Investigating microbial antagonism by probiotics using *Caenorhabditis elegans* as a model organism

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Abstract

There are over 175 species of *Lactobacillus* and many of them can be found in our bodies. For example, *Lactobacillus* can be found in our intestines as they attach to the cells and mucous of the intestinal lining. Due to the health benefits that *Lactobacillus* brings, it is ubiquitous to consider the use of *Lactobacillus* as a cure for several medical conditions, such as diarrhoea, lactose intolerance and eczema. This project aims to investigate if *Lactobacillus plantarum* and *Lactobacillus acidophilus* have antimicrobial effects against the bacteria *Serratia marcescens* and *Staphylococcus epidermidis,in* the *in vitro* well diffusion and colony count tests, and if these *Lactobacillus* strains are able to restore survival and locomotion of *C. elegans* infected with *S. marcescens* and *S. epidermidis* in the *in vivo* test. The results indicate that *L. plantarum* and *L. acidophilus* do possess antimicrobial effects against the bacteria *S. marcescens* and *S. epidermidis* and are able to restore survival and locomotion of *C. elegans* infected with *S. marcescens* and *S. epidermidis*. The results of this study have shown the potential application of lactic acid bacteria in antagonism against opportunistic pathogens. Hence, they provide a natural and more cost effective alternative to using antibiotics.

1. Introduction

There are over 175 species of *Lactobacillus* and many of them can be found in our bodies. For example, *Lactobacillus* can be found in our intestines as they attach to the cells and mucous of the intestinal lining, becoming part of our gut microbiome. Since humans provide nutrients and shelter for *Lactobacillus*, in turn they help in digestion of food, absorb nutrients, and fight off harmful bacteria that might cause diseases, resulting in a mutualistic relationship. Due to the health benefits that *Lactobacillus* brings, it is ubiquitous to consider the use of *Lactobacillus* as a cure for several medical conditions, such as diarrhoea, lactose intolerance and eczema. *Lactobacillus* is used in the production of yogurt, cheese and soy products.

There is growing awareness of the health-promoting effects of probiotics for humans in pharmaceutical industries. According to Guantario et al. (2018), the definition of “probiotic” was formulated by international scientists working on behalf of FAO/WHO (Food and

Agriculture Organization/World Health Organization), and refers to viable, non-pathogenic microorganisms, that, when ingested in adequate amounts, are able to reach and colonize the gastrointestinal tract and to confer health benefits to the host. Karimi, Rashidian, Birjandi, and Mahmoodnia (2018) reported that probiotics are beneficial bacteria which are natural flora of the digestive system, and in controlled amounts, can be beneficial to human health. Probiotics have been associated with immunomodulation, digestion, production of short-chain fatty acids and essential vitamins, and competitive exclusion of pathogenic microorganisms (Kim et al., 2019).

According to a study done by Canganella et al. (1997), one of the probiotic strains that produced the best results in terms of growth rate, pH and bile salt tolerance was *Lactobacillus rhamnosus*. The antagonism of a mixed culture of *L. acidophilus* and *Enterococcus faecium* against the pathogen *Yersinia enterocolitica* was demonstrated and attributed to either acid or antimicrobials production (Canganella et al., 1997).

One key application of probiotics in microbial antagonism is in the prevention of diseases of the digestive tract such as diarrhoea, which is one of the leading death causes in children in developing countries. With the development of more effective probiotics, more lives can be saved. According to a study by Choi, Patra, Kim, and Kang (2018) antagonistic ability of the lactic acid bacteria is an important factor for the evaluation of probiotics. The antagonistic ability of the lactic acid bacteria is measured by its adhesion to the intestine, reduction of pathogenic bacterial adhesion to the intestine, aggregation and coaggregation as well as production of antimicrobial substances such as bacteriocins. To this end, research on *Lactobacillus spp.* has displayed great potential in the management of urinary tract infections (UTIs). Clinical studies have found that the oral administration of certain *Lactobacillus spp.* was capable of resolving UTIs through providing patients with *Lactobacillus spp.* suspended in skim milk twice a day for 14 days (Reid et al., 2006).

The nematode *Caenorhabditis elegans* presents itself as a suitable model host to uncover new antimicrobial drugs due to its genetic ortholog similarity to humans, display of extensive host immune responses and ease of use in laboratories. According to Baumeister and Ge (2002), *C. elegans* is chosen as a model organism due to its genotypic similarity to humans, which makes it a very useful model representation for human diseases. We therefore propose the use of *C. elegans* nematodes as an in vivo host platform to investigate the antimicrobial effectiveness of *Lactobacillus casei* and *L. acidophilus* on *S. marcescens* and *S. epidermidis.*

2. Objectives and hypotheses

The objectives of this project are to determine if culture supernatants of *L. plantarum* and *L. acidophilus* have antimicrobial effects against the bacteria *S. marcescens* and *S. epidermidis* in the in vitro well diffusion and colony count tests, and if *L. plantarum* and *L. acidophilus* are able to restore survival of *C. elegans* infected with *S. marcescens* and *S. epidermidis* in the i*n vivo* antibacterial test.

It is hypothesized that culture supernatants of *L. plantarum* and *L. acidophilus* have antimicrobial effects against the bacteria *S. marcescens* and *S. epidermidis* in the *in vitro* well diffusion and colony count tests, and *L. plantarum* and *L. acidophilus* are able to restore survival of *C. elegans* infected with *S. marcescens* and *S. epidermidis* in the *in vivo* antibacterial test.

3. Methods and Materials

Growth of pre cultures of microorganisms

Escherichia coli OP50 and the test organisms (*S. epidermidis* ATCC12228 and *S. marcescens* Carolina) were inoculated into 10 ml of LB (Luria-Bertani) broth. Lactic acid bacteria were inoculated in 10 ml of MRS broth. The cultures were grown overnight at 30°C in a shaking incubator. The absorbance of the pre cultures at 600 nm was measured using a UV-vis spectrophotometer and standardised at 0.8.

Antibacterial test using the *in vitro* well diffusion method

The test bacterial culture was spread evenly on Mueller-Hinton agar plate with a sterile swab. A sterile pipette was used to create wells in the agar.

The culture supernatants of lactic acid bacteria culture were obtained by centrifugation at 8000 rpm for 10 min. 80 µl of culture supernatant was added to the well. The positive control was 10% bleach and the negative control was MRS broth. The plates were incubated at 30°C overnight and the diameter of the zone of inhibition was measured the next day. Five replicates were conducted.

Antibacterial test using the *in vitro* colony count method

In the test setup, 0.1 ml of bacterial culture was added to 1.0 ml of lactic acid bacteria culture supernatant and 3.9 ml of LB broth. In the control setup, 1.0 ml of MRS broth was added instead of culture supernatant. Five replicates for each setup were prepared.

The mixtures were incubated with shaking at 30°C overnight. Serial 10-fold dilutions were performed with 0.85% sodium chloride solution to the appropriate dilution factor, and 0.1 ml of the diluted culture was spread on LB agar. Plates were incubated at 30°C overnight. The number of colonies was determined the next day.

Preparation of NGM (Nematode Growth Medium)

0.9 g NaCl, 7.5 g agar, 0.75 g bacto peptone was added to 291.6 ml water. After autoclaving at 15 psi for 15 minutes, 0.3 ml of cholesterol (5 mg/ml), 0.3 ml of $MgSO_4$ solution (1 M), 0.3 ml $CaCl_2$ solution (1 M), 7.5 ml of KH_2PO_4 solution pH 6.0 (1M) were added.

***C. elegans* survival assay (*in vivo* antibacterial test)**

The NGM plate was added with the following and grown overnight at 30°C.

50 µl of *E. coli* OP50 culture (control 1)
50 µl of lactic acid bacteria culture (control 2)
25 µl of *E. coli* OP50 culture mixed with 25 µl of test organism culture (control 3)
25 µl of lactic acid bacteria culture mixed with 25 µl of test organism culture (test)
A block of agar containing *C. elegans* wild type strain N2 was cut and placed in the centre of the NGM plates. The plates were incubated at 20°C for 2 days. Five replicates were performed. The percentage survival of worms were determined after 2 days. Worms were considered dead if they do not move.
In all tests, temperature of growth of bacteria, absorbance of bacterial pre-cultures at 600 nm, and duration of growth of bacteria were kept constant.

4. Results and Discussion

Antibacterial test using the *in vitro* well diffusion method

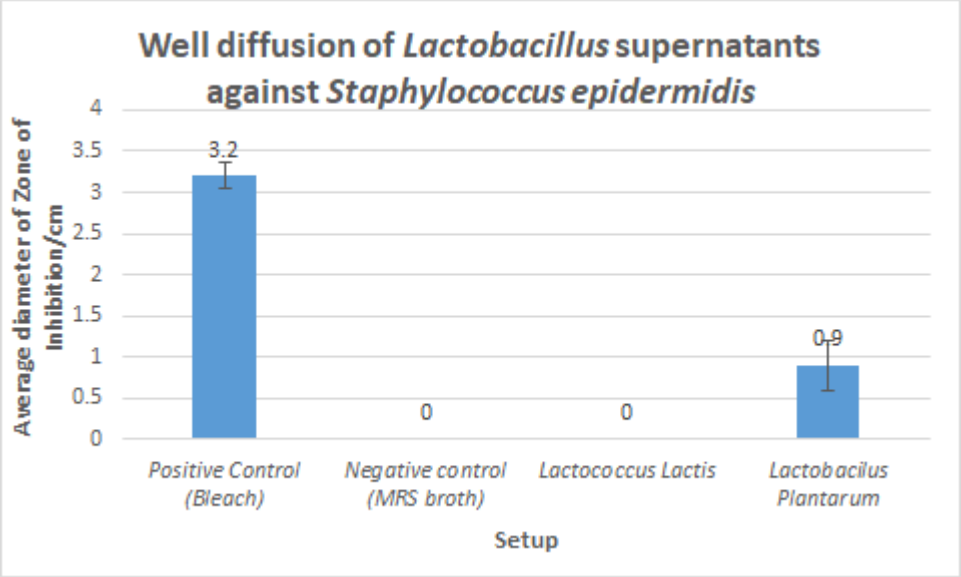

Fig 1: : Graphs showing the diameter of inhibition of supertanatants of *L. lactis* and *L.plantarum* against *S.epidermidis*.

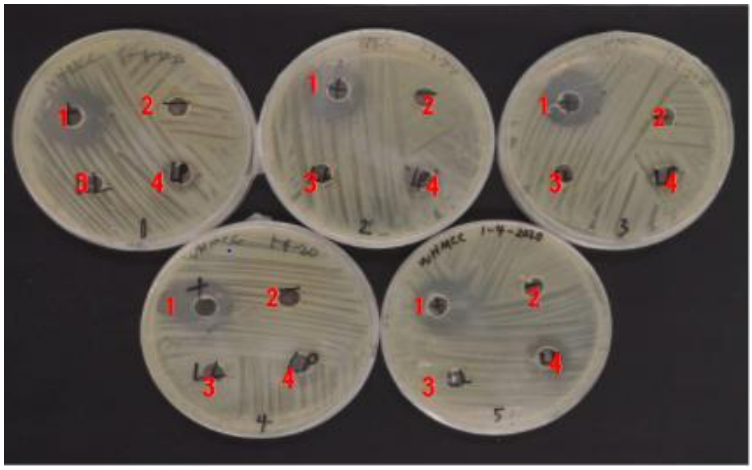

Fig 2: Well diffusion test against *S. epidermidis*. Wells were filled with culture supernatants of *L. lactis*(3) and *L. plantarum*(4). The positive control (1) for all setups were 10% bleach while the negative control (2) was MRS broth.

Fig.1 shows that *L. plantarum* displays antibacterial properties against *S. epidermidis* while *L.lactis* does not. As such, *L.acidophilus* was used in following experiments instead of *L.lactis*.The Kruskal-Wallis test showed that the difference between the *L.plantarum* setup and the control is significant, with p=0.007 which is <0.05.

Antibacterial test using the *in vitro* colony count method

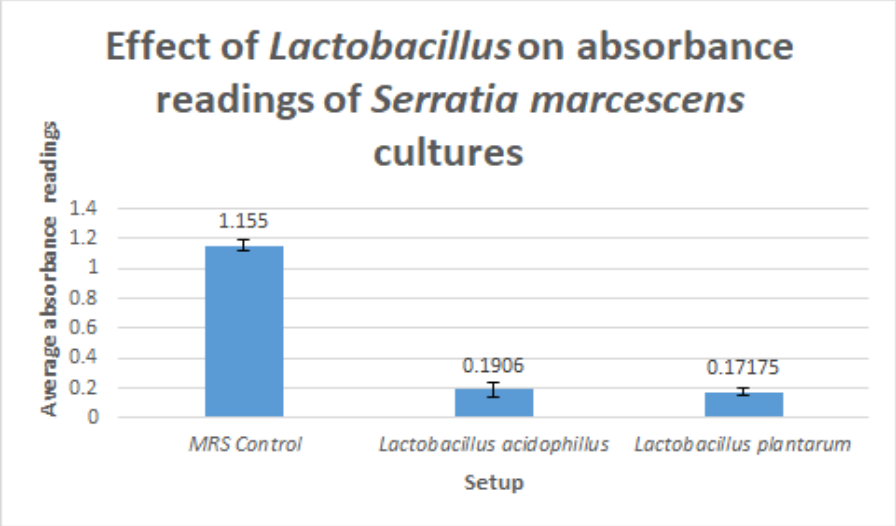

Fig. 3(a)

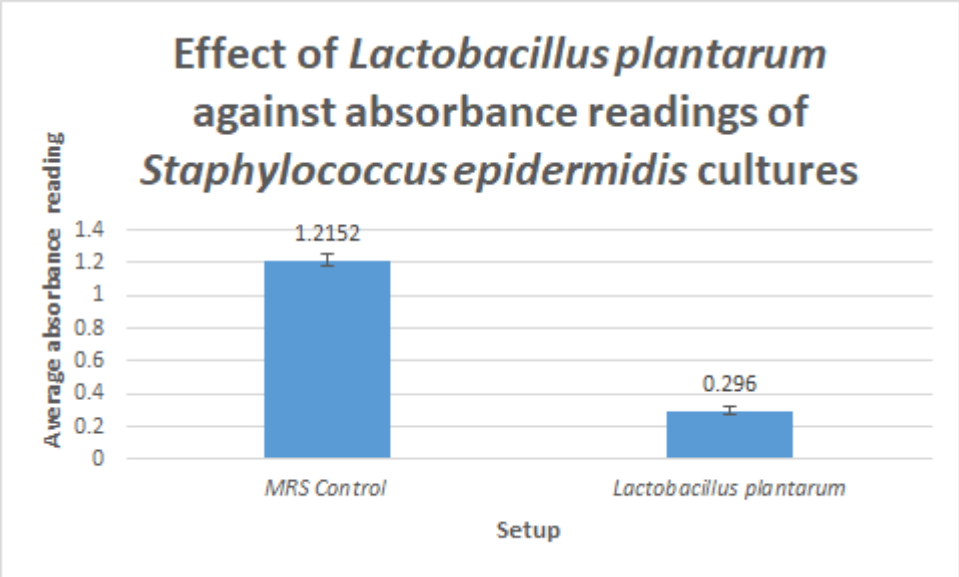

Fig.3(b)

Fig.3: Graph showing the effect of *Lactobacillus* on absorbance readings of (a)*S.epidermidis* (b)*S. marcescens* cultures.

Fig.3(a) shows that *L.plantarum* has inhibitory effect on *S.epidermidis*. Mann-Whitney U test showed that the difference between test and control setup is significant, with p=0.007937 which is <0.05.

Fig.3(b) shows that *L. plantarum* showed a greater inhibitory effect against *S. marcescens* than *L. acidophilus*. Kruskal-Wallis test showed that the difference between the test setups and the control is significant, with p=0.00675 which is <0.05.

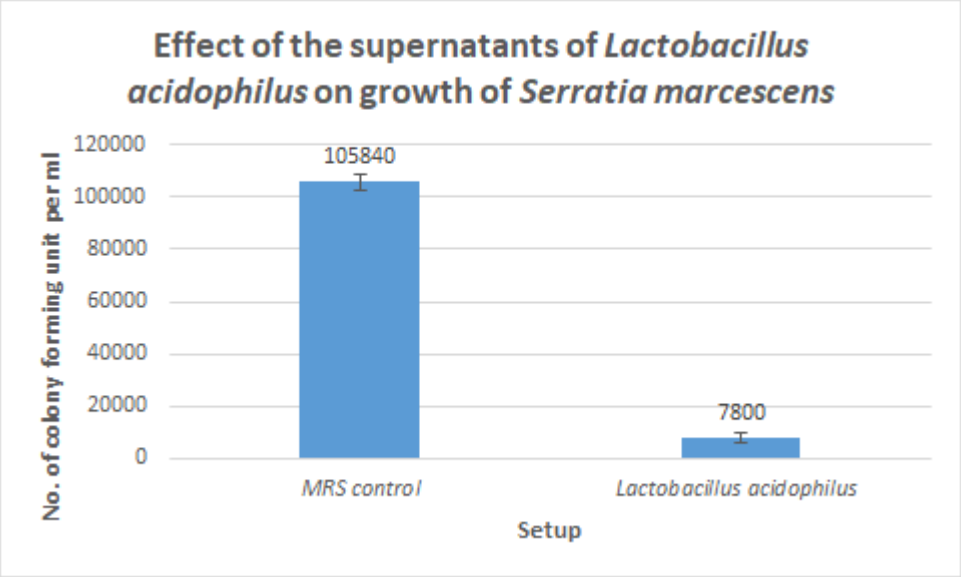

Fig.4: Graph showing effect of supernatants of *L.acidophilus* on growth of *S. marcescens.*

The results of the antibacterial well diffusion test are shown in Fig.5 below.

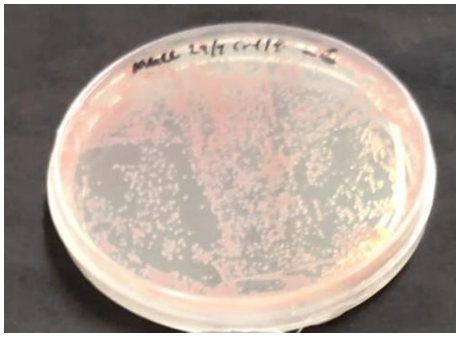

Fig.5(a)

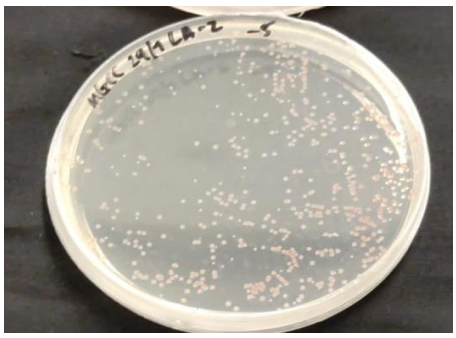

Fig.5(b)

Fig.5: Colony count test against *S. marcescens*.Culture with *S. marcescens* and (a) MRS Broth, (b) *L.acidophilus* supernatant were diluted by a factor of (a)10^{-6},(b) 10^{-3} before being spread on LB agar plates.

Graph shows that *L.acidophilus* supernatants had a significant inhibition on the growth of *S. marcescens*. Mann-Whitney U test showed that the difference between test and control setup is significant, with p=0.01208 which is <0.05.

***C. elegans* survival assay (*in vivo* antibacterial test)**

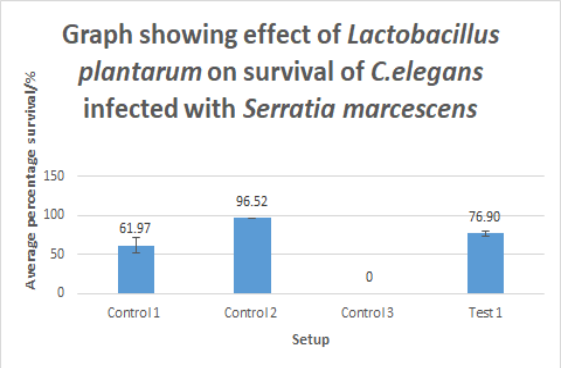

Fig.6(a)

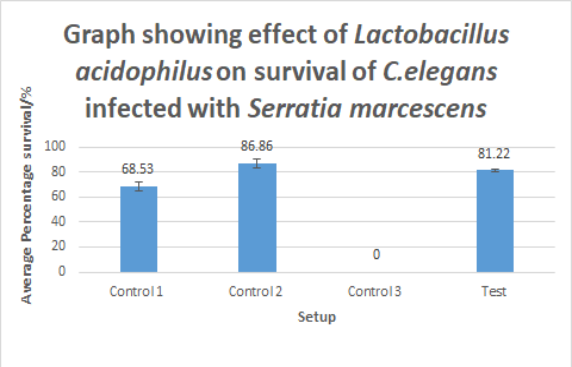

Fig.6(b)

Fig.6: Graph showing the effect of (a) *L.acidophilus* and (b) *L.plantarum* on survival of *C. elegans* infected with *S. marcescens*.

In control 1, 50 µl of *E. coli* OP50 culture was spread on the agar plates.

In control 2, 50 µl of lactic acid bacteria culture was spread on the agar plates.

In control 3, 25 µl of *E. coli* OP50 culture mixed with 25 µl of *S. marcescens* culture was spread on the agar plates.

In the test set-up, 25µl of lactic acid bacteria culture mixed with 25µl of *S. marcescens* culture was spread on the agar plates.

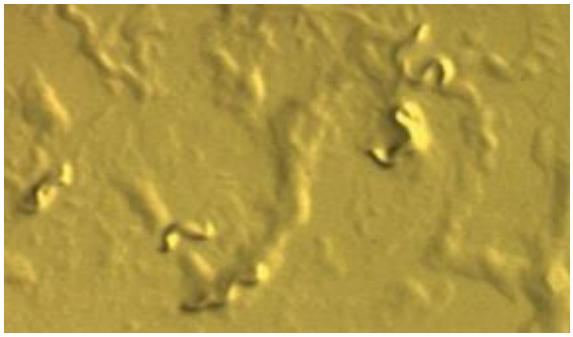

Fig.7(a): *C.elegans* fed with *L.plantarum* and *S. marcescens* culture

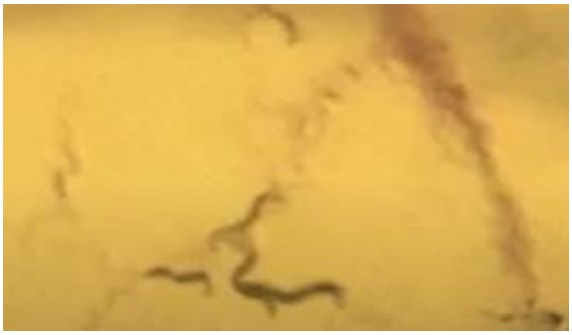

Fig.7(b): *C.elegans* fed with *L.acidophilus* and *S. marcescens* culture

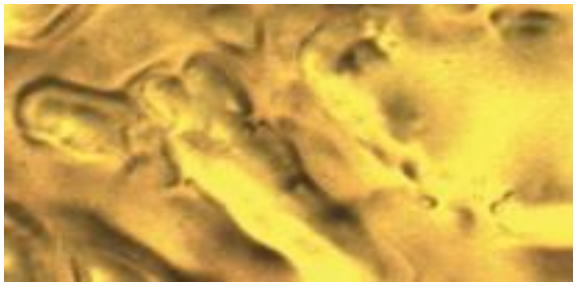

Fig 7(c): C. elegans fed with *E. coli* OP50 and *S. marcescens* culture

As hypothesized,culture supernatants of *L. plantarum* and *L. acidophilus* showed antimicrobial effects against the bacteria *S. marcescens* and *S. epidermidis* in the *in vitro* well diffusion and colony count tests, and *L. plantarum* and *L. acidophilus* were able to restore survival of *C. elegans* infected with *S. marcescens* and *S. epidermidis* in the *in vivo* antibacterial test. We generally note that both cultures of *C. elegans* nematodes inoculated separately with *L.plantarum* and *L.acidophilus* extracts, respectively, saw a dramatic decrease in death rate or increase in survival rate when infected with *S.marcescens*,suggesting that *L.plantarum* and *L.acidophilus* drastically inhibited the pathogenicity of bacteria.

Interestingly, *L.acidophilus* showed better competitive exclusion of pathogenic microorganisms in the in-vivo tests with a lower absorbance reading in the colony count test, but *L.plantarum* showed better results in the in-vivo bacterial tests with a higher percentage of *C.elegans* having survival restored. Using the Kruskal-Wallis test, both graphs showed that the differences between the mean values were significant with $p=0.00081$ which is <0.05 Fig.7(a), and $p=0.00149$ which is <0.05 for Fig.7(b).

According to (Stapleton, 2017), *Lactobacillus* strains secrete lactic acid and hydrogen peroxide. This is the key ingredient behind the antibacterial effects of the lactic acid bacteria supernatant, as they can disrupt the outer membrane of bacteria (Alakomi et al., 2000), and increase the acidity of the surroundings (Dhewa, 2009), allowing it to inhibit growth of bacteria.

Conclusion and Recommendations for future work

From our study, we can conclude that different strains of *Lactobacillus* exhibit antimicrobial effects against the bacteria *S. marcescens* and *S. epidermidis* to varying degrees. Most notably *L. plantarum*, which exhibited antimicrobial effects against the different bacteria in all three of our tests, the antibacterial test using the in vitro well diffusion and colony count method, and *C. elegans* survival assay . *L. acidophilus* was also able to restore survival of *C. elegans* in the *in vivo* antibacterial test and displayed antimicrobial effects against the bacteria in the *in vitro* colony count test, suggesting a high potential of this species in antimicrobial therapy.

Some limitations to our study that can be improved on in future work include standardising the stage of growth of the *C. elegans*, to ensure that they have the same susceptibility to infection and thereby making the results more accurate.As further work, increasing the range of *lactobacillus* and test organisms tested would increase the reliability of the results in proving that *lactobacillus* are able to exhibit antimicrobial effects.

Studies in the restoration of locomotion of *C. elegans* infected with pathogenic bacteria at varying temperatures and pH can be carried out.

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