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Inhibitory effects of *Lactobacillus fermentii* and *Lactococcus lactis* against *Pseudomonas aeruginosa* biofilms

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Abstract

Probiotics and their derivatives are becoming increasingly popular in the fight against pathogenic biofilms. This research work explores the use of probiotic bacteria and their potential as antimicrobial agents against *Pseudomonas aeruginosa* biofilms and the infections they cause. *P. aeruginosa* strains were grown on LB (Lysogeny Broth) for 24 hours and characterized using biochemical tests. The biofilm-forming strains were quantified using polystyrene microtiter-plates in a spectrophotometric assay supplemented with M63 minimal media. LAB (*Lactobacillus fermentii* and *Lactococcus lactis*) were isolated from fermented maize paste (*ogi*), Cheese (*Waara*), *Fura* and fermented milk (*Nunu*) on Skim milk agar and were characterized based on colony morphology, cell morphology and biochemical tests. From the isolates, two strains of LAB were selected as probiotics. They were tested for their inhibitory potential against biofilm-forming *P. aeruginosa* using liquid co-culture assay. The Lactic acid content and Hydrogen Peroxide was estimated by titration for three days. *Lactococcus lactis* had more stable lactic acid production than *Lactobacillus fermentii* (2.70 g/L) although both peaked on Day 2 with *Lactococcus lactis* producing slightly more lactic acid (2.72 g/L). With liquid co-culture assay, there was a 63% decrease observed in the optical density of biofilms.

Keywords: Probiotics, Lactic acid bacteria, Lactic acid, *Lactobacillus fermentii*, *Lactococcus lactis*, *Pseudomonas aeruginosa*.

Introduction

Probiotics are dietary supplements containing viable microorganisms capable of transiently colonizing or persisting in the intestinal tract of human thereby conferring valuable influence on the host's physiology, such as improving the health of the host (Sharifi-Rad *et al.*, 2020; Babakulov *et al.*, 2022). These probiotics produce antimicrobial substances such as bacteriocins or organic acids which help in reducing the risk of the development of an allergy, decreasing the adhesion of pathogens, regulating immune

responses by the secretion of IgA against possible pathogens, modulating the expression of the host genes, releasing the functional proteins such as natural enzymes, promoting the recovery or increasing the stability of the commensal microflora when disturbed and improving the functions of the intestinal mucosal barrier (Plaza-Diaz *et al.*, 2019; Sharifi-Rad *et al.*, 2020)

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It was estimated globally that 40–80% of all prokaryotes live in biofilms. Biofilms are microbial communities encased in a protective exo-polymeric substances adhering to surfaces (Verderosa *et al.*, 2019). They are also responsible for some 80% of bacterial infections such as conjunctivitis, urethritis, vaginitis, otitis, and colitis which are often extremely difficult to treat due to the specific protection mechanisms provided by the biofilm (Verderosa *et al.*, 2019; Câmara *et al.*, 2022). The formation of biofilm is significant in the virulent mechanism of the pathogenesis of medically important pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Verderosa *et al.*, 2019). *Pseudomonas aeruginosa* is a major health challenge that causes recalcitrant multi-drug resistant infections, especially in immunocompromised and hospitalized patients. It persists in some antiseptic solutions used for disinfecting surgical instruments and contact lenses, infecting patients with weak immune system leading to serious diseases, for example, urinary tract infections, bacteremia, pneumonia and ulcerative. A major setback in the treatment of biofilm-related infections is the ineffectiveness of existing antibiotics due to the protective layers built by cells in the biofilm (Verderosa *et al.*, 2019; Penesyan *et al.*, 2021).

In this research Gram positive mesophilic *Lactococcus lactis* was used for the inhibition of *Pseudomonas aeruginosa* biofilm. The utilization of probiotics for clinical health benefits is an exciting area of research that has yet to be thoroughly explored in the modern era. The aim of this work was to determine the effectiveness of Lactic Acid Bacteria (LAB) as an antibacterial agent against *Pseudomonas aeruginosa* biofilm.

Materials and Methods

Collection of Samples

The fermented maize (Ogi) used for this study was prepared at Fountain University's Laboratory while Waara, Fura and Nunu were collected directly from the Fulani's living in Oke-Osun, Osogbo, Osun State Nigeria. The *P. aeruginosa* strains used in this study were obtained from the Microbiology Laboratory of Fountain University.

Isolation of LAB and *Pseudomonas aeruginosa*

Stock solutions of 10% of fermented Fura and Waara was prepared by dissolving 1 ml each of the samples in 9ml of sterile distilled water in a test tube and was serially diluted while nunu and ogi samples were first soaked for 48 hours in a tightly sealed container and 1ml was added into 9 ml of sterile distilled water and serially diluted before they were inoculated into sterile Skim milk agar plates using pour plate method. The plates were incubated anaerobically at 32°C for 48hrs and observed for microbial growth. Concurrently, an already identified clinical isolate of *P. aeruginosa* was sub-cultured from the Microbiology Laboratory of Fountain University.

Identification and Characterization of LAB

The lactic acid bacteria strains isolated were identified by morphological, phenotypic and biochemical characteristics using Bergey's Manual of Systemic Bacteriology as a reference (Holt *et al.*, 1994).

The numbers of microbial colonies were counted. The total viable count was then determined using the expression:

$$\text{Total Viable Count} = \frac{\text{Number of Colonies} \times \text{Volume Inoculated}}{\text{Dilution Factor}}$$

Distinct colonies were sub-cultured on fresh plates then purified and maintained on appropriate slants at 4°C for further analyses.

Quantitative determination of Lactic acid production by isolates

The production of lactic acid was determined by titrating 10 ml of the homogenized sample against 0.25 mol/l NaOH using 1 ml of phenolphthalein indicator (0.5% in 50% alcohol). The titratable acidity was calculated as percentage lactic acid (v/v). Each millilitre of 1 N NaOH is equivalent to 9.008 mg of lactic acid (Sarayza & Hemashenpagam, 2011).

Determination of Hydrogen Peroxide production by isolates

Twenty-five millilitres of the fermenting samples

and 20 ml of diluted H₂SO₄ were titrated against 0.1 N potassium permanganate. 1 ml is equivalent to 1.70 mg of H₂O₂ (Sarayza & Hemashenpagam, 2011).

pH determination

The pH of the fermenting substrates was measured daily with the electrode of a pH meter standardized with distilled water.

Identification and Characterization of *Pseudomonas aeruginosa*

Biochemical tests were used to authenticate the identity of the *P. aeruginosa* isolate. The tests included: Gram stain, catalase test, oxidase test, citrate test motility test.

Categorization of Acquired Resistance Profiles in *P. aeruginosa* Strain

Pseudomonas aeruginosa strains were inoculated onto Mueller-Hinton agar plates (0.5 McFarland) by streaking. Antibiotic paper disks for gram negative bacteria were then placed on the plates in aseptic conditions and the plates were incubated at 35 °C for 18h. All data were entered and analyzed using WHONET 5.6 software (Magiorakos *et al.*, 2012).

Biofilm formation using Microtitre-plate test

Biofilm formation by *P. aeruginosa* strains was assayed using a 96-well polystyrene microtitre plate. These strains were grown at 37 °C in Luria–Bertani (LB) medium containing 0.25% glucose for 24 h. Using M63 medium, the cultures were diluted (1:100) and then 200 µL of the bacterial suspension was added to sterile 96-well microtitre plates and then incubated for 24 h at 37 °C. The wells were washed three times with 300 µL distilled water, dried and then stained with 200 µL of 0.1% crystal violet for 15 min. The wells were washed three times using distilled water and were stained with 200 µL of 30% acetic acid in water. In the de-staining solution, the absorbance was measured at 570 nm using a spectrophotometer (Stepanovic *et al.*, 2000).

Quantitative Biofilm Detection

Biofilm formation in microtitre-plate was carried out in triplicates and the average optical density was calculated for each bacterial strain.

For the purposes of comparative analysis of test, we introduced classification of adherence capabilities of tested strains into four categories as follows;

OD ≤ OD_c non-adherent

OD_c < OD ≤ 2 x OD_c weakly adherent

2 x OD_c < OD ≤ 4 x OD_c moderately adherent

4 x OD_c < OD strongly adherent

The cutoff optical density (OD_c) for biofilm formation was defined as three standard deviations (SD) above the mean OD of the negative control (Swedan *et al.*, 2019).

Assessment of Antibiofilm Activity of LAB Strains; (Liquid Co-culture Assay in Microtiter Plate)

The capability of LAB strains to interfere with the growth of *P. aeruginosa* strains was evaluated by co-culture method in microtiter plate. The isolated *Lactobacillus fermentii* and *Lactococcus lactis* strains and the *P. aeruginosa* strains were grown on Skim milk broth and Lysogeny broth (LB), respectively. The 0.5 McFarland turbidity (1.5×10^8 CFU/mL) of these suspensions was prepared and 100 µL of cell-free supernatant of LAB strains and 100 µL of the *P. aeruginosa* strains were added together to a 96-well microtiter plate and incubated for 24 h at 37 °C. Positive controls were prepared by inoculating the same medium with the *P. aeruginosa* strains alone. To check whether the pathogens were inhibited or killed, 50 µL of co-culture suspension was seeded on Mueller-Hinton agar medium and incubated at 37 °C for 24–48 h. By comparing the growth with a negative (100% inhibition) and a positive control (0% inhibition), the growth inhibition values of pathogens were estimated by looking at the growth under microscope to evaluate the predominant cells on agar plates of the co-culture after the 24-h incubation (Shokri *et al.*, 2017).

Results

Morphological Identification of Isolated Bacteria

Observation of colonies on the different agar plates indicated that different microorganisms were present in the samples as shown in Table 1.

Biochemical Characteristics and Identification of the Isolated Bacteria

A total of 20 bacterial isolates were isolated from *Fura*, *Nono*, *Waara* and *ogi* samples.

Bacteria colonies were identified based on their morphological and biochemical characteristics using standard references organisms. The bacterial isolates of interest were identified based on the microscopic characteristics of Gram's reaction as described in Bergey's manual of Determinative Bacteriology. From the total isolates, *Lactococcus lactis*, *Lactobacillus fermentii*, *Enterococcus* sp., *Staphylococcus* sp., were identified in varying frequencies from *Fura*, *Nono*, *Waara* and *Ogi*. *Pseudomonas aeruginosa* isolate was also subjected to biochemical tests. Results of biochemical tests for LAB and *P. aeruginosa* are shown in Table 2.

pH determination

The pH was measured at daily intervals from 0 hours of incubation to 72 hours after incubation. pH levels ranged from 5.9 - 4.66 in *L. fermentii* and 5.21 - 4.55 in *Lactococcus lactis*. Reduction in pH during fermentation is due to the fermentative transformation of carbohydrates to lactic acid. The pH development during fermentation by the LAB isolates is represented in Figure 2.

Quantitative determination of Lactic acid production by isolates

Figure 3 shows the lactic acid produced by the LAB strains. It ranged within 2.25-0.9 g/L in *L. fermentii* from day 0 to day 3. *Lactococcus lactis* had the highest (2.72g/L) at 36 hrs after incubation.

Determination of Hydrogen Peroxide production by isolates

Figure 4 shows the hydrogen peroxide produced by the LAB isolates, the highest (1.05 g/L) was produced by *Lactococcus lactis* at 0 hrs after incubation. The Hydrogen peroxide

production subsequently remains somewhat constant between *Lactococcus lactis* and *Lactobacillus fermentii*.

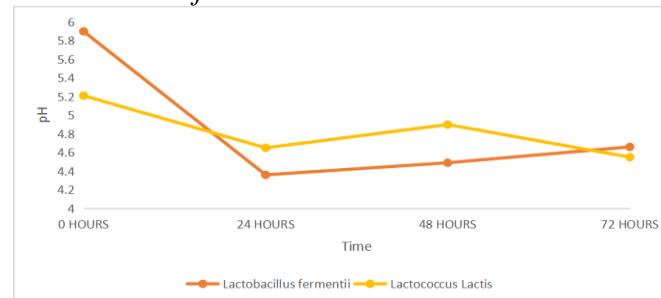


Figure 2: pH determination

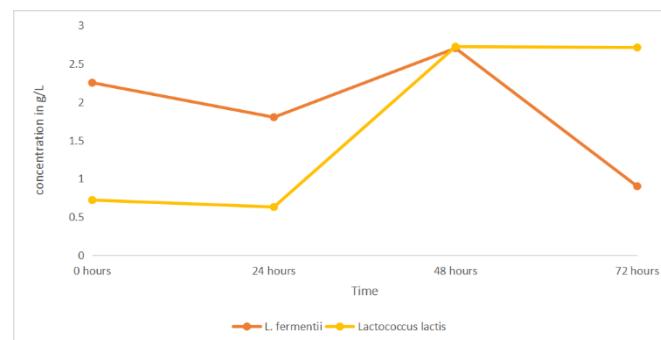


Figure 3: Concentration of Lactic acid by LAB isolates

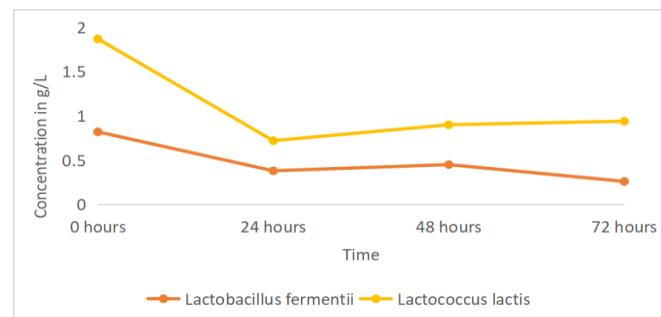


Figure 4: Concentration of H₂O₂ by LAB isolates

Table 1: Colony Morphology of Pure Isolates

Isolate	Shape	Edge	Elevation	Surface	Pigment	TVC(cfu/mL)
O1	Filamentous	Undulate	Umbonate	Smooth	Creamy	2×10^1
N2	circular	Entire	Flat	Smooth	Creamy	TNC
F4	Irregular	Entire	Raised	Rough	White	4.2×10^4
F5	Circular	Entire	Raised	Rough	White	TFC
W2	Irregular	Undulate	Umbonate	Rough	White	6.7×10^2

Key: O1: *Ogi* isolate, dilution factor 10^{-1} , N2: *Nono* isolate dilution factor 10^{-2} , F4: *Fura* isolate dilution factor 10^{-4} , F5: *Fura* isolate dilution factor 10^{-2} , W2: *Waara* isolate dilution factor 10^{-2} . TVC: Total Viable Count

Table 2: Biochemical characterization of isolates

Isolate	Gram' s Stain	Endospore test	Acid fast test	Catalase test	Glucose fermentation	Mannitol Test	Motility test	Hemolysis test	Oxidase Test	Citrate test	Presumed Organism
N2	+ve rods	-ve	-ve	-ve	Acid & gas	-	-	-	-	-	<i>L. fermentii</i>
W4	+ve rods	-ve	-ve	-ve	Acid & gas	-	-	-	-	-	<i>L. fermentii</i>
O1	+ve cocci	-ve	-ve	-ve	-	-	non motile	γ	-	-	<i>Lactococcus lactis</i>
F4	+ve cocci	-	-	+ve	-	+ve	-	β	-	-	<i>S. aureus</i>
F5	+ve cocci	-	-	-ve	-	-	non motile	γ	-	-	<i>Enterococcus</i> sp.
P1	-ve rods	-	-	+ve	-	-	motile	-	+ve	+ve	<i>P. aeruginosa</i>

Key: γ: Gamma hemolysis, β: Beta hemolysis, P1: *Pseudomonas aeruginosa* isolates

Categorization of Antibiotic Resistance Profiles in *P. aeruginosa* strains

Result analysis using WHONET 5.6 software showed that all 5 isolated strains were multidrug-resistant (MDR). MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. The most effective antibiotic against the *P. aeruginosa* strains was Gentamicin with only 5% resistance and high percentages of resistance observed against other studied antibiotics, shown in Figure 5.

Quantitative biofilm detection using 96-well microtitre plate assay

Using comparison with established OD_c value, the five *P. aeruginosa* isolates are categorized according to adherence capabilities. P1 was a moderately adherent biofilm producer, 2 were weakly adherent and 2 were non-adherent and did not form biofilms. At 570nm, the spectrophotometric results obtained were averaged and expressed as numbers. The average OD values for all tested strains are shown in Table 3.

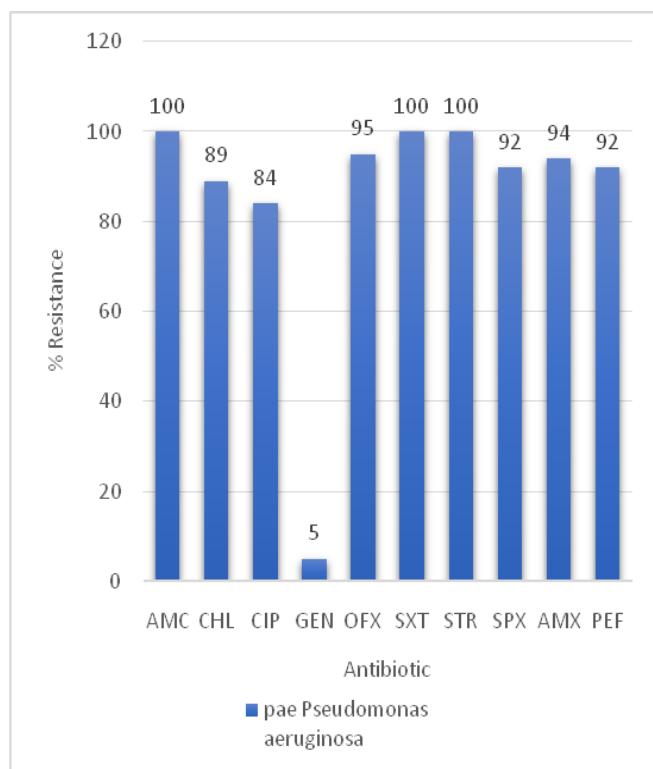


Figure 5: Antibiotic resistance profile results of *Pseudomonas aeruginosa* strain

Table 3: Biofilm quantification results

Isolates	OD _{570nm}	Biofilm producing capability
P1	0.547	Moderate producer
P2	0.253	Weak producer
P3	0.200	Weak producer

Key: P1: *P. aeruginosa* isolate 1, P2: *P. aeruginosa* isolate 2, P3: *P. aeruginosa* isolate 3.

[Calculated OD_c value: 0.25]

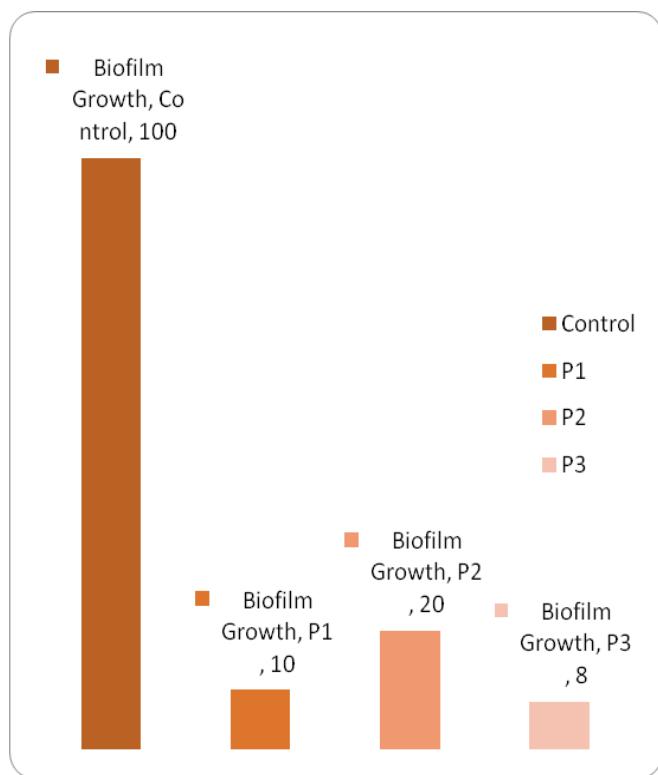


Figure 6: Inhibitory Activity of Probiotics against the growth and Formation of Biofilms

Discussion

Morphological and Biochemical Identification of isolated bacteria

In the current research, strains of lactic acid bacteria showing antimicrobial activity to biofilm forming *Pseudomonas aeruginosa* as the test organism were isolated. *Nono* had higher lactic

acid bacteria counts than *Waara*, *Fura* and *Ogi*. This is in conformity with the report of Oyeleke *et al.*, (2006) that in the locally fermented foods analyzed, *Nono* had higher LAB count than *Waara*.

pH determination

The results obtained in this research showed that during the spontaneous fermentation of Maize *Ogi* and *Nono*, the pH decreased from 5.9 to 4.6 within 48hrs. The pH of fermented *Ogi* decreased from 5.2 to 4.5 within 48 hrs. These results are in agreement with the results of Olaoluwa *et al.* (2013). The fermenting organisms do contribute to acidity attributable to the production of lactic acid and acetic acid during the processes which exert a depressive effect on the pH of the fermenting materials.

Quantitative determination of Lactic acid and H₂O₂ production by isolates

LAB produce large amounts of lactic acid, which results in a marked pH decrease during the fermentation. In the current study, the amount of Lactic acid was shown to range from 0.72 to 2.71 g/L in *Lactococcus lactis*. Dahiya & Speck (1968) reported that the presence of a carbohydrate source was necessary for the formation of H₂O₂ by *Lactobacilli*. In another study, Price & Lee (1970) reported that H₂O₂ was bacteriostatic at 2–8 ppm and bactericidal at 25–40 ppm against *Pseudomonas aeruginosa* at 30°C. As H₂O₂ is a strong oxidizing agent, the antibacterial effects of LAB isolates in this study are justified.

Quantitative biofilm assay using 96-well microtitre plate

A modification of the microtiter-plate method was used in which addition of acetic acid enables indirect measuring of bacteria attached both to the bottom and walls of the wells. Acetic acid resolubilizes dye bound to the adherent cells at the bottom of the well as well as to the cells attached to the walls of the wells. Microtitre dish assays while convenient are less well suited to studies of biofilm structure. As the cells are immobilized, it does not depict an accurate description of wild type biofilms. The results obtained in this study

however are similar to the research by Maldonado *et al.*, (2007) on *Klebsiella* biofilm forming strains.

Conclusion

Pseudomonas biofilms are known to be antibiotic-resistant, that is the use of antibiotics hasn't been effective in their control. Based on the findings in this study, it was revealed that Probiotics are found in most dairy products, which are of great benefits to the Human health. Probiotics (*Lactobacillus*) due to their broad antibacterial activity are more effective in the treatment and prevention of biofilm Infections than the use of antibiotics, which was tested and confirmed in this study. Lactic acid bacteria as probiotics possess important functional attributes that contribute to preventing and treatment of diseases related to pathogenic microbes as well as tenacious biofilms such as *Pseudomonas aeruginosa*.

Recommendations

The use of Probiotics could be encouraged in clinical treatment of biofilm infections. Probiotics (*Lactobacillus spp*) are considered safe and poses no harm whatsoever to human and animal health. Probiotics are more effective and works faster compared to antibiotics, more reason why they should be encouraged. Humans could consume more Dairy products containing *Lactobacillus* as they have great health benefits.

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