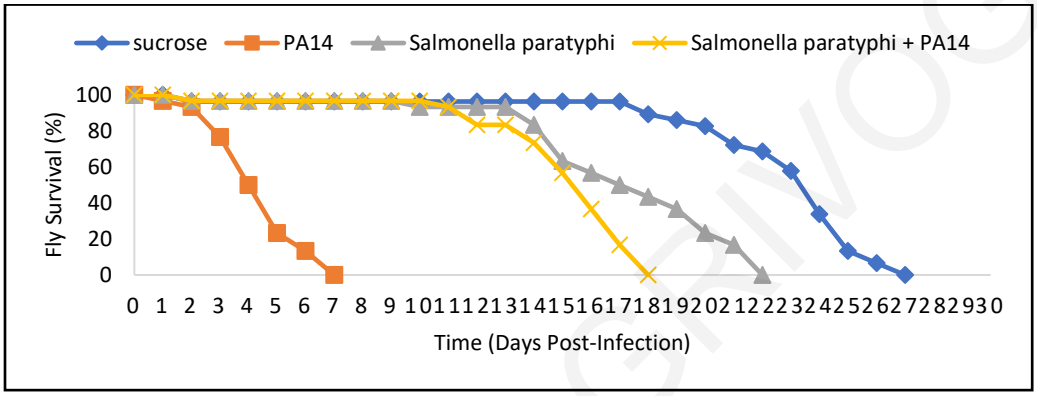

Figure 10.

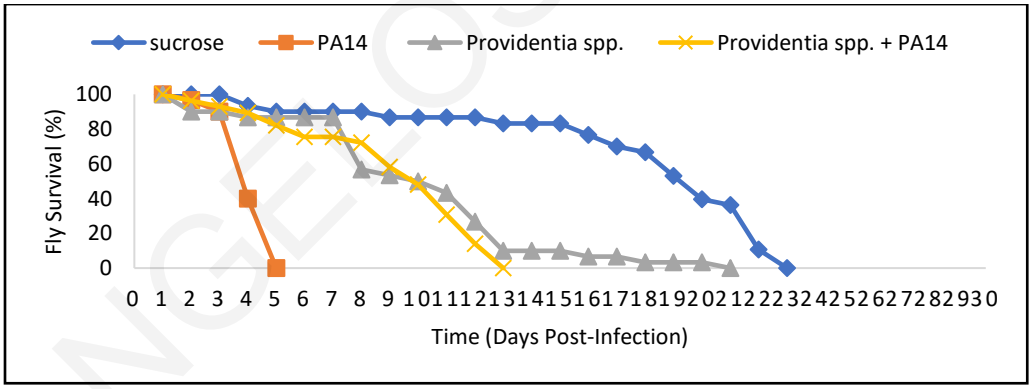

Figure 11.

Figures 6-11. Antagonistic bacterial interaction with strong negative effect. In all figures, orange line marked with boxes represents PA14 (most pathogenic strain), grey line with triangles represents the less pathogenic strain (Serratia marsescens, Klebsiella spp., Enterobacter aerogenes, Acinetobacter baumannii, Salmonella paratyphi and Providentia spp., respectively) and yellow line marked with X represents combination of PA14 and less pathogenic strain. When survival rates of the bacteria combination are much higher than those of the more pathogenic bacterium or even equal to those of the less pathogenic strain, the less pathogenic strain exerts a strong negative effect on PA14. (Michael Christina, 2019, University of Cyprus).

AIM

As previously mentioned, *in vivo* experiments conducted in our lab revealed 15 antagonistic relationships between *Pseudomonas aeruginosa* strain PA14 and 15 bacteria that colonize the human intestine. However, only 6 of them (*Serratia marcescens, Klebsiella* spp., *Enterobacter aerogenes, Acinetobacter baumannii, Salmonella paratyphi* and *Providentia* spp), exerted a strong negative effect, resulting in extending the fly life expectancy.

The main purpose of this follow-up study was to examine whether the same kind of interactions can also occur in isolation *in vitro*, when PA14 is co-cultured either with supernatant or pellet of the 6 bacteria, under both normal and hypoxic (5% O_2) oxygen conditions that may mimic the mammalian intestine environment. The strains tested were cultured alone in bacterial media in the presence and absence of glucose to also evaluate the impact of sugar fermentation by the bacteria on each other. PA14 growth and pyocyanin production, a key virulence factor produced by this species, were assessed upon in culture interaction between *P. aeruginosa* and the six aforementioned species.

In addition, two different *in vivo* studies were also performed by using mice and *Drosophila melanogaster* respectively, as model organisms.
In the first one, we wanted to check whether bacterial interactions can also save mice from lung infection. Mice were co-infected with *P.aeruginosa* strains PA14 and B136-33 along with *E. coli* MGH and *Bifidobacterium infantis.* As for the second study, we tried to replicate the previous survival fly experiment upon hypoxic conditions (5% O_2). Flies were co-infected with PA14 and *Serratia marcescens*, *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, *Providentia* spp., *Lactobacillus plantarum*, *Bifidobacterium infantis* and *E. coli* MGH.

2. Materials and Methods

2.1 Bacterial Strains

Bacterial strains used for the experiments include *Pseudomonas aeruginosa* strains UCBBP 14 (PA14), MTB-1 and B136-33, *Serratia marcescens*, *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, *Providentia* spp., *Lactobacillus plantarum*, *Bifidobacterium infantis and E. coli* strain MGH. All bacterial strains were maintained at glycerol stock and stored at -80°C.

2.2 Mice Handling

Female CD1 5-week-old mice were obtained from 'The Cyprus Institute of Neurology and Genetics' (CING) and reared 5 individuals per cage at 24°C in a 12-hour day and night cycle. Mice were adapted to their new environment for 3 days before being used for our experiments. Standard chow diet (a complete balanced diet containing mainly starch 35.18%, sucrose 5.66%, crude protein 22% and crude oil 3.5%) was obtained from Mucedola s.r.l Italy.

2.3 Intranasal Mouse Lung Infection Assay

The intranasal infection achieves the spreading of the bacteria from the upper airways to the intestine and low airways., thus mimics the pathology seen in acute bacterial pneumonia. Bacterial strains used were *P. aeruginosa* strains PA14 / MTB-1 and B136-33, *E. coli* strain MGH and *Bifidobacterium infantis*. PA14 / MTB-1 and B136-33 strains were grown in LB (Luria Broth) cultures overnight (~ 16 hours), then diluted 1:100 and grown over day for about 5-6 hours (depending on the strain) in LB to OD_{600nm}: 3.0 (~$3x10^9$ CFUs/ml). MGH is grown in LB liquid cultures overnight, then diluted 1:100 and grown over day in 4% Glucose (LB) or 4% Sucrose (LB) to OD_{600nm}: 3.0 (~$3x10^9$ CFUs/ml). *B. infantis* was grown for 48 hours under hypoxic conditions (5% O_2) in 4% Glucose (LB) or 4% Sucrose (LB). One ml of each bacteria culture was centrifuged for 2 min at 4610 rcf and the supernatant was removed. (MGH and *B. infantis* supernatant was also kept in order to infect the mice). The pellet was washed twice (except for *B. infantis*, which washed only once) and finally diluted in 2.5% Glucose (ddH_2O), 4% Sucrose (ddH_2O) or 4% Sucrose (LB). Mice were intranasally infected under very light anaesthesia (100

μl solution containing anaesthetics Ketamine-100mg and Xylazine-20mg), as previously described (Liu, J. et al. 2011; Teichgraber, et al., 2008) by placing 10μl of a bacterial suspension in each nostril (20μl in total) to reach the desired infectious dose of $2x10^7$ CFUs per mouse. Mortality counts were taken twice a day for a week.

2.4 Ethics Statement

Animal protocols were approved by the Cyprus Veterinary Service inspectors under the license number CY/EXP/PR.L6/2018 for the Laboratory of Prof. Apidianakis at the University of Cyprus. The veterinary services act under the auspices of the Ministry of Agriculture in Cyprus and the project number is CY.EXR101. These national services abide by the National Law for Animal Welfare of 1994 and 2013 and the Law for Experiments with Animals of 2013 and 2007. All experiments were performed in accordance with these guidelines and regulations.

2.5 Drosophila melanogaster Maintenance

Oregon R flies were used as a standard wild-type strain and reared on a standard agar/cornmeal diet (1% Agar, 3% Yeast, 5% Sugar, 6% Cornmeal, supplemented with 2.56% Tegosept and 0.38% Propionic Acid) and kept in plastic bottles approximately with 50ml of fresh fly food in a 12-hour day and night cycle at 25°C with 65% humidity. The flies were transferred in new bottles with fresh fly food every 3 to 4 days.

2.6 Fly Feeding Assay

Flies were subjected to bacterial infection with the so-called feeding assay, a standard method being generally used. As its name implies, it 'allows' bacteria to be readily entered to fly's intestine through food, leading to infection. Here, we fed our flies with bacterial strains mentioned before, either alone or in combinations with PA14, i.e., 10 different conditions.

Bacterial cultures were prepared as follows: freshly LB agar-plated bacterial single colonies were inoculated in 3ml LB medium and cultured overnight at 37°C with shaking at 200 rpm. Then, the overnight cultures were diluted 1:100 in LB to prepare an over day 3ml culture to OD_{600nm}: 3.0. *Anaerobes *L.plantarum* and *B.infantis* on the other hand, were grown only in 3ml BHI (Brain Heart Infusion) for 2 days at 37°C without shaking, under hypoxic conditions (5% O_2). OD_{600nm} for the two bacteria was found to be ~0.89 and ~0.72, respectively.

In the meantime, the oral infection mix was prepared containing 1ml 20% sucrose, 3.5ml ddH_2O and 05.ml LB.

0.5ml from the bacterial cultures for each strain was collected, centrifuged for 2min at 6010rcf and the pellet was resuspended (either only for PA14 itself or the combinations of PA14 with the other bacteria) in 5ml of the aforementioned oral infection mix. 5ml of this mix was used to soak an autoclaved cotton ball in a narrow plastic fly vial, which was subsequently plugged with a dry cotton ball. Wild-type female Oregon R flies 3-7 days old were starved for 5-6 hours and then were transferred in the infection vials plugged with a piece of cloth securely closed with rubber bands. Vials were put in a jar, where hypoxic conditions (5% O_2) were created and then transferred to 29°C.Three vials with 10 flies each were used for every condition.

2.7 Fly Survival Assay

To determine the mortality rate of the infected flies, daily observation of the experimentations was necessary. Uninfected flies can live up to 60 days. However, when flies are infected with *P.aeruginosa* PA14 they are killed in less than 10 days. The percentage of dead flies was calculated daily as: (the number of dead flies per vial/total number of flies) x 100, until all flies were dead in each vial. The fly survival was calculated based on the total number of flies surviving.

2.8 Assessing PA14 Growth – CFUs (Colony Forming Units) Assay

Freshly LB agar-plated bacterial single colonies were inoculated in 3ml LB medium in the absence or presence of 4% Glucose and cultured overnight at 37°C with shaking at 200rpm.

*Anaerobic Gram-positive bacteria *L.plantarum* and *B.infantis*, were also grown in both mediums for 2 days at 37°C without shaking, under hypoxia (5% O_2).

Each bacteria culture was collected and centrifuged for 5min at 6010rcf. Two different experiments were performed simultaneously:

a) Firstly, bacteria supernatant was used after being filtered by 0.2μm filters to remove any bacteria cells' residues. Filtered bacteria supernatant were mixed in 1:1 volume ratio with fresh LB broth (1.5ml each) along with PA14 bacteria (30μl) in a glass test tube containing a final volume of 3ml and incubated for 24 hours at 37°C with shaking at 200rpm. 24-hour incubation for *L. plantarum* and *B. infantis* was conducted under hypoxia at 37°C.

b) Bacteria pellet was resuspended in 1.5ml fresh LB and then immediately heat-killed at 70°C for 7-10min using the heat-block. As previously, heat-killed bacteria were mixed in 1:1 volume ratio

with fresh LB broth, along with PA14 bacteria in a glass test tube and incubated for 24 hours at 37°C with shaking at 200rpm.

In both cases, after a series of dilutions were made in order to be able to count individual colonies (CFUs) 100μl from the bacteria suspension mix was streaked in selective LB agar plates containing the antibiotic rifampicin (50μg/ml). Only *P. aeruginosa* is resistant to rifampicin, making it easier to count PA14 colonies. PA14 colonies were enumerated after an overnight incubation at 37 °C.

2.9 PA14 Pyocyanin Measurement

Freshly isolated bacterial single colonies on LB agar plates were inoculated in 3ml LB medium either in the absence or presence of 4% Glucose and cultured overnight at 37°C with shaking at 200rpm. Overnight bacteria cultures were measured with the spectrophotometer (OD_{600nm}) and diluted appropriately in order to have the same spectrophotometric measurements. Each bacteria culture was collected and centrifuged for 5min at 6010rcf. Two different experiments were performed concomitantly.

a) For starters, bacteria supernatant was used after being filtered by 0.2μm filters to remove any bacteria cells' residues. Filtered bacteria supernatant were mixed in 1:1 volume ratio with fresh LB broth (15ml each) along with PA14 bacteria (300μl) in 250ml flask containing a final volume of 30ml and incubated for 24 hours at 37°C with shaking at 200rpm.

b) Furthermore, bacteria supernatant was thrown away, the pellet was resuspended in 15ml fresh LB and then immediately heat-killed at 70°C for 10-18min using the water-bath. As previously, heat-killed bacteria were mixed in 1:1 volume ratio with fresh LB broth, along with PA14 bacteria in 250ml flask and incubated for 24 hours at 37°C with shaking at 200rpm.

For both experiments, supernatants were collected after centrifugation for 10min at 3381rcf. 4.5ml of chloroform was added to 7.5ml of supernatant and vortexed really well. (Chloroform goes to the bottom of the sample). Each sample was centrifuged again for 10min at 3381rcf. 3ml of the resulting blue layer at the bottom was transferred to a new tube. 1.5ml of 0.2M HCl was added to each tube and vortexed 2 times for 10 seconds. Samples were centrifuged for 2min at 3381rcf, and one layer of the pink layer was transferred to cuvettes for pyocyanin measurement. Pyocyanin concentration (μg/ml) was calculated by multiplying the spectrophotometric measurements taken an OD_{520nm} by 17.07, then multiplying them again by 1.5 due to the chloroform dilution.

2.10 LB/BHI Broth Media Preparation

LB (Candalab – Madrid, Spain) as well as BHI (brain heart infusion) broth (HIMEDIA – Mumbai/India) were prepared according to manufacturers.

20g LB powder / 1 litre ddH_2O and 37g BHI powder / 1 litre ddH_2O, respectively, were produced and autoclaved.Both media were kept under room temperature conditions and used for all experiments.

2.11 LB Agar Plates / LB Agar + Rifampicin Plates Preparation

LB agar (Invitrogen – Massachusetts, USA) plates were prepared according to manufacturers.

32g LB agar powder / 1 litre ddH_2O was autoclaved and used in plain petri dishes.

LB agar + Rifampicin plates were prepared as previously. In addition, 1ml of Rifampicin (1M) was added to the mixture after it was autoclaved. Plates were kept at 4° C until usage.

3. Results

3.1 The growth of *P. aeruginosa* strain PA14 is inhibited by supernatants of *Enterobacter aerogenes, Klebsiella* spp., *Acinetobacter baumannii* and *Providentia* spp., when cultured in the presence of glucose.

To assess whether *Serratia marcescens, Klebsiella* spp., *Enterobacter aerogenes, Acinetobacter baumannii, Salmonella paratyphi* and *Providentia* spp. can have a negative effect on PA14 similar to one found *in vitro*, supernatant of the 6 bacteria were cultured together with PA14 alive cells. CFUs were thoroughly calculated in LB plates specifically designed for PA14 with the addition of antibiotic Rifampicin, as PA14 is resistant to it. All bacteria tested were cultured in either the presence or absence of glucose to evaluate the impact of sugar fermentation by the bacteria on each other and each experiment was replicated twice in order to display consistency for our results. Figure 12 shows the total number of PA14 CFUs and how these can be affected upon interaction with the 6 bacteria. Consistently with previous experiments conducted, bacteria's primary growth seems to be a very important aspect to take into consideration. Absence of glucose does not seem to inhibit PA14 growth in any bacteria in both replicates as colony number per ml are approximately the same (Fig. B, 1-2). On the other hand, when 4% glucose is added to the culture media, PA14 growth is slightly impaired by *Providentia* spp. in both replicates, resulting in a ~1.30-fold inhibition. More promising results were shown upon PA14 interaction with *Klebsiella* spp, *Acinetobacter baumannii* and especially *Enterobacter aerogenes.* Despite the fact that in replicate 1 no proper dilutions were used, meaning that although we could see some inhibition, we could not measure it properly, we speculated that the PA14 CFUs can be less than 10^7 / ml. Use of lower dilutions to detect PA14 colonies in replicate 2, clearly showed that numbers of PA14 colonies had been decreased in half upon PA14 interaction *Klebsiella* spp with and *Acinetobacter baumannii*. As for *Enterobacter*, further dilutions (but still not the correct ones), manifested a striking inhibition in PA14 numbers from 'less than 10^7 to less than 10^3 per ml.

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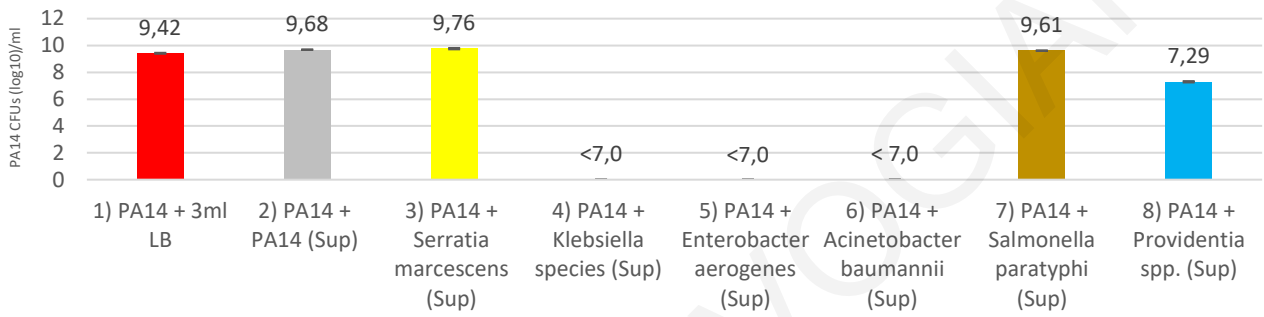

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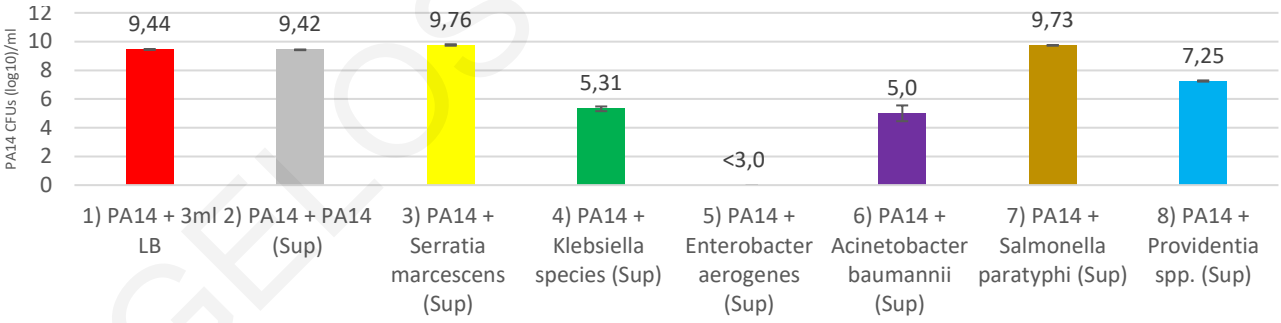

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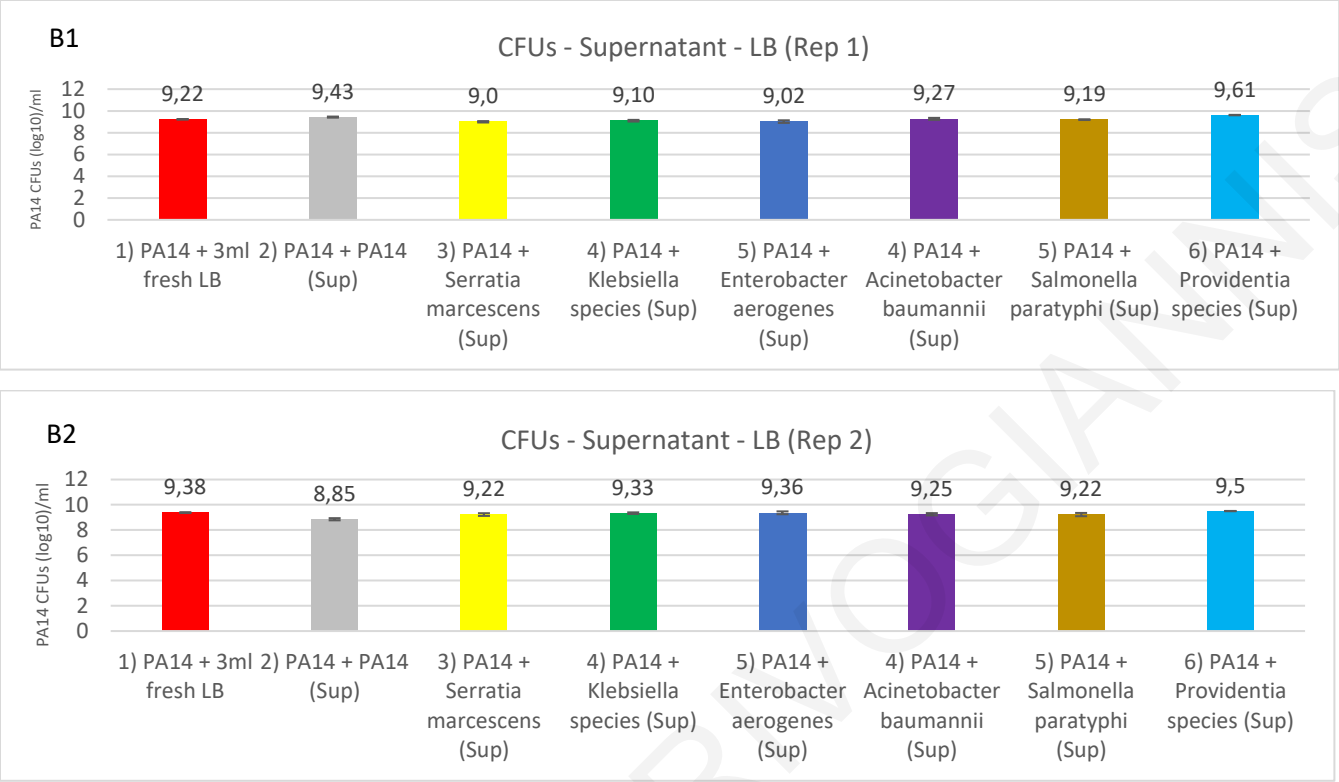
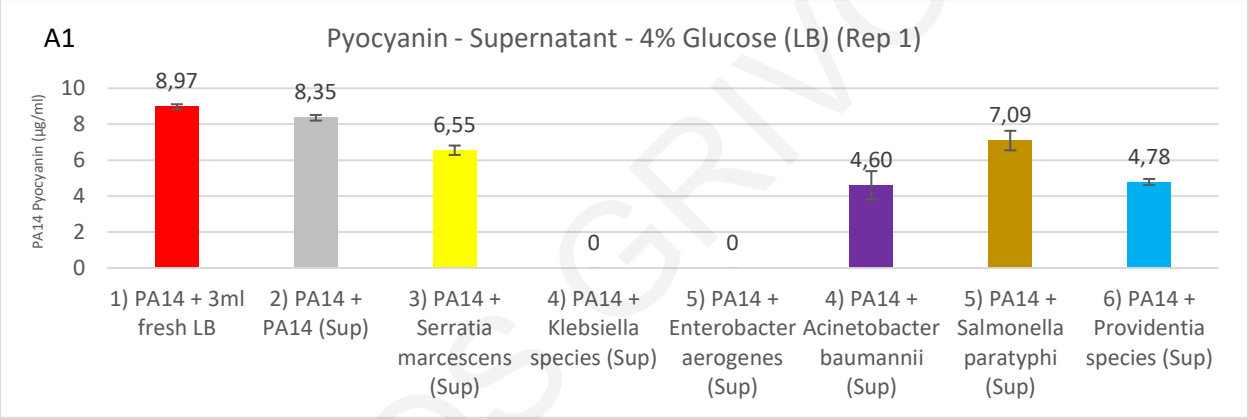


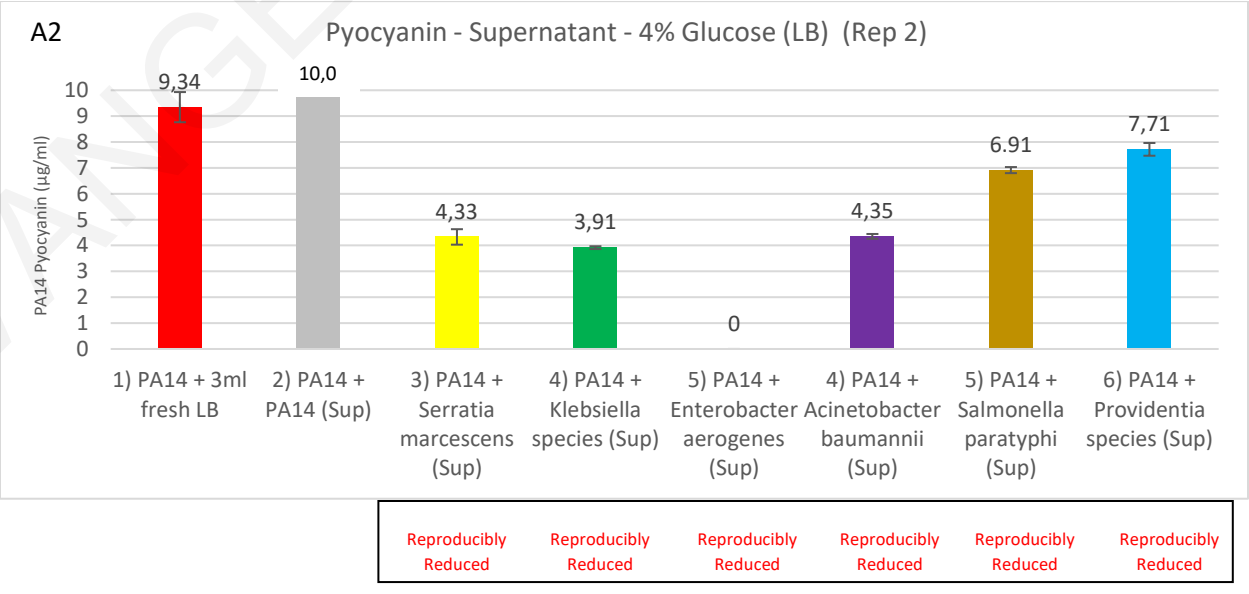
Figure 12. PA14 growth is inhibited by the supernatant of *Enterobacter aerogenes, Klebsiella* spp., *Acinetobacter baumannii* and *Providentia* spp., when cultured in the presence of glucose. Total numbers of CFUS count upon PA14 interaction with supernatant of *Serratia marcescens, Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii, Salmonella paratyphi* and *Providentia* spp. either in the absence or presence of glucose (A and B respectively). Each experiment was done twice (A1-,2 B1-2) [n=3] Error bars represent standard deviation of the mean.

3.2. Pyocyanin production by PA14 is inhibited by the supernatant of all virulent species tested, when cultured both in the presence and absence of glucose.

Counting PA14 CFUs was not the only method used for investigating bacterial interactions. In fact, the production pf phenazine Pyocyanin (μg/ml), produced only by *P.aeruginosa* was also being evaluated upon PA14 interaction with the other 6 virulent species (Figure 13). As previously, bacteria were grown both in presence of glucose or not and experiments were performed twice. As a general remark, all 6 of the bacteria seemed to exert a negative effect on PA14, resulting in decreased Pyocyanin levels. In contrast to the previous experiment, primary bacteria growth does not seem to be an issue, as the negative effect was manifested either when bacteria were cultured in medium with or without glucose. For the former case and to our surprise, *Enterobacter*

aerogenes seemed to be the most potent strains, as they completely inhibit Pyocyanin production. Not only that, but strikingly, the result was also being replicated the second time we performed the test. (fig. 13. A 1-2). After those, the most potent bacteria, are suggested to be *Klebsiella* spp. and *Acinetobacter baumanni* and *Serratia marcescens.* Unfortunately, we were unable to demonstrate twice that *Klebsiella* spp also, exhibited a detrimental effect on PA14, such as the one presented for *Enterobacter*, but it still produced an approximately 50% decrease in Pyocyanin production from 9,34 to 3,90 μg/ml. Lastly, interactions with both *Providentia* spp. and *Salmonella paratyphi* also resulted in a slight decrease of the PA14 toxin. When glucose was absent from the culture media, same 'potency' trend as previously was observed. In this case however, neither bacteria interaction resulted in a complete Pyocyanin production.





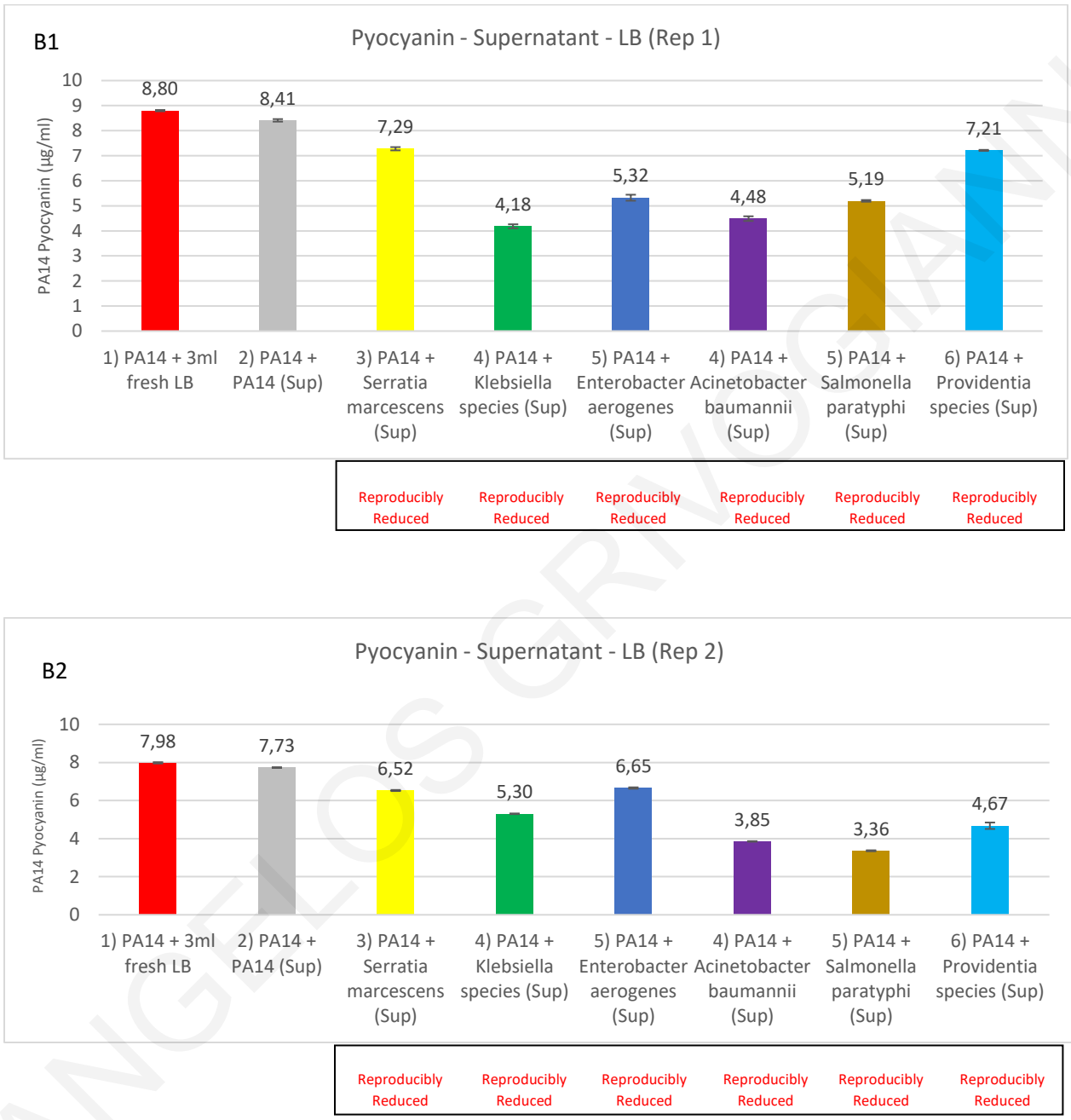
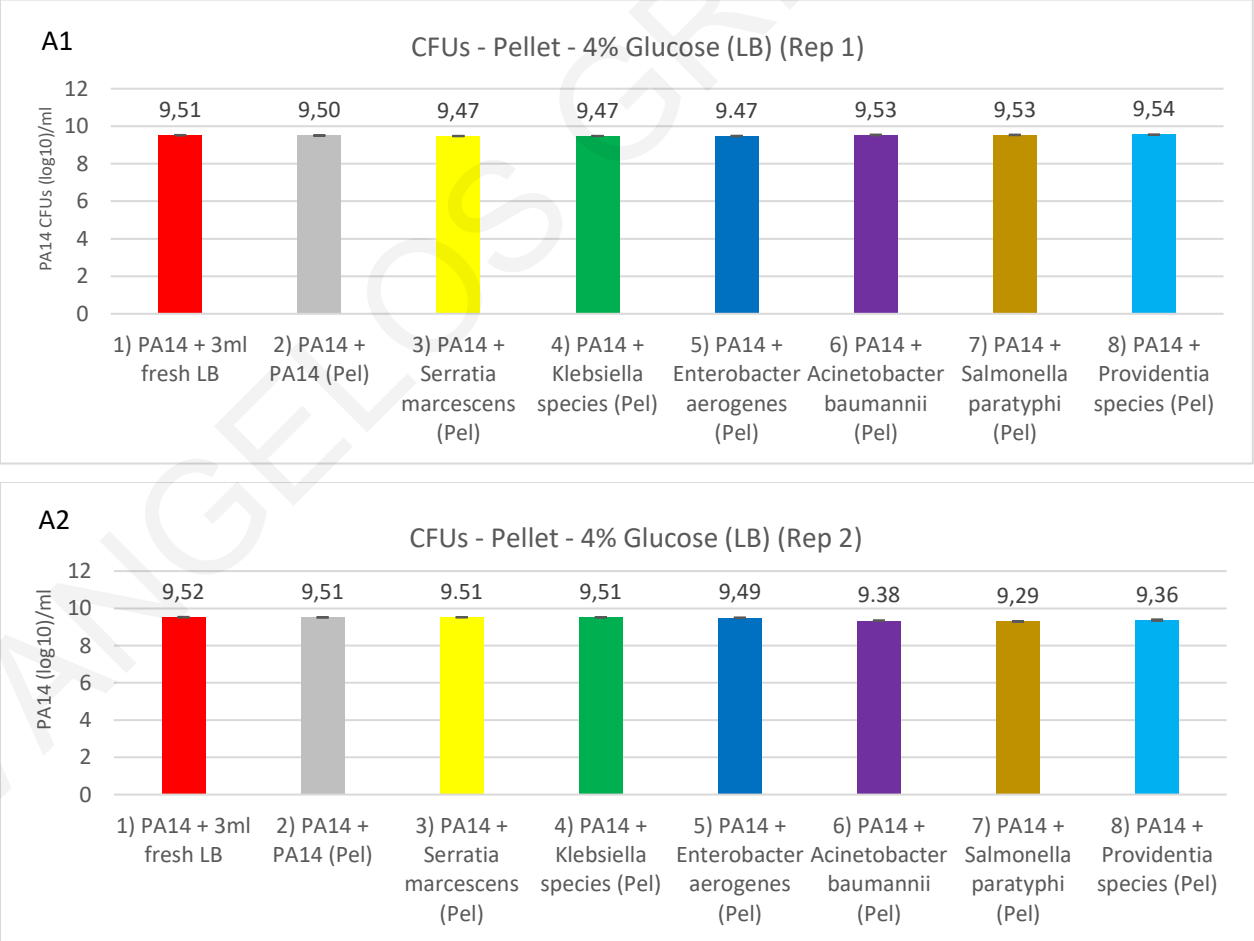


Figure 13. PA14 Pyocyanin production is impaired upon interaction of PA14 and supernatant of all the virulent species tested. Pyocyanin production was measured (µg/ml) upon different bacterial interactions, either in the absence or presence of glucose (A and B respectively). Each experiment was done twice (A1-,2 B1-2) [n=3] Error bars represent standard deviation of the mean.

3.3 PA14 growth is not inhibited by heat-killed bacteria (pellet) in the presence or absence of glucose.

Apart from the use of bacteria supernatants as a tool for assessing antagonistic interactions with PA14, due to secreted factors, we also wanted to examine the possibility that physical cell-to-cell contact might actually play a role in revealing new antagonist relationships. Thus, bacteria were heat-killed and interacted with alive PA14 cells in test tubes. As usually, bacteria were cultured in both presence and absence of glucose and each experiment was done twice. Interaction between PA14 and 'cell debris' from the 6 bacteria did not have any effect on PA14 growth, as the numbers for each case were approximately the same (~ 9.5(log 10)/ml) (fig 14). The same result was observed when pellet had been previously cultured in media both in the absence and presence of glucose.



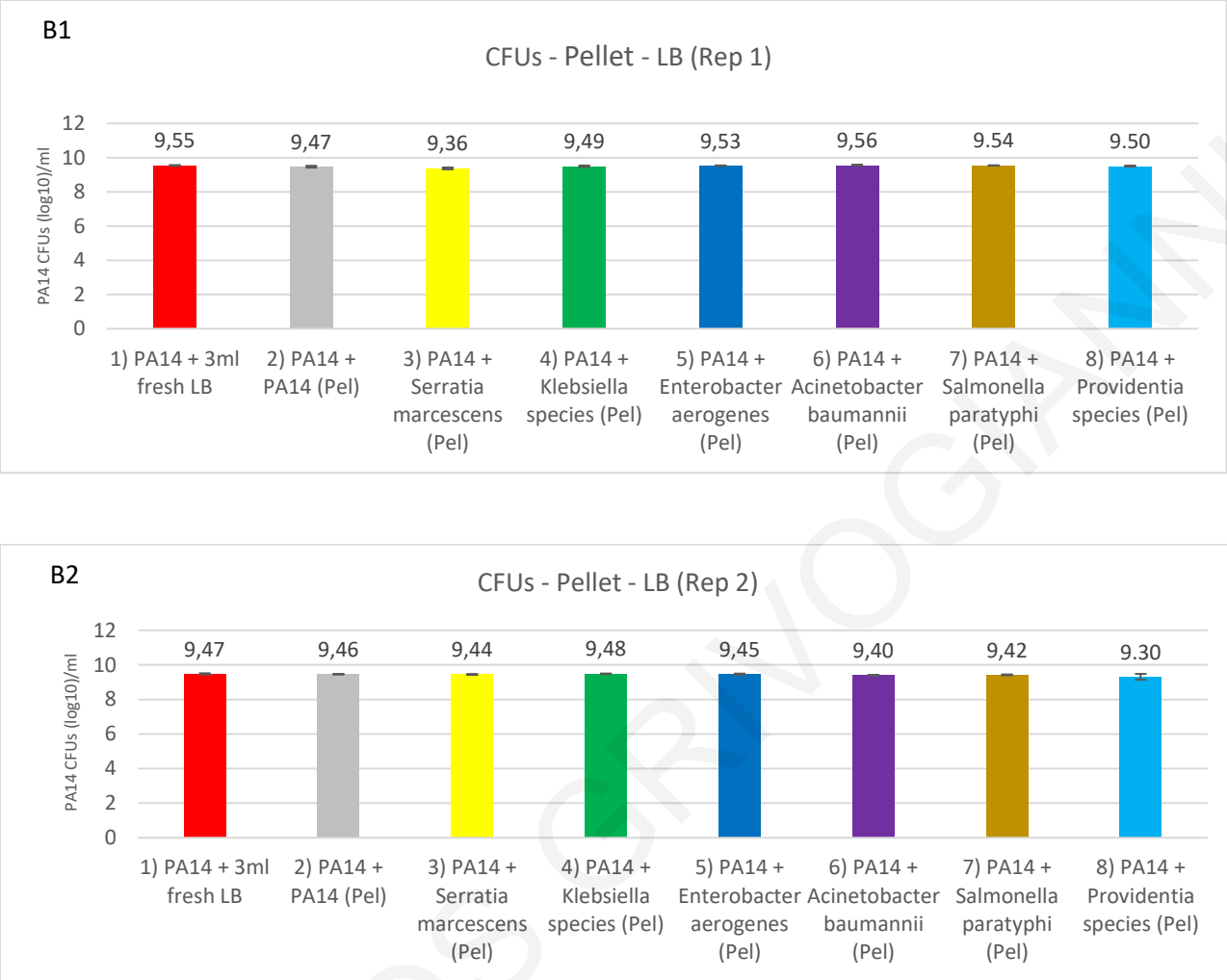
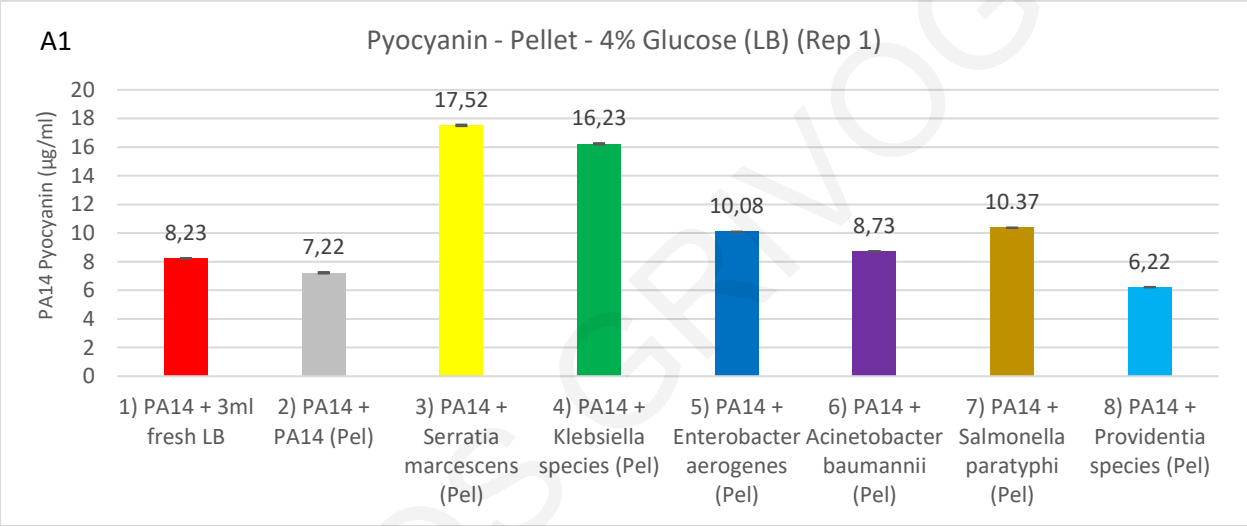


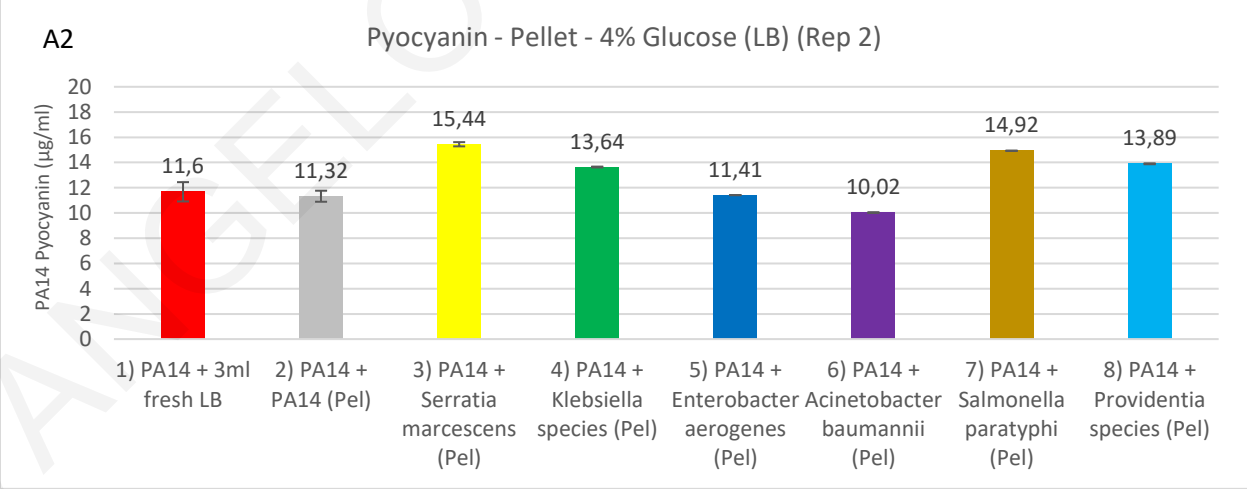
Figure 14. PA14 growth is not inhibited by heat-killed bacteria (pellet) in the presence or absence of glucose. PA14 CFUs count upon interaction of PA14 and heat-killed bacteria in the presence and absence of glucose. (A and B respectively). Each experiment was done twice (A1-2, B1-2) [n=3] Error bars represent standard deviation of the mean.

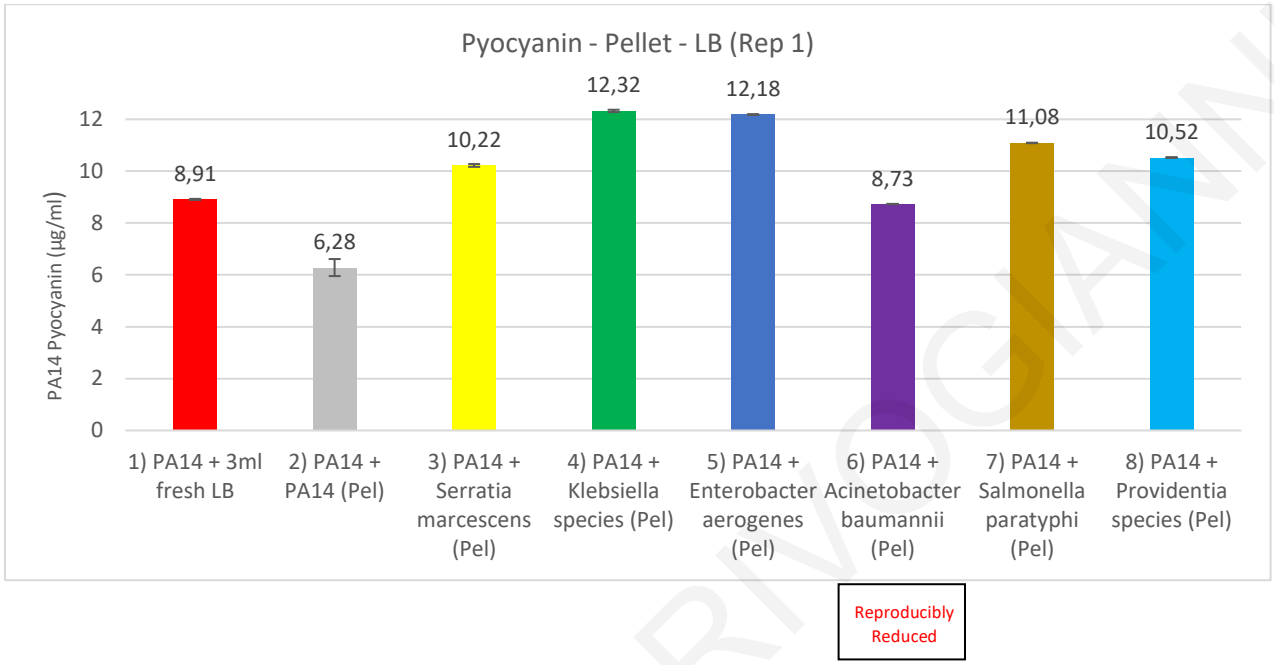
3.4 PA14 produced pyocyanin is slightly impaired by heat-killed bacteria of *Providentia* spp, and *Acinetobacter baumannii*, when cultured in the presence and absence of glucose, respectively.

The potential negative effect upon PA14 was also assessed by the measurement of its virulent toxin Pyocyanin , when killed heated bacteria interacted with PA14. Bacteria were cultured in both the presence and the absence of glucose, and each study was done twice. Unfortunately, as shown in

figure 15, our results in both conditions (with and without glucose), show great inconsistencies between the two replicates that performed. However, what could be extracted from these results is that *Providentia* spp. seemed to exert a small effect on PA14 as Pyocyanin production is slightly reduced (unfortunately shown only in replicate 1) when glucose is present (fig. 15 A1). When glucose is absent, PA14 interaction with *Acinetobacter baumannii* resulted to a modest Pyocyanin decrease.







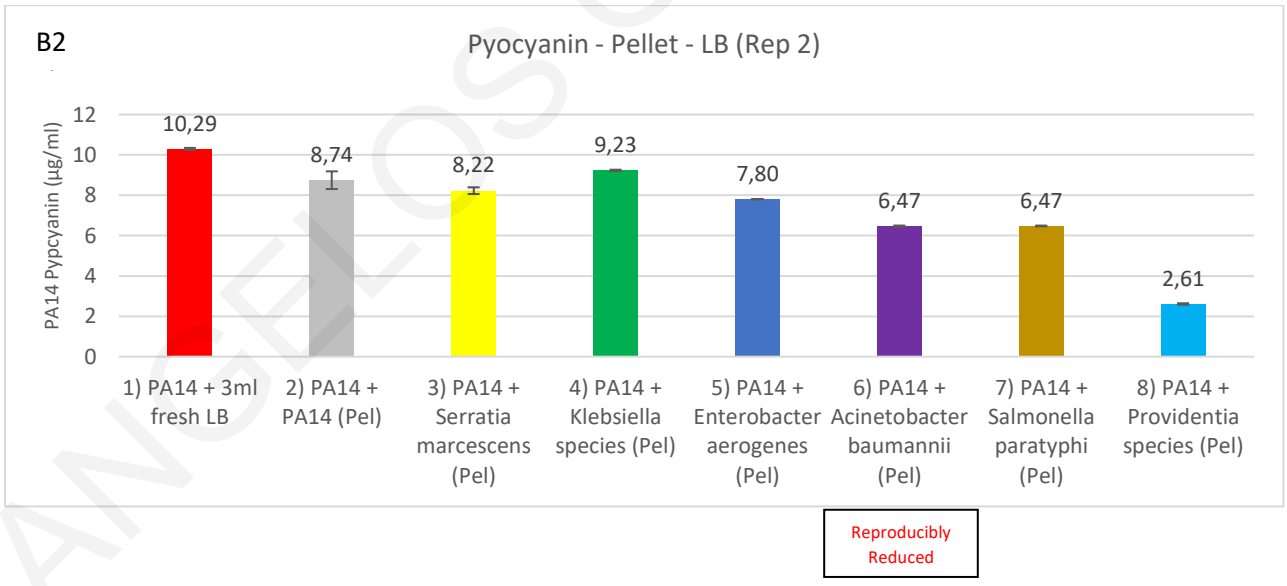


Figure 15. Pyocyanin is slightly reduced when heat-killed bacteria of *Providentia* spp. and *Acinetobacter baumannii* interact with PA14, in the presence and absence of glucose. Pyocyanin production was measured (µg/ml) upon PA14 and heat-killed bacteria interactions, either in the absence or presence of glucose (A and B respectively**).** Each experiment was done twice (A1-2, B1-2) [n=3]. Error bars represent standard deviation of the mean.

3.5 Under hypoxic conditions, PA14 growth is perturbed by the supernatants of *Bifidobacterium infantis, Lactobacillus plantarum* and *E. coli* MGH in the presence of glucose. Lastly, PA14 growth was also investigated, upon PA14 interactions with supernatants of *Klebsiella* spp., *Enterobacter aerogenes* and *Acinetobacter baumannii* along with anaerobe and Gram-positive bacteria *Bifidobacterium infantis, and Lactobacillus plantarum*, as well as the facultative anaerobe *E.coli* MGH, under hypoxic (5% O_2) conditions. Bacteria were once again grown with or without glucose and each experiment was done twice. Figure 16 shows how PA14 colonies are affected. In the absence of glucose (fig. 16 B1-2) all 6 bacteria do not exert any effect on PA14 as CFUs remain unchanged, and the same result is observed consistently in both replicates. Inversely, presence of glucose resulted in perturbation of PA14 growth by the supernatants of *Bifidobacterium infantis, Lactobacillus plantarum* and *E. coli* MGH. Both replicates show a consistent (approximately 50%) decrease of PA14 CFUs when interacted with *Bifidobacterium infantis*, making it a promising candidate for antagonistic relationships. In addition, a medium inhibition was also manifested twice upon PA14 interaction with *E. coli* MGH. Regrettably, the 50% perturbation of PA14 growth due to *Lactobacillus plantarum*, was not reproducible in the second replicate. Nevertheless, we succeeded in showing a medium inhibition, similar to the one exerted by *E. coli* MGH.

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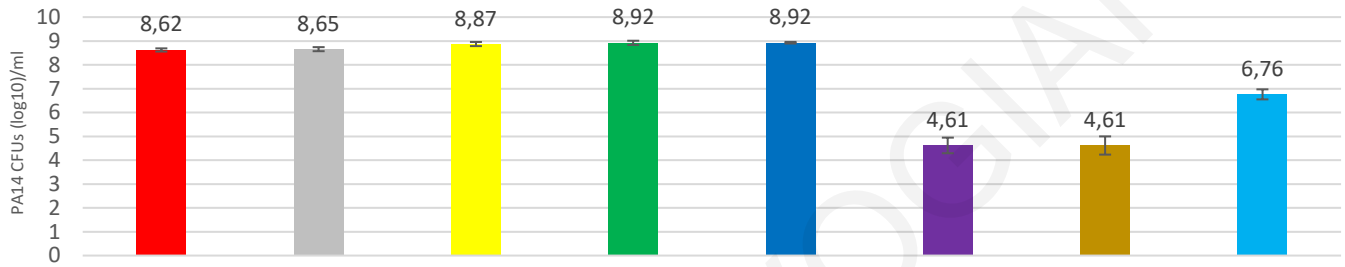

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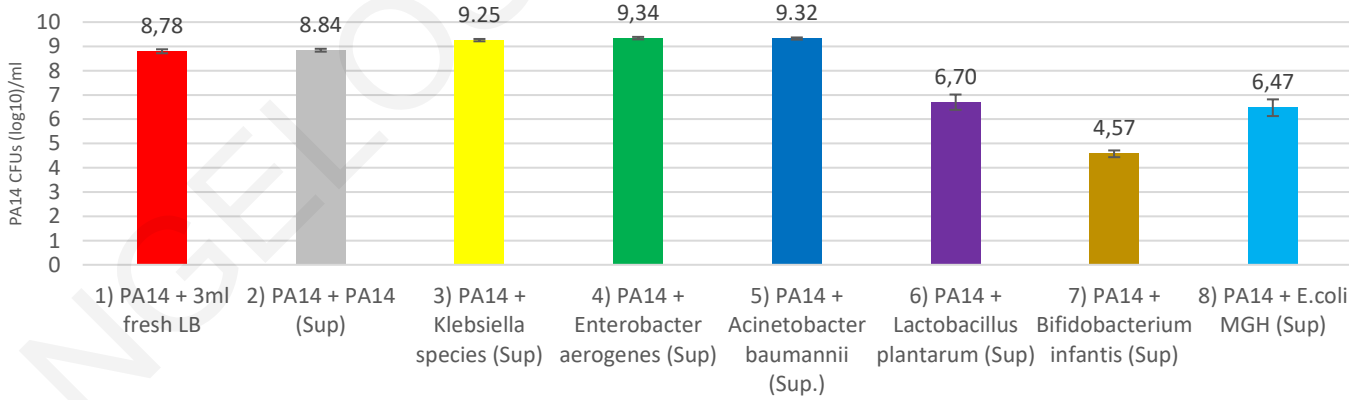

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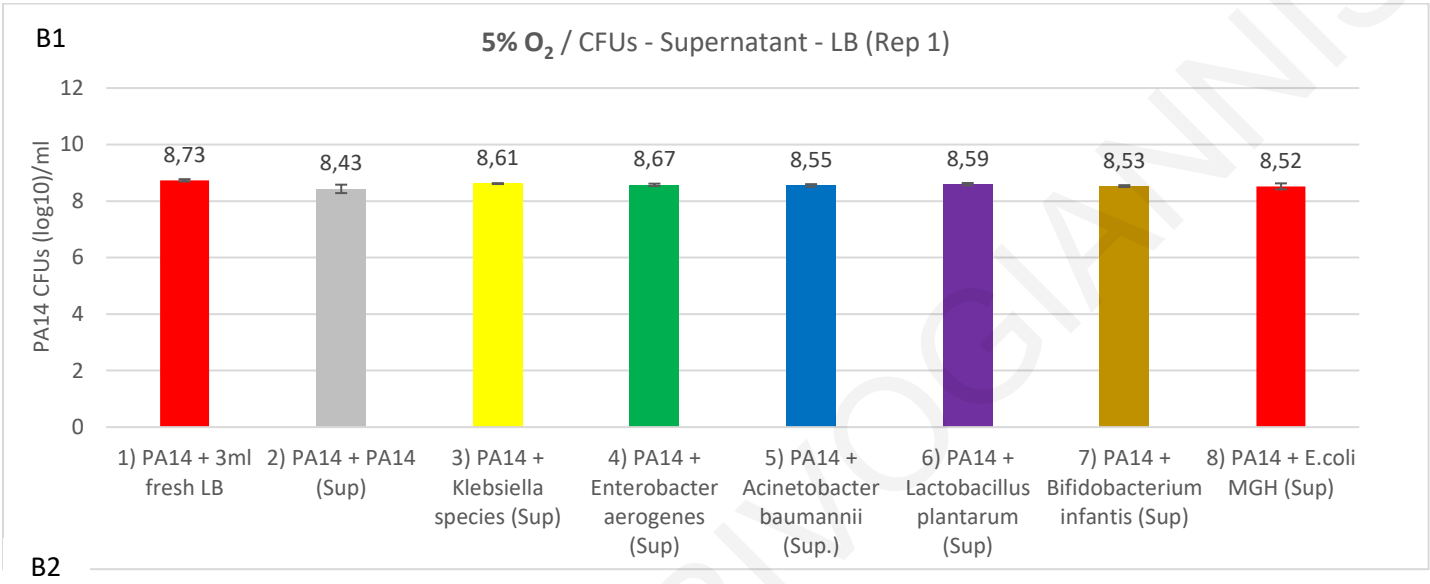

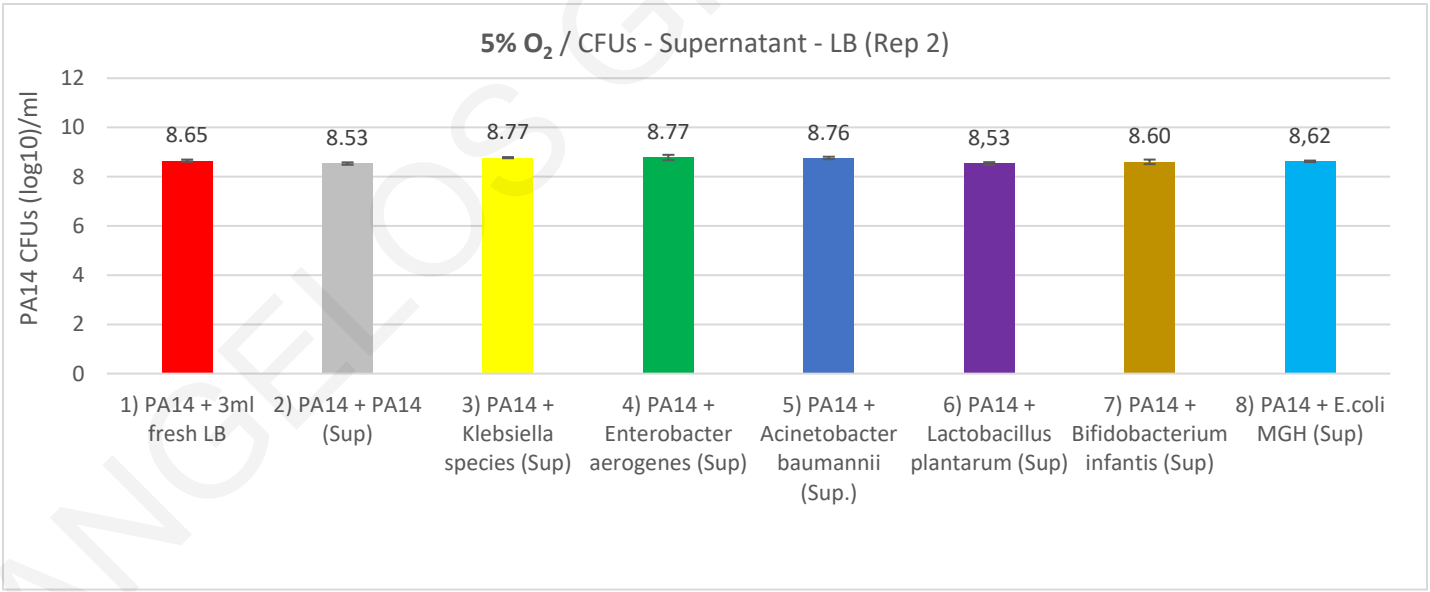

Figure 16. PA14 growth is inhibited under hypoxia by the supernatant of *Bifidobacterium infantis, Lactobacillus plantarum* and *E. coli* MGH in the presence of glucose. PA14 CFUs count upon interaction of PA14 and bacteria supernatant in the presence and absence of glucose, at 5% O_2 (A and B respectively). Each experiment was done twice (A1-2, B1-2). (n=3). Error bars represent standard deviation of the mean.

3.6 Mouse survival

Apart from our primary *in vitro study* to evaluate potential antagonistic interactions between PA14 and 6 different bacteria that exerted a strong negative effect *in vivo,* we also wanted to examine the possibility that the same bacterial interactions can affect mice's health in a positive, negative, or neutral manner. CD1 female mice were intranasally infected with *P. aeruginosa* strains PA14 with 2.5% glucose (ddH_2O), 4% sucrose (ddH_2O) and 4% sucrose (LB) as a vehicle, or B136-33 with 2.5% glucose (ddH_2O) and 4% sucrose (ddH_2O) as a vehicle, in order to assess mouse lung infection-driven mortality.

To investigate potential bacterial interactions, mice were co-infected with PA14 with both 4% sucrose (ddH_2O) and 4% (LB) sucrose used as vehicles along with supernatants and pellets, of either *E. coli* MGH or *Bifidobacterium infantis*. Co-infection between B136-33 with and 4% sucrose (ddH_2O) as a vehicle and *E. coli* MGH (pellet only) was also tested. LT50, the time it takes for half of the samples to be killed was used to have a better understating of the results.

3.6.1 Mouse lung infection with *P. aeruginosa* strain PA14 with 4% Sucrose (ddH_2O) as a vehicle is avirulent, with 4% Sucrose (LB) is virulent, while the *P. aeruginosa* strain B136-33 is highly virulent in 4% Sucrose (ddH_2O).

Figure 17.1 clearly shows that when mice were infected with PA14 and 4% sucrose (ddH2O), saves the mice from lung infection, while combination of PA14 with 4% sucrose (LB) results in death of all mice approximately 45 hours post-infection, with an LT50 (the time it takes for half of the mice to be killed) to be around 40 hours. On the other hand, B136-33 is very potent in in 4% sucrose (ddH2O). and kills the mice in half the time compared to PA14 (20 hours post-infection with an LT50 close to 15 hours). Furthermore, we found that combination of PA14 and 2.5% glucose (ddH_2O) is also avirulent, while the same combination with B136-33 resulted in marginal mortality (1 mouse death in a week)- data not shown.

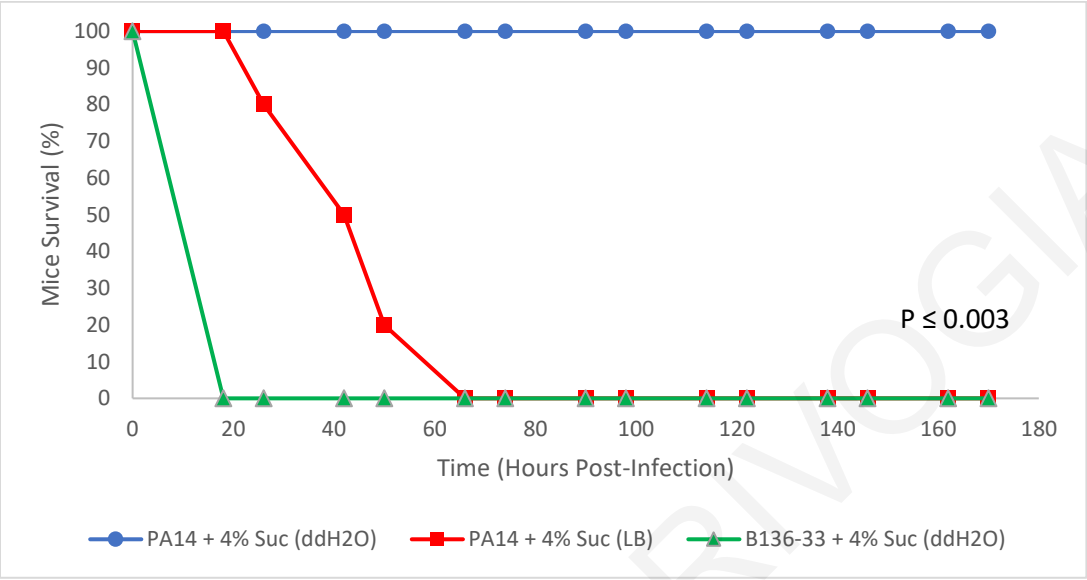

Figure 17.1. Mouse lung infection with *P. aeruginosa* PA14 with 4% Sucrose in ddH_2O as a vehicle is avirulent, with 4% Sucrose in LB is virulent, while the *P. aeruginosa* strain B136-33 is highly virulent in 4% Sucrose in ddH_2O. Mice survival upon infection with *P.aeruginosa strains* PA14 and B136-33. PA14 / B136-33 + 4% Sucrose (ddH_2O) [n=5]. PA14 + 4% Sucrose (LB) [n=10]. The P values between PA14 in 4% Sucrose (ddH_2O), PA14 in 4% Sucrose (LB), as well as B136 in 4% Sucrose (ddH_2O) are 0.001 and 0.003, respectively. The log-rank test was used to calculate P values.

3.6.2 Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose (LB) as a vehicle is alleviated when the vehicle contains supernatant of *E. coli* MGH grown in 4% Sucrose (LB).

Interestingly enough, when supernatant of *E.coli* MGH, cultured in the presence of 4% sucrose in LB, combined with PA14 with 4% sucrose (LB) as a vehicle, and co-infecting the mice, resulted in alleviation of mice mortality. The time it took for half of the mice to be killed by lung infection (LT50) was about 60 hours post-infection, compared to PA14 alone (approximately 40 hours).

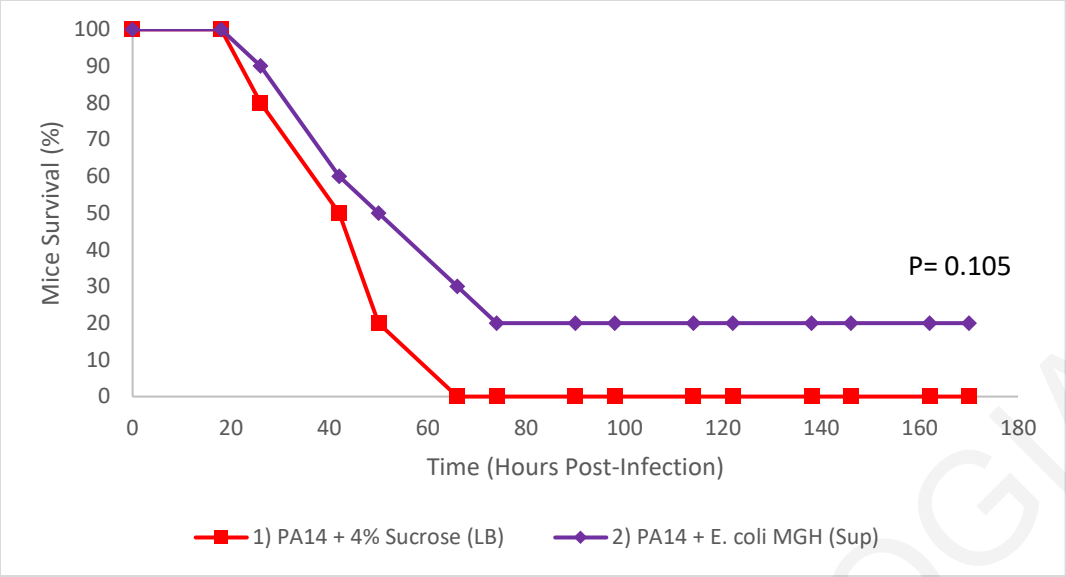

Figure 17.2. Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose in LB as a vehicle is alleviated when the vehicle contains supernatant of *E. coli* MGH grown in 4% Sucrose in LB. Survival of mice upon co-infection with *P.* PA14 and *E. coli* MGH. [n=10]. P value is 0.105. The log-rank test was used to calculate P values.

3.6.3 Mouse lung infection-driven mortality by *P. aeruginosa* B136-33 with 4% Sucrose (ddH$_2$O) as a vehicle is alleviated when the vehicle contains pellet of *E. coli* MGH.

In addition to the previous experiments, combination of pellets of MGH along with B136-33 was coinfected in the mice. Approximately half the mice remained alive after a week (figure 17.3) LT50 of the B136-33 with 4% sucrose (ddH$_2$O) as vehicle, was about 50, while the vehicle contains *E. coli* MGH pellet LT50 was 40.

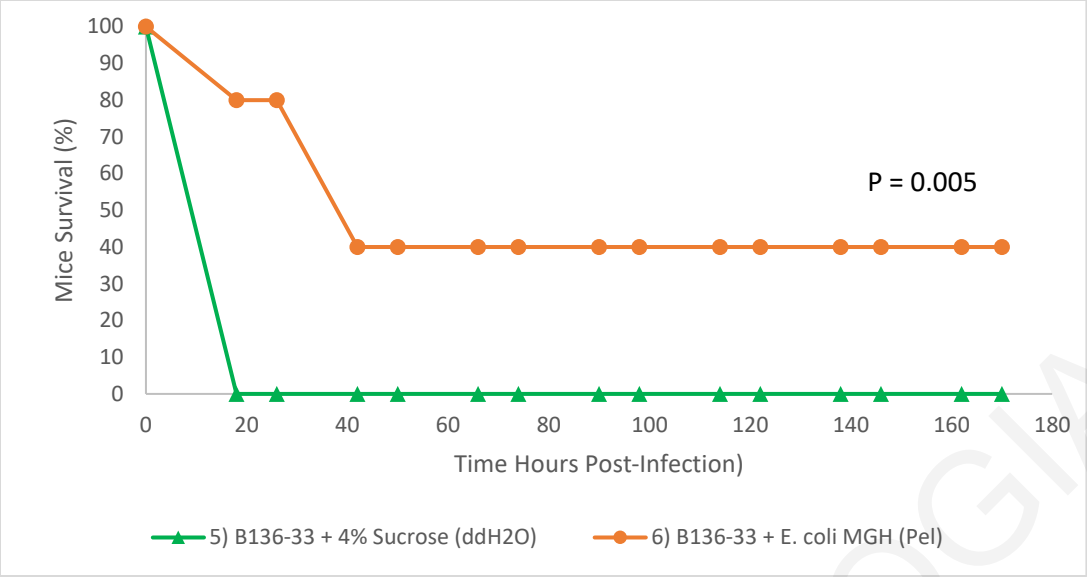

Figure 17.3 Mouse lung infection-driven mortality by *P. aeruginosa strain* B136-33 with 4% Sucrose in ddH_2O as a vehicle is alleviated when the vehicle also contains pellets of alive *E. coli* MGH. Survival of mice upon infection with B136-33. B136-33 + 4% Sucrose (ddH_2O) [n=5]. B136-33 + E. *coli* MGH (Pel) [n=10]. P value is 0.005. The log-rank test was used to calculate P values.

3.6.4 Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose (LB) as a vehicle is alleviated when the vehicle contains supernatant of *E. coli* MGH grown in 4% Sucrose (LB), but not when the vehicle contains supernatant of *B. infantis.*

Mice when co-infected with PA14 with 4% sucrose (LB) and the anaerobe bacteria *Bifidobacterium infantis*, as it exhibited a striking inhibition of PA14 CFUs *in vitro*. Unfortunately, the combination could not alleviate the mice from lung infection, showing an LT50 about 20 hours. On the other hand, when the vehicle contains supernatant of *E. coli* MGH PA14 mortality is alleviated. LT50: approximately 40 hours post-infection.(Figure 17.4).

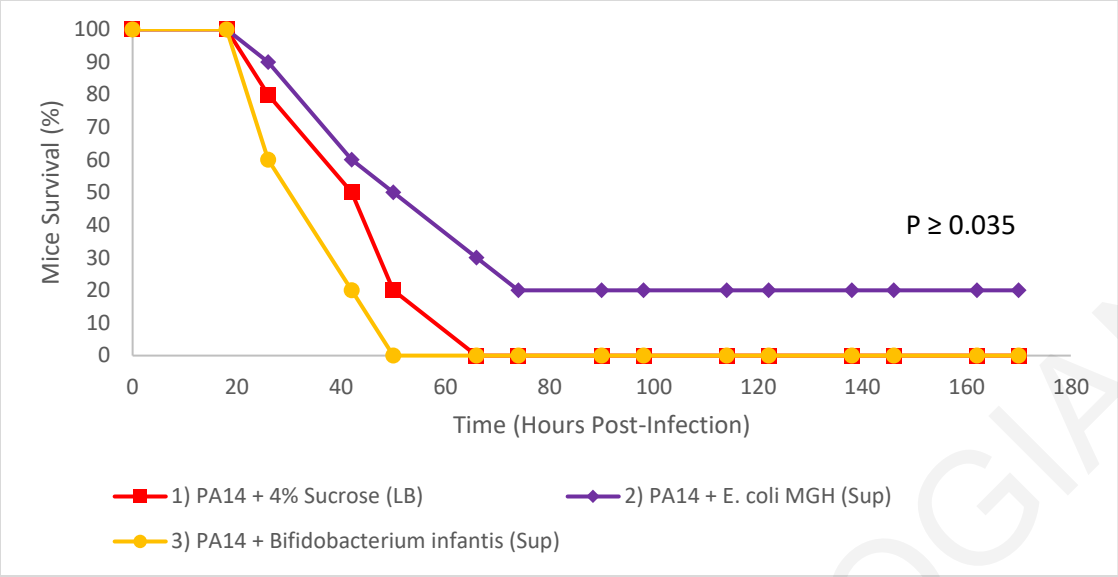

Figure 17.4. Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose in LB as a vehicle is alleviated when the vehicle contains supernatant of *E. coli* MGH grown in 4% Sucrose in LB, but not by supernatant of *B. infantis.* Survival of mice upon co-infection with PA14 along with *E. coli* MGH and *Bifidobacterium infantis*. PA14 + *Bifidobacterium infantis* [n=5]. PA14 + 4% Sucrose (LB) and PA14 + *E. coli* MGH (Sup) [n=10]. The P values between PA14 in 4% Sucrose (LB) and PA14 combined with *E. coli* MGH (Sup) as well as between the latter and PA14 along with *Bifidobacterium infantis* (Sup) are 0.105 and 0.035, respectively. The log-rank test was used to calculate P values.

3.6.5 Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose (LB) as a vehicle is not alleviated when the vehicle contains pellets of alive *B. infantis*, and it becomes more virulent with pellets from *E. coli* MGH.

Co-infection of mice with PA14 and pellets of *Bifidobacterium infantis* did not have the ability to diminish mouse lung infection (LT50 was about 40 hours). Surprisingly, co-infection with PA14 with 4% sucrose (LB) as vehicle and pellets of *E. coli* MGH seemed to be more virulent than the one containing PA14 as a vehicle, with an LT50 close to 18 hours post-infection (fig. 17.5).

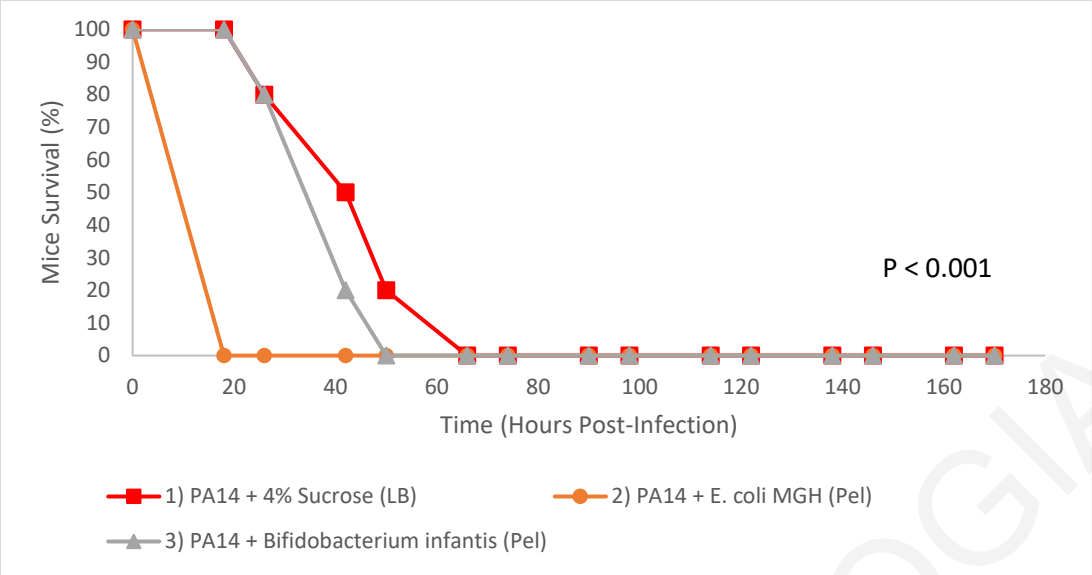

Figure 17.5. Mouse lung infection-driven mortality by *P. aeruginosa* PA14 with 4% Sucrose in LB as a vehicle is not alleviated when the vehicle contains pellets of alive *B. infantis,* and it becomes more virulent with pellets from *E. coli* MGH. Mice survival upon co-infection with PA14 along with *E. coli* MGH *and* Bifidobacterium infantis. PA14 + 4% Sucrose (LB) and PA14 + *E. coli* MGH (pel) [n=10]. PA14 + *Bifidobacterium infantis* (pel) [n=5]. The P value between PA14 in 4% Sucrose (LB) and PA14 combined with *E. coli* MGH (Pel), as well as, between the latter and PA14 along with *Bifidobacterium infantis* (Pel) is less than 0.001. The log-rank test was used to calculate P values.

3.7 Fly Survival

Flies infected with PA14 and pellets of *Klebsiella* spp., *Enterobacter aerogenes* or *E. coli* MGH, exhibit a significantly delay in fly mortality, while co-infection with PA14 along with pellets of *Acinetobacter baumannii, Salmonella paratyphi* or *Providentia* spp. results in only a slight delay.

Last but not least, another *in vivo* study by the use of *Drosophila melanogaster* was also conducted. Here, we tried to replicate the previous interesting survival fly experiment, in order to reveal any promising antagonistic interactions. The only difference is that it was performed upon hypoxic conditions (5% O_2). Flies were infected with PA14 alone and combination of PA14 and *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, *Providentia* spp., *Lactobacillus plantarum* and *E. coli* MGH. LT50 (lethal time 50), the time it takes for 50% of the flies to be killed was used as a tool. Figure 18.1 specifically depicts that when flies were fed a combination of PA14 and pellets of *Klebsiella* spp., *Enterobacter aerogenes* or *E. coli* MGH, significantly delay fly mortality for up to 5 days more. *Enterobacter aerogenes*, *Klebsiella* spp and *E. coli* MGH delayed death for 3, 4 and 5 days, respectively. Furthermore, LT50 for PA14 alone

was two days, while on the contrary, the LT50 for the 'rescuers' bacteria *Enterobacter aerogenes*, *Klebsiella* spp and *E. coli* MGH was approximately 4 days for the first two bacteria and approximately 6 days for MGH. Figure 18.2 proves that when flies co-infected with PA14 and pellets of *Acinetobacter baumannii, Salmonella paratyphi* or *Providentia* spp fly mortality also got a slight delay, but not as much as the previous one (0ne to two days- we called those bacteria 'marginal' effectors). The LT50 for those bacteria was close to 3. Finally, co-infection of PA14 with pellets both of *Bifidobacterium infantis* and *Lactobacillus plantarum* surprisingly revealed a slight increase in fly mortality compared to the one caused by PA14 alone. We called those bacteria 'No-effectors'. Their LT50 was 2.5 to 3, similar to PA14 when infected flies alone (figure 17.3).

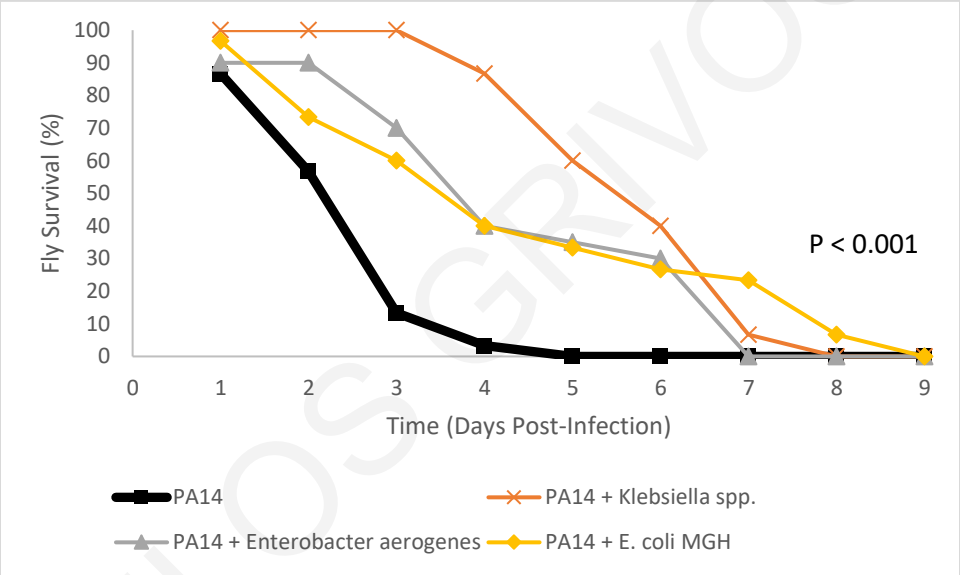

Figure 18.1. Flies infected with PA14 and pellets of *Klebsiella* spp. *Enterobacter aerogenes* and *E. coli* MGH *significantly* delay fly mortality caused by PA14 infection alone. Fly survival upon co-infection of PA14 with *Klebsiella* spp., *Enterobacter aerogenes* and *E. coli* MGH [n=20]. The P value between PA14 alone and all the rest PA14 combinations with *Klebsiella* spp., *Enterobacter aerogenes* and *E. coli* MGH) is less than 0.001. The log-rank test was used to calculate P values.

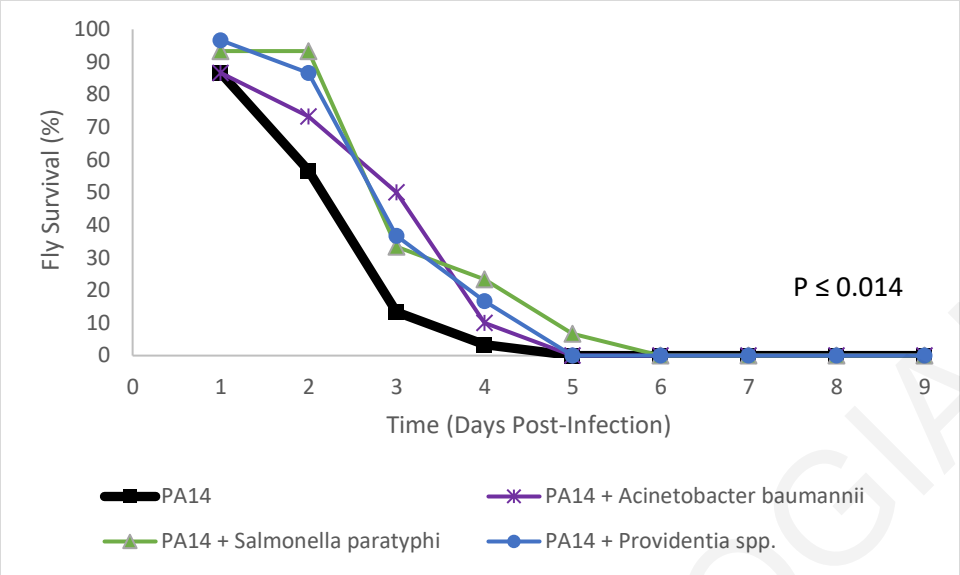

Figure 18.2. Flies infected with PA14 and *Acinetobacter baumannii*, *Salmonella paratyphi,* as well as *Providentia* spp. produces a slight delay in fly mortality caused by PA14 infection alone. Fly survival upon co-infection of PA14 with *Salmonella paratyphi, Acinetobacter baumannii* and *Providentia* spp. [n=30]. The P values between PA14 alone and PA14 combinations with *Salmonella paratyphi, Providentia* spp. and *Acinetobacter baumannii* are 0.001, 0.004 and 0.014, respectively. The log-rank test was used to calculate P values.

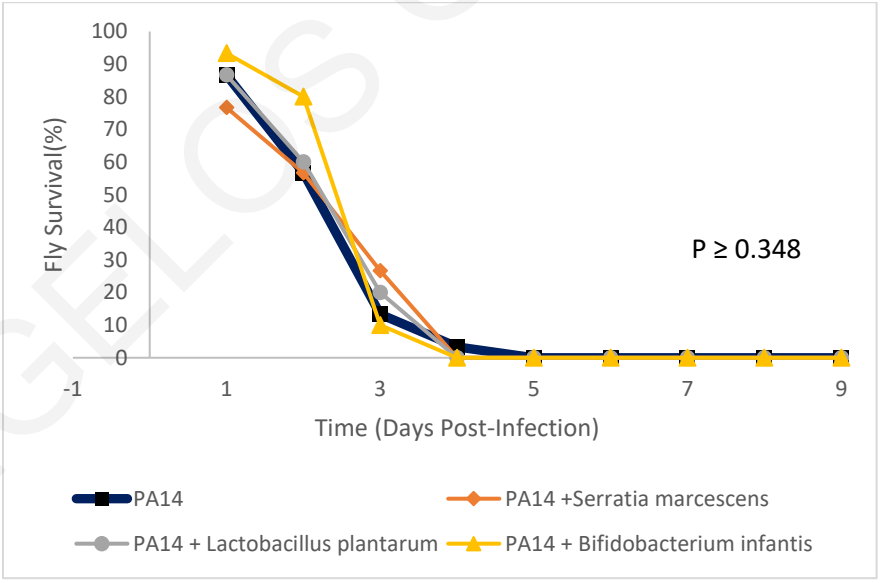

Figure 18.3. Flies infected with PA14 and *Serratia marcescens, Lactobacillus plantarum* and *Bifidobacterium infantis* slightly increase fly mortality caused by PA14 infection alone. Fly survival upon co-infection of PA14 with *Serratia marcescens, Lactobacillus plantarum* and *Bifidobacterium infantis.* [n=30]. The P values between PA14 alone and PA14 combinations with *Bifidobacterium infantis, Serratia marcescens* and *Lactobacillus plantarum* are 0.348, 0.744 and 0.771, respectively. The log-rank test was used to calculate P values.

4. Discussion

Microbial species including bacteria, archaea, fungi, protozoa, algae, and viruses, rarely live as 'independent' microorganisms both in nature and in potential hosts they affect. In contrast, all those different microbes live together and develop the so-called polymicrobial community, where constant interaction between all the different members occurs, in order to adapt better in the environment. Especially for bacteria, it has been found that the mechanism of quorum sensing is critical in cell-cell communication, in order to coordinate different actions, thus achieving 'goals' that they could not have the possibility to do if working alone, such as be more virulent to a specific host, or be more resistant to antibiotics for example. To make matters more complex, in some cases, bacteria can also interact with other microbes as well (Zhou et al., 2014), This makes it imperative to study extensively both (poly)microbe-microbe interactions, as well as the effect of those upon the host, since nowadays, studies have proven that most infections produced in humans are in fact polymicrobial, and not only related to only one pathogen. It has been distinctly manifested that there are 4 different types of microbe-microbe interactions, mainly additive, synergistic, neutral all even antagonistic, each proving to be important in different aspects for host's health.

Despite the fact that all 4 types of interactions are crucial for better understanding of the microbiome in general, we decided to focus mainly on revealing potential antagonistic interactions specifically between bacterial strains that colonize the human intestine. We also wanted to evaluate how these interactions can specifically affect different hosts, such as *Drosophila melanogaster* Based on previous *in vivo* studies in our lab revealing antagonistic interactions with strong negative effect between *Pseudomonas aeruginosa* strain PA14 and *Serratia marcescens*, *Klebsiella* spp., *Enterobacter aerogenes*, *Acinetobacter baumannii*, *Salmonella paratyphi*, and *Providentia* spp, the primary goal of this project was to investigate whether similar interactions can also occur *in vitro.* PA14 growth and Pyocyanin production (one of the most virulent PA14 factors) were substantially examined when PA14 interacted with both supernatants and heat-killed bacteria. An effect due to bacteria supernatant (indirect interaction) would mean that a specific factor of the antagonizing bacteria is secreted, leading to the effect itself. So, a future experiment could focus on the other 6 strains in finding which substrates are actually important for the interaction and

maybe design a new antimicrobial drug for example. Furthermore, direct cell-cell contact (pellet) was investigated, as there is a possibility that this physical contact with alive cells and cell debris can also exert an antagonistic effect. As another potential experiment we could also have co-cultures of both alive cells *in vitro* in order to assess potential PA14 growth or Pyocyanin impairment. All bacteria tested were also cultured in different media. One containing only the standard LB and the other one containing 4% glucose in LB. This was done to assess any potential involvement of sugar fermentation from bacteria. Interestingly, recently our lab has shown that *E. coli* can be used as a 'health promoting microbe against intestinal *P. aeruginosa* (Christofi et al., 2019), when *E.coli* is able to ferment glucose to lactic and acetic acid, either aerobically or anaerobically.

More specifically indirect PA14 interaction with all the 6 bacteria tested, do not exert any effect on PA14 when sugar is absent. Conversely, adding sugar to the medium resulted in PA14 growth inhibition by *Enterobacter aerogenes, Klebsiella* spp., *Acinetobacter baumannii* and *Providentia* spp. As a previous lab study has revealed, bacteria glucose fermentation to lactic and acetic acid is crucial for the production of antagonistic effect. In order to validate those results we could reproduce the whole experiment, by interacting PA14 with mutant bacteria strains of the above bacteria, to see whether those strains could exert the same result or not (in case fermentation is not the only key aspect to consider). As it was the first time to handle bacteria in general, in replicate 1 only 1:10000000 dilution was tested in an LB Agar+Rifampicin plates. This is why we could not detect the exact numbers of PA14 CFUs and rather just speculate about it. On the contrary, in replicate 2 different dilutions (up to 1:1000) were tested in order to count PA14 colonies. While it gave definitive numbers for *Klebiella* spp. and *Acinetobacter baumanni*, the exact PA14 colony numbers after interaction with the most promising candidate (*Enterobacter aerogenes*) still remained elusive. As a potential future work, we could use a less diluted sample from *Enterobacter* on the plate to count the colonies, resulting in exact numbers. Pyocyanin, the most virulent factor of PA14 produced during quorum sensing, seems to be affected by supernatant of all bacteria tested both in the absence and presence of glucose 4%. The fact thar presence of glucose resulted in Pyocyanin inhibition was further validated with a previous experiment done in our lab reported that facultative anaerobe *E. coli* MGH inhibits Pyocyanin production in the presence of glucose. However, in contrast to our aerobic bacteria that we used extensively, MGH cannot inhibit Pyocyanin when glucose is not being fermented to lactic and acetic acid as mentioned previously.

Enterobacter aerogenes seems to inhibit Pyocyanin completely, making it a very promising tool, in the designing of new antibiotics for inhibit PA14.
Cell to cell 'physical' contact between alive and heat-killed bacteria, either with or without glucose does not seem to affect PA14 growth. This strange result was validated as the second replicates for both absence and presence of glucose 4% showed the same outcome as in replicate 1.

Investigating whether Pyocyanin is impaired when PA14 interacts with pellets seemed a little trickier than before, as this time was out first to implement the protocol that we were given. This can clearly be shown in both in presence and absence of 4% glucose where too many inconsistencies occur are viable. (*There were some samples that were approximately the same). At least, we were able to show that Pyocyanin is slightly inhibited heat-killed bacteria of *Providentia* spp, and *Acinetobacter baumannii*, when cultured in the presence and absence of glucose, respectively. Due to time constrains we did not have the time to do another replicate properly, so that would be an imminent experiment.

Finally, when bacteria supernatants were interacted with PA14 under hypoxic conditions, only the two Gram-positive anaerobic bacteria *Lactobacillus plantarum* and *Bifidobacterium infantis*, as well as the facultative anaerobe *E. coli* MGH, exerted a negative effect (inhibition of PA14 growth), in the presence of glucose only, (making sugar fermentation an important aspect once again.

Apart from our primary study of this project that contained *in vitro* experiments we also wanted to investigate potential antagonistic microbe-microbe interactions and their effect in host directly, by the use of *Mus musculus a*nd *Drosophila melanogaster.*

As far why mouse is a great model organism to study *P. aeruginosa*, PA14 has been shown to exert chronic lung infection (Bayes et al., 2016; Hoffmann et al., 2005) that leads to mice mortality. On top of that, Pyocyanin is critical for this kind of infection. (Lau et al., 2004). Our results revealed that when mice were infected only with *P. aeruginosa s*trains PA14 and B136-33 with different media as vehicle, different outcomes occur. Addition of LB to the medium, results in a more rapid death of mice. Most importantly, when supernatant of *E. coli* MGH is co-infected with PA14 and B136-33, the mice are rescued in both cases, as opposed combination of PA14 with pellet of *E. coli* MGH, which makes Mouse lung infection-driven mortality by *P. aeruginosa* PA14,

much more virulent. Thus, two new mice survival studies can be easily designed and performed. The first design would include 4 different conditions. Mice will be infected with 1) Mice will be infected B136-33 with 4% sucrose (ddH_2O) as a vehicle, in order to replicate the high virulence that found in the previous experiment, 2) *E. coli* MGH (pel) with 4% sucrose (ddH_2O) as a vehicle, 3) Co-infection with B136-33 and *E. coli* MGH (pel), and 4) B136-33 co-infection along with *Bifidobacterium infantis* The second study would include 4 new conditions including PA14 and *E. coli* strain BW25113, as well as mutant strains of *E coli* that cannot ferment glucose, such as pgi and ldhA. Mice will be infected with 1) PA14 in 2.5% glucose LB as a vehicle, 2) PA14 co-infected with BW25113, 3)PA14 co-infected with pgi and 4) PA14 co-infected with ldhA.

As far as for the second *in vivo* study with the use of *Drosophila melanogaster* is concerned , we did manage to show some promising results under hypoxic conditions, replicating some antagonistic interactions from the previous test. However, due to time constraints two limitations have to be resolved in order to be surer of our results. Firstly, as it was the first time to handle and maintain flies, we can speculate that something might got wrong during the process of the survival assay. Thus, we have to repeat the experiment at least once more when we have time. Secondly, despite the fact that we were able to show that antagonistic interactions occur likewise *in vivo*, by finding rescuers, marginal effectors, and no effectors whatsoever, we could not 'quantify' the negative effect that the bacteria exerted upon PA14. In order to do that properly, flies have to infected alone with PA14 / each bacteria we want to test in order to know how virulent they are under hypoxic conditions. After that, flies would be co-infected with PA14 along with each of the bacteria to see the effect of combination and quantify how strong or medium this effect can b

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