Investigating the antibacterial and antioxidant effects of *Lactobacillus* spp. on the prevention of Urinary Tract Infections

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Abstract

This study aims to investigate the antibacterial and antioxidant effects of various species of *Lactobacillus*. Antibacterial properties were tested by conducting the antibacterial well-diffusion and colony count tests on *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus gasseri*, and *Lactobacillus plantarum* against the uropathogens *Escherichia coli* and *Serratia marcescens*. The swimming and swarming motility assay was also conducted on the four species of *Lactobacillus* against the same uropathogens to investigate the ability of *Lactobacillus* to inhibit the formation of biofilms by the uropathogens. Antioxidant tests were tested by performing the standard DPPH assay. All species of *Lactobacillus* showed antibacterial effects against both uropathogens. They also showed significant inhibition towards the formation of biofilms and displayed significant antioxidant properties. Hence, *Lactobacillus* has potential to be used as a treatment for urinary tract infections (UTIs) caused by the uropathogens.

Introduction

Urinary tract infections (UTIs) are defined as the microbial infiltration of any part of the otherwise sterile urinary tract (Barber, Norton, Spivak, & Mulvey, 2013). Such infections affect over 150 million people per year worldwide (Stamm & Norrby, 2001). Pathogens associated with UTI include the Gram-negative bacteria *Escherichia coli* and *Serratia marcescens* -- *E. coli* alone represents over 80% of UTIs (Barber et al., 2013). According to Shareef and Yagoub (2006), these two species are also responsible for most incidences of nosocomial UTIs.

Microorganisms tend to accumulate at interfaces to form microbial aggregates such as biofilms. Biofilms are microbial communities of surface-attached cells embedded in an extracellular

matrix. They are the result of complex communication among bacterial cells, regulated by a complex quorum sensing regulation system. Quorum sensing (QS) allows pathogenic bacteria to coordinate virulence factors expression for escaping the host immune response. Biofilm development allows for the long-lasting persistence of bacteria in the genitourinary tract (Delcaru et al., 2016). Flagella motility-dependent swimming is regulated by QS. A reduction in the swimming area suggests the presence of anti-QS compounds. Likewise, anti-QS compounds result in a reduction in swarming motility, which promotes bacterial adhesion to different surfaces (Chu et al., 2013).

Current treatment for UTIs predominantly involve antibiotics, *inter alia* co-trimoxazole, which inhibits nucleic acid synthesis by inhibiting folate synthesis (Barber et al., 2013), and tetracyclines, which inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA (Chopra & Roberts, 2001). Bacteria do, however, have a manifest genetic ability to rapidly develop resistance to antibiotics and fundamentally undermine their effectiveness (Zaman et al., 2017). For instance, *S. marcescens* has been reported to be uniformly resistance to tetracycline among other antibiotics (Moradigaravand, Boinett, Martin, Peacock, & Parkhill, 2016), highlighting the need for novel and sustainable alternative medical solutions to UTIs.

To this end, research on *Lactobacillus* spp. has displayed great potential in the management of UTIs. Clinical studies have found that the oral administration of certain *Lactobacillus* spp. was capable of resolving UTIs through providing clients with *Lactobacillus* spp. suspended in skim milk twice a day for 14 days. (Reid et al., 2006) It has also been observed that supernatants of *Lactobacillus* spp., *inter alia L. plantarum*, display significant levels of antibacterial activity against uropathogenic *E. coli*, and *L. acidophilus* was observed to inhibit the growth and biofilm formation of uropathogens including *S. marcescens* (Shokouhfard, Kermanshahi, Shahandashti, Feizabadi, & Teimourian, 2015). Indeed, a study by Zuccotti et al. (2008) stated that probiotics including *Lactobacillus* species display potential in resolving UTIs given their ability to secrete biosurfactant and also bind to uroepithelial cells, inhibiting the growth of uropathogens.

Therefore, this study seeks to compare the antioxidant and antibacterial properties of various *Lactobacillus* species, and to evaluate their potential in resolving UTIs and inhibiting the formation of biofilms by uropathogens.

Objectives and Hypotheses

The objectives of this study are to investigate whether *L. plantarum, L. rhamnosus, L. gasseri* and *L. acidophilus* show antibacterial property against *E. coli* and *S. marcescens* and are able to remove biofilms of these uropathogens by reducing their swarming and swimming motilities. This study also aims to investigate whether the different species of *Lactobacillus* exhibit antioxidant properties.

It is hypothesised that *Lactobacillus* species are antibacterial towards *E. coli* and *S. marcescens*; are able to remove biofilms of *E. coli* and *S. marcescens* by reducing their swarming and swimming motilities; and display antioxidant activity to varying degrees.

Methods

Preparation of Bacterial Cultures

Bacterial colonies of *L. acidophilus* ATCC 4356, *L. plantarum* ATCC 8014*, L. casei* ATCC 334*,* and *L. gasseri* ATCC 19992 were each removed from bacterial culture using a sterile wire loop, inoculated in 10ml of De Man, Rogosa and Sharpe (MRS) broth, and were incubated in a shaking incubator at 30°C for 24 hours.

Bacterial colonies of *E. coli* ATCC 25922 and *S. marcescens* Carolina were inoculated in Luria-Bertani (LB) broth, and incubated in a shaking incubator at 30°C for 24 hours. A UV-vis spectrophotometer was used in standardising the absorbance of each culture at 600nm at 0.8.

Preparation of Cell Pellet and Supernatant

Lactobacillus cultures were centrifuged at 4500rpm for 10 minutes. The supernatant, containing

extracellular secreta, was collected and filter-sterilised using a sterile microfilter. The cell pellet was collected and mixed with MRS agar, which was prepared and autoclaved beforehand.

Antibacterial Well Diffusion and Colony Count Test

Mueller-Hinton and LB agar was prepared. A sterile cotton swab was immersed in either *E. coli* or *S. marcescens* broth culture, and was streaked across the Petri dish.

For the well-diffusion, four wells were created in MH agar of each petri dish. 80µl of cell pellet-MRS mixture was added to two wells in each Petri dish, with each of the two wells containing the cell pellet of a different *Lactobacillus* species. 80µl of 10% bleach and MRS agar were added to the remaining wells as the positive and negative controls respectively. The agar plates were incubated at 30°C for 24 hours and the zone of inhibition for each well was measured.

As for the colony count test, for the test setup, 0.5ml of *E. coli* or *S. marcescens* broth culture and 5 ml of the supernatant of a *Lactobacillus* species was added to 4.5ml of LB broth. The *Lactobacillus* supernatant was replaced by MRS broth for the test control. Five replicates were prepared for both the test setup and test control.

The absorbance of all samples at 600nm was measured. 10-fold serial dilutions were then performed using 0.85% saline. 0.1ml of the diluted culture was then spread on petri dishes with LB agar and incubated at 30°C for 24 hours. The number of colonies formed on each Petri dish thereafter was counted.

Swarming and Swimming Motility Assay

For the swimming motility test, which measures individual cell movements in aqueous environments, filter-sterilised *Lactobacillus* supernatant was added to nutrient broth supplemented with 0.3% agar. For the swimming motility test, which measures multicellular movement in semi-solid surfaces, the nutrient broth used was supplemented with 0.5% agar

instead. For both tests, filter-sterilised *Lactobacillus* supernatant was replaced by MRS broth for the control setup.

The agar mixture was then poured into Petri dishes. Afterwards, *E. coli* or *S. marcescens* broth culture was inoculated at the centre of each Petri dish using a toothpick. The diameter of the colony caused by swimming or swarming motility was recorded the following day.

Antioxidant Test

For the test setup, 0.1ml of *Lactobacillus* supernatant was mixed with 1.9ml of methanol and 1.0ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. For the test control, the 0.1ml of *Lactobacillus* supernatant was replaced by 0.1ml of methanol. For the blanks for both the test setup and test control, the 1.0ml of DPPH solution was replaced by 1.0ml of methanol. Five replicates were prepared for all samples. The samples were left in the dark for 10 minutes, and the final absorbance readings were then measured.

The Percentage Radical Scavenging Activity was calculated as follows:
[(Final absorbance of control – Final absorbance of test) / Final absorbance of control] x 100%

Results and Discussion

Antibacterial Well Diffusion Tests

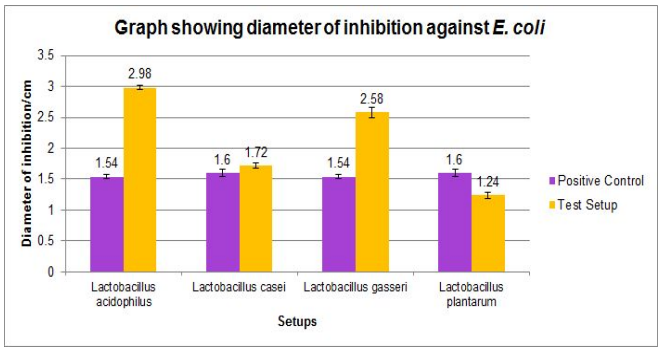

Fig. 1(a)

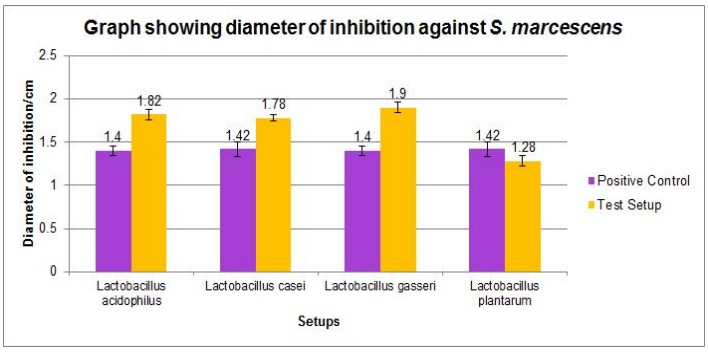

Fig. 1(b)

Fig. 1: Graphs showing the diameter of inhibition against (a) *E. coli* and (b) S. marcescens of the cell pellets of various species of *Lactobacillus*.

The results of the antibacterial well diffusion test are shown in Figure 2 below.

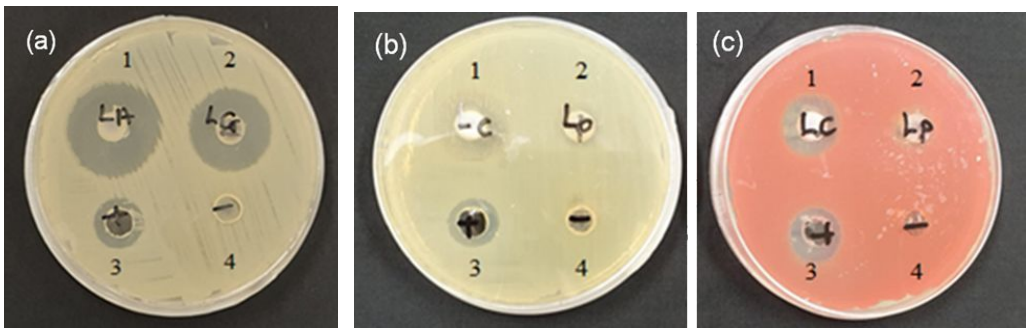

Fig. 2: Well diffusion test against (a, b) *E. coli* and (c) *S. marcescens*. Wells were filled with (a) cell pellets of *L. acidophilus* (1) and *L. gasseri* (2) or (b, c) cell pellets of *L. casei* (1) and *L. plantarum* (2). The positive control (3) for all setups were 10% bleach while the negative control (4) was MRS agar.

Both graphs show that the cell pellets of certain *Lactobacillus* species display antibacterial properties against uropathogens. With the exception of the *L. plantarum* against *S. marcescens* and *L. casei* against *E. coli* setups, Mann-Whitney U tests for the remainder indicate a significant difference between the pairs of groups of data with $p < 0.05$.

L. acidophilus showed the greatest inhibitory effect against *E. coli*, followed by *L. gasseri*, *L. casei*, and *L. plantarum*. *L. gasseri* showed the greatest inhibitory effect against *S. marcescens*, followed by *L. acidophilus*, *L. casei.,* and *L. plantarum*.

Antibacterial Colony Count Test

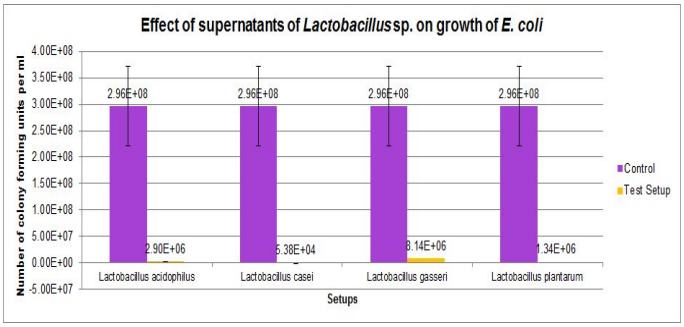

Fig. 3(a)

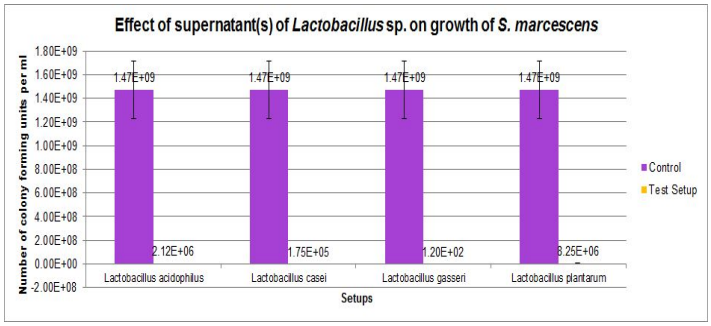

Fig. 3(b)

Fig. 3: Graph showing the effect of the supernatant of various species of *Lactobacillus* on the number of colony forming units (CFU) of (a) *E. coli* and (b) *S. marcescens*.

The results of the antibacterial well diffusion test are shown in Figure 4 below.

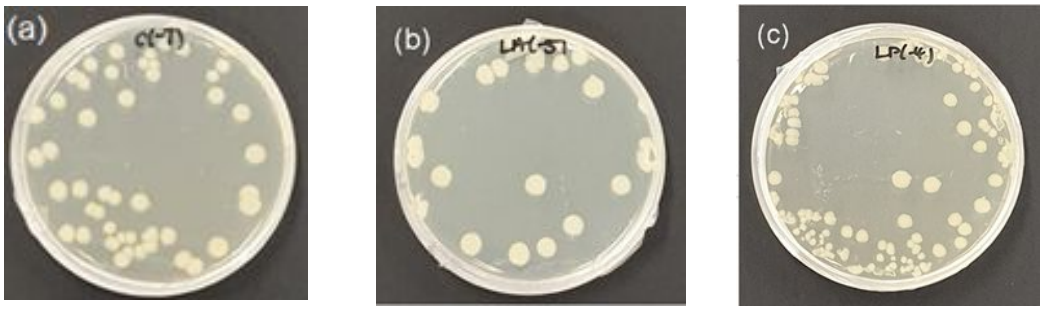

Fig. 4: Colony count test against *E. coli*. Culture with *E. coli* and (a) MRS Broth, (b) *L. acidophilus* supernatant and (c) *L. plantarum* supernatant were diluted by a factor of (a) 10^-7, (b) 10^ -5 and (c) 10^-4 before being spread on LB agar plates.

Both graphs show that *Lactobacillus* supernatants had a significant impact on the growth of uropathogens. Mann-Whitney U tests show that the difference between the test setup and the control is significant, with p=0.012.

Swarming and Swimming Motility Assay

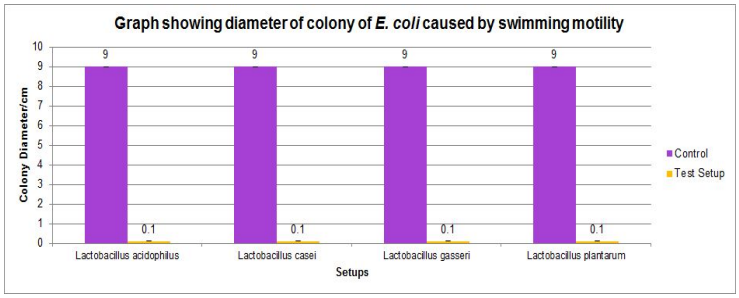

Fig. 5(a)(i)

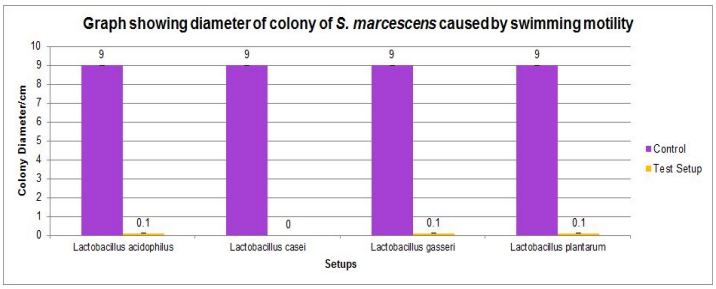

Fig. 5(b)(i)

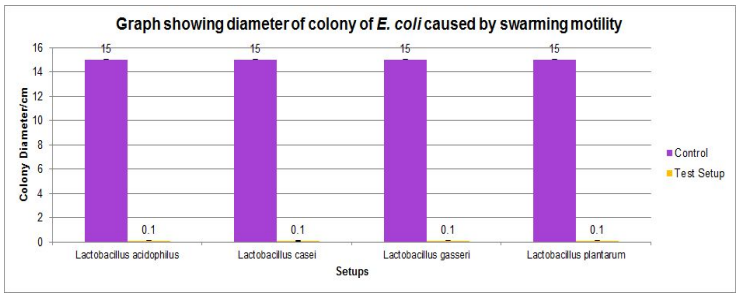

Fig. 5(a)(ii)

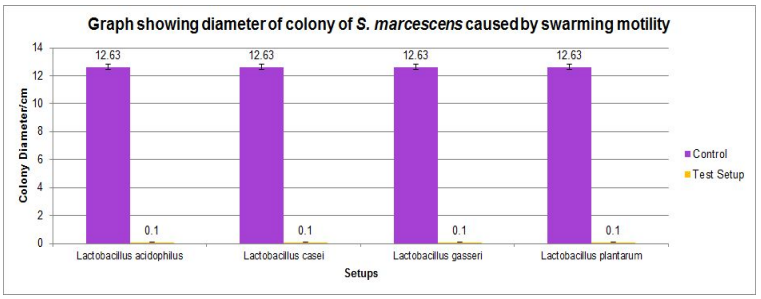

Fig. 5(b)(ii)

Fig. 5: Graph showing the effect of the presence of *Lactobacillus* supernatant on colony diameter of (a) *E. coli* and (b) *S. marcescens* in nutrient broth supplemented with (i) 0.3% agar and (ii) 0.5% agar.

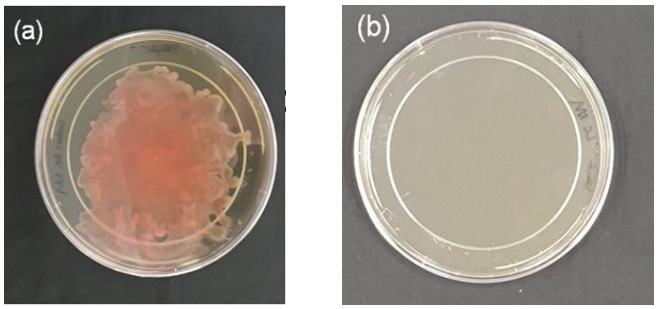

Fig. 6: Swarming motility assay against *S. marcescens*. *S marcescens* was inoculated in 0.5% agar with (a) 25% MRS v/v to act as the control setup (b) 25% *L. casei* supernatant v/v.

It is shown in Fig. 5a - 5d that the supernatants of all species of *Lactobacillus* could significantly reduce the colony diameter caused by swimming and swarming motility. Hence, there is potential for the supernatant of *Lactobacillus* to prevent the formation of biofilms of uropathogens in the urinary tract of humans.

Antioxidant Test

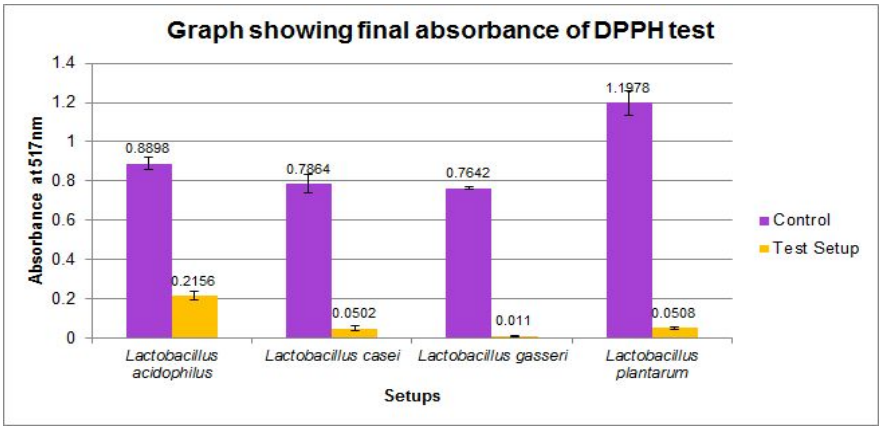

Fig. 7: Graph showing the final absorbance at 517nm of the DPPH assay.

The graph shows that *Lactobacillus* supernatants display significant antioxidant activities. Mann-Whitney U tests show that the difference between the test setup and the control is significant, with p=0.012. The Percentage Radical Scavenging Activity for each *Lactobacillus* species, from left to right in the order shown in the graph, is 75.8%, 93.6%, 95.8%, and 98.6%.

Conclusions and Discussion

Different species of *Lactobacillus* display antibacterial activity against *E. coli* and *S. marcescens* to varying degrees and are able to remove biofilms of these uropathogens to varying degrees by reducing their swarming and swimming motilities. Different species of *Lactobacillus* also display antioxidant properties to varying degrees.

The antibacterial properties shown by *Lactobacillus* cell pellets in this study is in line with reports by Ołdak, Zielińska., Rzepkowska, and Kołożyn-Krajewska (2017) and Coman et al.

(2014). Given that there was significant inhibition of both pathogens, the *Lactobacillus* cells most likely did synthesise antibacterial substances resulting, and it is likely that these substances were able to diffuse well, as was the case in Coman et al. (2014). The hydrogen peroxide and lactic acid secreted by *Lactobacillus* species (Stapleton, 2017) could be the active ingredient behind the antibacterial effects of the supernatant, considering that they can disrupt the outer membrane of bacteria (Alakomi et al., 2000), and increase the acidity of the environment (Dhewa, 2009), thereby inhibiting bacterial growth. The compound present in the supernatant of *Lactobacillus* that displays antioxidant property is believed to be lactic acid.

The positive results from the swimming and swarming motility assay suggest that *Lactobacillus* can inhibit the formation of biofilms by uropathogens. One possible explanation of such a phenomenon is that supernatant of *Lactobacillus* contains anti-Quorum Sensing (anti-QS) compounds, and these compounds disrupt the complex communication between uropathogenic cells, making them not able to coordinate virulence factors, which disrupts the swimming and swarming motility caused by the moving of the flagella (Delcaru et al., 2016). Hence this causes a decrease in the swimming and swarming motility.

However, in this study, all antibacterial, anti-biofilm and antioxidant assays were performed *in-vitro*. However, these results might not be reproducible for *in-vivo* tests and hence cannot accurately determine the effects of *Lactobacillus* in the human microbiota. This is because conditions like pH and temperature in the human body differ from conditions in the experiment.

Future work is necessary to determine and isolate the compound(s) that cause the antibacterial activity of *Lactobacillus* species, and to understand their antibacterial mechanism of action.

In addition to this, further tests as to the antioxidant properties of *Lactobacillus* species so as to understand the cause of their antioxidant properties, and to explore whether they can protect other living cells from oxidative stress.

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