Eradication of Stenotrophomonas maltophilia biofilm by combination of antibiotics and lactobacillus casei filtrate

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**Abstract**

*Stenotrophomonas maltophilia* is a multi-drug resistant pathogen that has been isolated with increasing frequency from the hospitalized patients. Most clinical isolates of *S. maltophilia* efficiently form biofilms on biotic and abiotic surfaces, making this bacterium resistant to a number of antibiotics treatments. In the present study, the clinical specimens were gathered from patients suffering from urinary tract infections (UTI). After collected specimens were taken from patients, they were on MacConkey agar and incubated for 24h at 37°C. After that, only 4 suspected bacterial isolates from urine were identified by Vitek 2 belong to *Stenotrophomonas maltophilia*. By using the Microtiter plate technique All identified isolates were screened their ability to produce biofilm and the results showed that all isolates were able to form moderate biofilms. Later the *Lactobacillus casei* filtrate and each of antibiotics Ciprofloxacin and Gentamicin and ceftazidime and Amikacin and their combination were investigated to eradicate of biofilms formed by the four clinical isolates, results showed that combinations of filtrate and antibiotics had significant efficiency in the eradication of biofilm.

**Introduction:**

*Stenotrophomonas maltophilia* is a Gram-negative, non-fermenting organism, widespread in many environmental sources. It is increasingly recognized as an opportunistic pathogen responsible for nosocomial
infections in intensive care unit patients (Trifonova and Strateva, 2019). It was first isolated in 1943 with a scientific name of *Bacterium bookeri*, and then named *Pseudomonas maltophilia*. Its rRNA cistron analysis determined that it was more likely to be named *Xanthomonas maltophilia*. By 16S rRNA genes classification and naming of *X. maltophilia* it was finally named as *Stenotrophomonas maltophilia* (Denton and Kerr, 1998).

Despite that *S. maltophilia* is associated with aqueous sources; it was isolated from soil, plant rhizosphere and animals. It can be found in hospital settings and other medical and nonmedical equipment. It is the only species of the genus Stenotrophomonas connected with human infections compared to other species which are plant pathogens (Looney, 2005). *S. maltophilia* has a distinctive feature of its ability to form biofilms on biotic and abiotic surfaces, which could lead to development of the infection and can lead to natural protection against host immune defenses and different antimicrobial agents (Nicodemo and Paez, 2007). Demiraslan *et al.* (2013) declared that this bacterium can cause many infections including respiratory tract, urinary tract, skin, soft tissues, eye and abdomen in addition to cystic fibrosis, bacteremia, endocarditis, and meningitis. Looney (2005) mentioned that the main risky patients for infection by *S. maltophilia* and other hospital bacteria are those who suffer from immunodeficiency or on immunosuppressive therapy especially with malignancy, chronic diseases, long hospitalization in the intensive care units using urinary catheter, transplantation of artificial implants and overusing of broad spectra antibiotics. Furthermore, *S. maltophilia* usually causes pneumonia mostly in patients of chronic respiratory diseases with long period-applied mechanical ventilation. On the other hand, it is a rare cause of community-acquired infections. Lopes *et al.* (2017) mentioned that probiotics can be used for treatment infectious biofilms. It can be regarded as a new class of anti-virulence agents. Probiotics can prevent
pathogenic bacteria from adhering to surfaces. Furthermore, biofilm formation has an effect on biofilm integrity and consistency, and eventually leads to biofilm eradication. Barzgeri et al. (2020) clarified that the mechanisms of probiotics include the secretion of surfactants, bacteriocins, exopolysaccharides (EPS), and lactic acid, fatty acids.

Material and methods

Collection and cultivation of clinical samples:

All clinical samples were gathered from patients suffering from urinary tract infection (UTI) form the period of November 2019 to February 2020. They samples were transferred quickly to the laboratory for isolation and identification of *Stenotrophomonas maltophilia* isolates.

Isolation of *Stenotrophomonas maltophilia*:

Each of the clinical specimens was cultured on MacConkey agar and incubated for 24h at 37 °C. After incubation, suspected *S. maltophilia* colonies were taken for identification according their morphology (Amoli et al., 2017).

Identification of *Stenotrophomonas maltophilia* isolates by Vitek 2 system:

Identification of *Stenotrophomonas maltophilia* isolates were applied Vitek 2 cards (bioMe´rieux) which consists of 64 tests and is automated. The colonies were inoculated in sterile saline in test tubes. Turbidity were Measured by the DensiChek meter. Later, the cassette of GN was loaded to the Vitek 2 chamber together with the adjusted specimen suspension (Pinot et al., 2011).

Determination of *Stenotrophomonas maltophilia* biofilm production:

(Stepanović et al., 2007)

The production of biofilm by *S maltophilia* isolates was measured by 96-well microtiter plate assay based on the crystal violet staining method. The clinical isolates were cultured on MacConkey agar at 37°C for 24h. later , bacterial suspension was prepared by inoculating part of a colony in Brain Heart Infusion Broth, then the concentration of isolate suspensions were adjusted to the 0.5 McFarland standards. The negative controls (containing
only sterile media) were enriched with 1% glucose to be used as blank. Later, 180 μl of the enriched media was put in each of the 96 wells, and 20 μl of bacterial suspension was added to it. The microtiter plate was incubated at 37°C for 24h. The isolates those formed biofilms on the walls of microplate wells were stained with 150 μl of CV for 15 min. Bacterial solutions in the wells were removed by washing twice with phosphate-buffered saline (PBS) of pH 7.2 before drying wells at 60°C for 1h. Then the planktonic cells which not produced biofilm in wells of microplate were discharged. After drying, dye of biofilms that lined the walls of the microplate was re-solubilized by 150 μl of 96% ethanol and then microplate was spectrophotometrically measured at 570 nm by the microplate reader. Absorbance values of the blanks were utilized to identify whether the biofilm formation of isolates exists or not. The wells of isolates which their OD values were higher than the blank were considered as biofilm formers.

**Activation and identification of probiotic bacteria:**

Activation of *Lactobacillus casei* solutions were performed by inoculating 1 ml culture of each separately into 10ml of MRS broth and incubating anaerobically in a candle jar at 37 °C for 48h. Later, probiotic was re-cultured on MRS agar and incubated under same conditions. Identification of both probiotic bacteria was performed due to their morphological characteristics, Gram staining, catalase test reaction and other biochemical tests (Shokri et al., 2018).

**Preparation of probiotic filtrates:**

The probiotic filtrates were prepared by inoculating 1ml of of *L. casei* isolates in 9ml MRS broth and incubated for 24h at 37°C anaerobically. Later, 2% of the grown culture was added to 100ml MRS broth and incubated in a candle jar at 37°C for 48h. After incubation, the fermented cultures were centrifuged at 4°C by 8000 rpm for 15 min and the suspensions were filtrated
through 0.22µm Millipore filters before stored at 4°C (Lin et al., 2014).

Three concentrated filtrates were prepared from the above unconcentrated culture (filtrate) by evaporating 100ml of the unconcentrated filtered suspension in the oven at 45°C to reduce the volume to 50ml for obtaining the one-fold concentrated filtrate. The test was repeated on the one-fold concentrated filtrate to obtain the two-fold (25ml) and the three-fold concentrated filtrates (12.5ml) (Izgü and Altinbay, 1997).

**Eradication of *Stenotrophomonas maltophilia* biofilm**

The eradication biofilm formed by four clinical isolates was determined by 96-well microtiter plate assay based on the crystal violet staining method. Briefly, each 96-well flat-bottomed sterile polystyrene microplate well containing 180µL of Brain Heart infusion broth were inoculated with 20µL from suspended bacterium of 0.5 McFarland. Microplates are incubated 24 h at 37°C. The liquid media was discarded, and the adherent cells were treated by:

1. Three fold concentration *L. casei* filtrate.
2. Each of antibiotics Ciprofloxacin, Gentamicin, Ceftazidime and Amikacin.
3. The combinations of each antibiotics Ciprofloxacin, Gentamicin, Ceftazidime and Amikacin and the three-fold concentrated filtrates of *L. casei* in ratios (50:50, 75:25 and 25:75).

Then Microplates are incubated 24 h at 37°C. The liquid media was discarded, and the adherent cells were washed twice with phosphate buffered saline (PBS) and wells are dried at 60°C for 1 hour or less. After that it was stained with 150µL of 2% of crystal violet for 15 min. Then the crystal violet -stained wells of microplates were washed twice with PBS to discharge crystal violet stain. After air drying process of wells of
microplate, dye of biofilms that lined the walls of the microplate is re-
solubilized by 150μL of 95% ethanol. After 5-10min microplate is 
measured spectrophotometrically at 570 nm by a microplate reader 
(Kirmusaoğlu, 2019).

Result and discussion

Isolation and primary identification of bacteria:

All clinical samples were cultured on MacConkey agar, 4 isolates 
were obtained from UTI specimens. Colonies of the isolates appeared as 
small, round, non-lactose fermenter, produce pigmented colonies (yellow) 
on MacConkey agar such characteristics are similar to those described by 
Adegoke et al. (2017) for Stenotrophomonas maltophilia. Amoli et al. 
(2017) obtained Stenotrophomonas maltophilia isolates when cultured on 
MacConkey agar from samples were collected from urine, blood and 
sputum of patients who were admitted to Imam Ali hospital in Amol. Gallo 
et al. (2015) obtained S. maltophilia isolates from the nosocomial 
environment, material, and equipment of a Brazilian hospital. The isolates 
were cultured on MacConkey agar.

Figure (1): Stenotrophomonas maltophilia grown on MacConkey agar
Identification of *Stenotrophomonas maltophilia* by VITEK 2 system:

VITEK 2 system which used for identification isolates showed that all the four bacterial isolates were identified as *Stenotrophomonas maltophilia* and gave positive results with 99% of probability.

Garcia-Garrote *et al.* (2000) mentioned that the VITEK 2 system is an easy to handle, provides a rapid result (during 4 to 15 h) and reasonably suitable for the identification of microbial species.

**Biofilm formation by *Stenotrophomonas maltophilia*:**

The results Microtiter plate (MtP) which used for determination of biofilm formation by *Stenotrophomonas maltophilia* isolates are shown in table. The absorbance of blank values are used to identify whether biofilm formation of isolates exists or not. Cut off value (ODc) can provide categorization of isolates as biofilm producer or not. After incubation, 4 isolates from urine were able to form biofilms. Among these, isolates No(215) was the strongest biofilm producer when its average optical density reached 0.42. However, isolate No 753 was recorded lowest an average optical density 0.33.

**Table (1): Detection of biofilm formation by *Stenotrophomonas maltophilia* isolates depending on microtiter plate method.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>OD1</th>
<th>OD2</th>
<th>OD3</th>
<th>Average</th>
<th>ODC</th>
<th>2*ODC</th>
<th>4*ODC</th>
<th>OD isolate</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>T776</td>
<td>0.33</td>
<td>0.36</td>
<td>0.38</td>
<td>0.35</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td>0.25</td>
<td>++</td>
</tr>
<tr>
<td>753</td>
<td>0.32</td>
<td>0.36</td>
<td>0.31</td>
<td>0.33</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td>0.23</td>
<td>++</td>
</tr>
<tr>
<td>754</td>
<td>0.37</td>
<td>0.35</td>
<td>0.39</td>
<td>0.37</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td>0.27</td>
<td>++</td>
</tr>
<tr>
<td>215</td>
<td>0.42</td>
<td>0.41</td>
<td>0.45</td>
<td>0.42</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td>0.32</td>
<td>++</td>
</tr>
<tr>
<td>NC</td>
<td>0.07</td>
<td>0.06</td>
<td>0.08</td>
<td>0.07</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0= No biofilm, + = Weak biofilm, ++= Moderate biofilm

To interpret results, categorization can be done as no biofilm production (0), weak (+), moderate (++), and strong biofilm production (+++) by the calculation of cutoff value (ODc) as shown below:
OD \leq ODc \text{ no biofilm production} \\
ODc < OD \leq 2^* \text{ ODc weak biofilm formation.} \\
2^* \text{ ODc} < OD \leq 4^* \text{ ODc moderate biofilm formation.}

**Eradication of biofilm formed by isolate *Stenotrophomonas maltophilia* (215):**

The performed biofilm of strain ST.A.F were treated by *L. casei* filtrate and each of antibiotics ciprofloxacin (CIP), Gentamicin (GM), Amikacin (AK) and Ceftazidime (CAZ) and their combination in ratio 50:50, 75:25, 25:75 as show in fig (2). The result showed that highest eradicating of biofilm by treated with combination of *L. casei* and each of ciprofloxacin and gentamicin in ratio 50:50 were 0.03 and 0.06 respectively. While *L. casei* filtrate recorded (0.07), and no effect recorded by uses of antibiotics alone.

![Figure 2](image_url)

**Figure(2):** Treatment of biofilm produced by strain *S. maltophilia* ST.A.F by *L. casei* and antibiotics ciprofloxacin (CIP), Gentamicin GM, Amikacin AK and ceftazidime CAZ and their combinations in ratio 50:50, 75:25, 25:75.

**Eradication of biofilm formed by *Stenotrophomonas maltophilia* clinical isolate(753):**
The biofilm produced by clinical isolate (753) were treated by each by *L. casei* each of antibiotics ciprofloxacin (CIP), Gentamicin (GM), Amikacin (AK) and Ceftazidime (CAZ) and their combination in ratio 50:50, 75:25, 25:75 as show in fig (3). The results showed that *L. casei* eradicated biofilm and recorded OD 0.07. While the combination of *L. casei* and Amikacin in ratio 50:50 showed highest effective 0.04.

![Figure (3): Treatment of biofilm produced by isolate 753 by *L. casei* and antibiotics ciprofloxacin (CIP), Gentamicin GM, Amikacin AK and ceftazidime CAZ and their combinations in ratio 50:50, 75:25, 25:75.](image)

Eradication of biofilm formed by *Stenotrophomonas maltophilia* clinical isolate (754):

The treatment of biofilm formed by clinical isolate (754) carried out by *L. casei* filtrate and each of antibiotics ciprofloxacin (CIP), Gentamicin (GM), Amikacin (AK) and Ceftazidime (CAZ) and their combination in ratio 50:50, 75:25, 25:75 as showed in fig (4). The result was recorded that *L. casei* had eradicated biofilm and recorded OD 0.06. However, the combination of *L. casei* and ceftazidime in ratio 50:50 showed highest...
eradicated of biofilm 0.03 and no effect of all antibiotics on performed biofilm.

Figure (4): Treatment of biofilm produced by *S. maltophilia* isolate (754) by *L. casei* and antibiotics ciprofloxacin (CIP), Gentamicin GM, Amikacin AK and ceftazidime CAZ and their combinations in ratio 50:50, 75:25, 25:75.

Eradication of biofilm formed by *Stenotrophomonas maltophilia* clinical isolate (T776):
The treatment of biofilm formed by clinical isolate (754) carried out in same way as the isolates above as showed in fig (5). The results showed that *L. casei* had eradicated performed biofilm and recorded OD 0.06 and the highest effective on performed biofilm were in combination of *L. casei* and Amikacin in ratio 50:50 which recorded OD 0.03.
Fig (5): Treatment of biofilm produced by S. maltophilia clinical isolate T776 by L. casei and antibiotics ciprofloxacin (CIP), Gentamicin GM, Amikacin AK and ceftazidime CAZ and their combinations in ratio 50:50, 75:25, 25:75.

Barzegari et al. (2020) mentioned that probiotics have ability to hinder the activity of pathogenic bacteria and their adhesion to surfaces. Moreover, they prevent quorum sensing, biofilm formation and the survival of biofilm pathogens, interfere with biofilm integrity/quality and finally lead to biofilm eradication. The authors also mentioned that Lactobacillus species can produce different exometabolites such as bacteriocins, and biosurfactants with anti-biofilm activity. Wasfi et al. (2018) investigated that whether Lactobacillus casei (ATCC 393), Lactobacillus reuteri (ATCC 23272), Lactobacillus plantarum (ATCC14917) and Lactobacillus salivarius (ATCC 11741) inhibit expression of Streptococcus mutans genes involved in biofilm formation, quorum sensing and stress survival using quantitative real-time polymerase chain reaction (qPCR). Pompilio et al., (2016) mentioned that the activity of minimal biofilm eradication concentration (MBEC) of levofloxacin (LVX) at clinically relevant
concentrations against preformed biofilm formed by *S. maltophilia* strain were significantly higher at least to 100-fold than MIC values. Fazlurrahman Khan *et al.* (2020) pointed out that the combinatorial strategies for treatment infectious biofilm include combination of multiple antibiotics, combination of antibiotics with non-antibiotic agents and loading of antibiotics onto a carrier. Abdulhasan *et al.* (2016) investigated the highest eradication of *K. pneumoniae* biofilm when treated with combination of antibiotics and essential oil.

**Conclusion:**

The ability of *S. maltophilia* to produce biofilm on surfaces and on host tissues has a clinical important due to difficult eradicate biofilm and less susceptibility to antimicrobial agents and it has been determined to be associated with 65% of hospital-acquired infections. The four producers clinical isolates of were treated with filtrate of *L. casei* and antibiotics ciprofloxacin (CIP), Gentamicin (GM), Amikacin (AK) and ceftazidime (CAZ) and their combinations in ratio 50:50, 75:25, 25:75. The result showed that most effective treatment were combination of filtrate of *L. casei* with antibiotics in ratio (50:50). Furthermore, biofilms of four clinical isolates eradicated by *L. casei* filtrate alone. We hypothesized from our result that combination treatments which consist of two or more antimicrobial agents have significant efficiency for eradication biofilm.

**References:**

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