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ANTIBIOFILM EFFECTS of *Citrus limonum* and *Zingiber officinale* Oils on BIOFILM FORMATION of *Klebsiella ornithinolytica, Klebsiella oxytoca* and *Klebsiella terrigena* SPECIES

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Abstract

Background: Microbial cells growing in biofilms, play a huge role in the spread of antimicrobial resistance. In this study, biofilm formation of *Klebsiella* strains belonging to 3 different *Klebsiella* species (*K. ornithinolytica, K. oxytoca and K. terrigena*), cooccurences' effect on biofilm formation amount and anti-biofilm effects of *Citrus limon* and *Zingiber officinale* essential oils on biofilm formations of highest biofilm forming *K. ornithinolytica, K. oxytoca and K. terrigena* strains were determined.
Materials and Methods: Anti-biofilm effects of *Citrus limon* and *Zingiber officinale* essential oils on biofilm formations of highest biofilm forming *K. ornithinolytica, K. oxytoca and K. terrigena* strains were investigated**.**
Results: 57% of *K. ornithinolytica* strains and 50% of *K. oxytoca* strains were found as Strong Biofilm Forming (SBF), there wasn't any SBF strain in *K. terrigena* species. In addition to this, clinical materials of urine and sperm were found as the most frequent clinical materials for strong biofilm forming *K. ornithinolytica* and *K. oxytoca* isolations respectively (63%; 100%) Secondly, all *K. ornithinolytica* strains isolated from surgical intensive care unit and all *K. oxytoca* strains isolated from service units of urology were found as SBF. Apart from these, although the amount of biofilm, formed by co-occurence of *K. ornithinolytica* - *K. oxytoca* and *K. oxytoca* - *K. terrigena* were more than the amount of biofilm formed by themselves separately, biofilm formation amount of co-occurrence of *K. ornitholytica* - *K. terrigena* strains was lower than biofilm formation amount of *K. ornithinolytica* but higher than biofilm formation amount of *K. terrigena.*
Conclusion: The antibiofilm effects of *Citrus limonum* and *Zingiber officinale* essential oils could be used against biofilm *Klebsiella* aquired infections.

Introduction

Klebsiella species are opportunistic pathogens, causing variety of infections such as urinary tract, pneumonia and abscesses (Podschun and Ullmann, 1998; Ullah et al., 2009). Besides these, by virtue of forming highly invasive hypermucoid colonies, the importance of *Klebsiella* strains' pathogenesis is on the increase daily (Ortega et al., 2011). In addition, the pathogenesis of *Klebsiella sp*. is largely affected by the growth of various bacteria and their biofilm formations on both living tissues and different surface materials. Due to its resistance to various antimicrobial agents, and the ability to acquire some new resistance traits genetically, microbial cells growing in biofilms, play a huge role in the spread of antimicrobial resistance and constitute a huge problem in medical cases (Watnick and Kolter, 2000; Donlan, 2002). When compared with *Klebsiella* species, *K. oxytoca* presents as a lead microorganism followed by *K. pneumoniae* in *Klebsiella* acquired infections. Again, *K. terrigena* and *K. ornithinolytica* are rarely isolated from human specimens (Podschun et al., 2000; Lehloenya and Christians, 2012).

Thus, in this study; investigation of biofilm formation levels of *K. ornithinolytica, K. oxytoca* and *K. terrigena* strains; examination of each *Klebsiella* strains' clinical information according to their biofilm formation results; observation of highest biofilm forming *K. ornithinolytica, K. oxytoca and K. terrigena* strains' co-occurrences on biofilm formation amount and determination of anti-biofilm effects of *Citrus limonum* and *Zingiber officinale* essential oils on biofilm formations of highest biofilm forming *K. ornithinolytica, K. oxytoca* and *K. terrigena* strains were aimed at in order to take precautions against biofilm acquired *Klebsiella* infections.

Materials and Methods

Bacterial strains

K. ornithinolytica, K. oxytoca and *K. terrigena* strains were obtained from clinical materials as identified in Avcioglu's previous study, were used (Avcioglu, 2015). All isolated strains were inoculated into the Brain Heart Infusion Broth media including 10% glycerol and stored at -20°C for later analysis.

Biofilm Formation

To investigate biofilm formation of *K. ornithinolytica, K. oxytoca* and *K. terrigena* strains, modified method of Crystal Violet Binding Assay described by O'Toole, was used (O'Toole, 2011). In this method, *Klebsiella* species were sub-cultured into the Brain Heart Infusion Broth at 37°C overnight. After the incubation period, these cultures were diluted to the proportion of 1:100 and transferred into the 24-well polystyrene plates and incubated for 24 hours at 37°C. Then, the wells were washed, dried and stained with 1% crystal violet and incubated for 30 min at room temperature. Following this, crystal violet were removed from the well by washing with distilled water. Finally, the absorbance of solubilized crystal violet for each well was measured at 540 nm and this experiment was performed in triplicate. *Klebsiella* strains having an OD value ≥ 0.4, were determined as biofilm producers and according to their biofilm formations, *Klebsiella* strains were classified into four categories as follows; 0≤OD<0.4 - None Biofilm

Former (NBF), 0.4≤OD<0.8 - Weak Biofilm Former (WBF), 0.8≤OD<1.2 - Intermediate Biofilm Former (IBF) and OD≥1.2 - Strong Biofilm Former (SBF).

Anti-Biofilm Effects of *Citrus limonum* and *Zingiber officinale* Essential Oils against *K. ornithinolytica, K. oxytoca* and *K. terrigena* Strains

To investigate the inhibitory effects of essential oils on biofilm formation of *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* strains, two different essential oils (*Citrus limonum* and *Zingiber officinale*) were purchased from NU-KA Defne Essencia, TURKEY, were used. Likewise, 0.2 ml of pure (none diluted) essential oils of *Citrus limonum* and *Zingiber officinale* and each dilution of them (ratio of 1:10 and 1:100) were added into the 24-well polystyrene plates seperately. Afterwards 0.5 ml of 1:100 diluted highest biofilm forming *K. ornithinolytica, K.oxytoca* and *K. terrigena* strains were inoculated into these plates and were incubated for 24hrs at 37°C. Positive controls were performed with the wells with the highest biofilm forming *K. ornthinolytica, K. oxytoca* and *K. terrigena* strains exist without any concentration of *Citrus limonum* and *Zingiber officinale* essential oils. The experiment was performed in triplicate. Finally, biofilm formation was analyzed by "Crystal Violet Binding Assay" which has been described previously. Percentage changes in biofilm formation amount were calculated by the following formula;

$$\% \text{ of Change} = \frac{[OD_{Control} - OD_{Treatment}]}{[OD_{Control}]}$$

Biofilm Formation by Co-occurrences of Highest Biofilm Forming *K. ornithinolytica, K. oxytoca* and *K. terrigena* Strains

In the last part of this study, according to the biofilm formation results of clinical strains of *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* strains; the highest biofilm forming strains of each *Klebsiella* species were determined. These strains were inoculated into Brain Heart Infusion Broth medium and were incubated at 37°C for 24 hrs. Afterwards, 0.5 ml of each highest biofilm forming strains' diluted inoculums were transferred into the 24-well polystyrene plates and cocultivations of *K.ornithinolytica - K.oxytoca, K.ornithinolytica - K.terrigena* and *K. oxytoca - K.terrigena* strains were incubated for 24 hrs at 37°C. The experiment was performed in triplicate. Biofilm formation by co-occurrences of highest biofilm forming *K. ornithinolytica, K. oxytoca* and *K. terrigena* strains were also analysed by "Crystal Violet Binding Assay" which has been described previously.

Results

In this study, the biofilm formation levels of 3 different species of *Klebsiella* species (*K. ornithinolytica*, *K. oxytoca* and *K. terrigena*) obtained from different clinical materials were examined and it was seen that whereas 57% of *K. ornithinolytica*, 50% of *K. oxytoca* and 40% of all *Klebsiella* strains were found as SBF, there weren't any SBF strain belonging to *K. terrigena* species (Fig. 1).

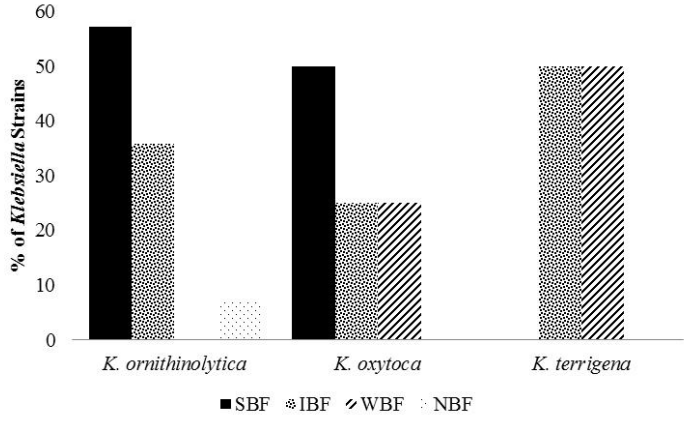

Figure. 1: The percentage of SBF, IBF, WBF and NBF of *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* species.

In addition, it was found that although sample of urine was the most SBF *K. ornithinolytica* isolated material, all *K. oxytoca* strains isolated from sample of sperms were observed as SBF (Fig. 2A and Fig. 2B).

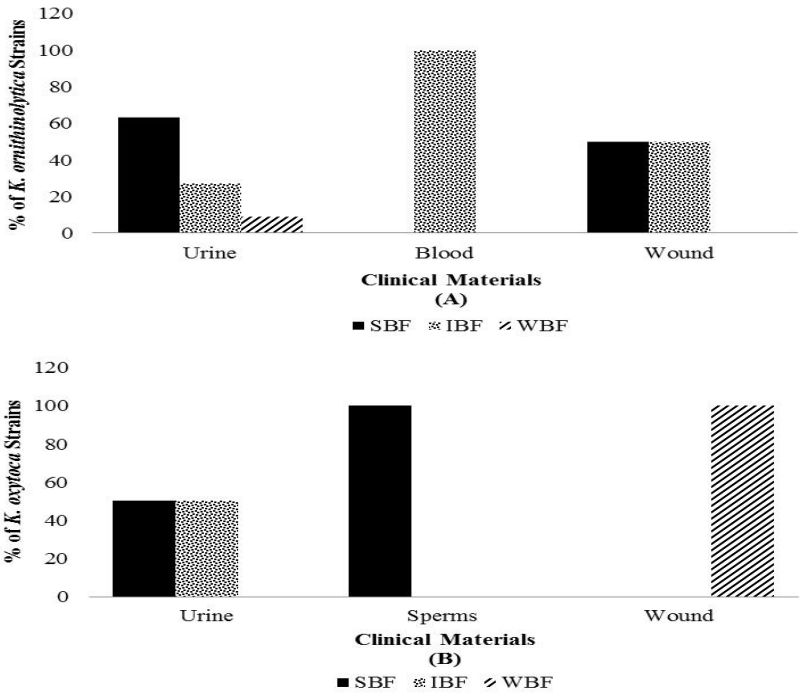

Figure 2: Percentage of WBF, IBF and SBF *Klebsiella* species in different clinical materials **(A)** *K. ornithinolytica* **(B)** *K. oxytoca.*

Furthermore, it was also observed that all *K. ornithinolytica* strains isolated from surgical intensive care unit and all *K. oxytoca* species isolated from urology were strong biofilm formers (Fig. 3A and Fig. 3B). Besides, when the percentage of clinical materials and services from which all SBF *Klebsiella* strains isolated, were examined together, it was seen that urine sample and the service impact of physical treatment and the rehabilitation unit were found as the most SBF isolated material and service respectively.

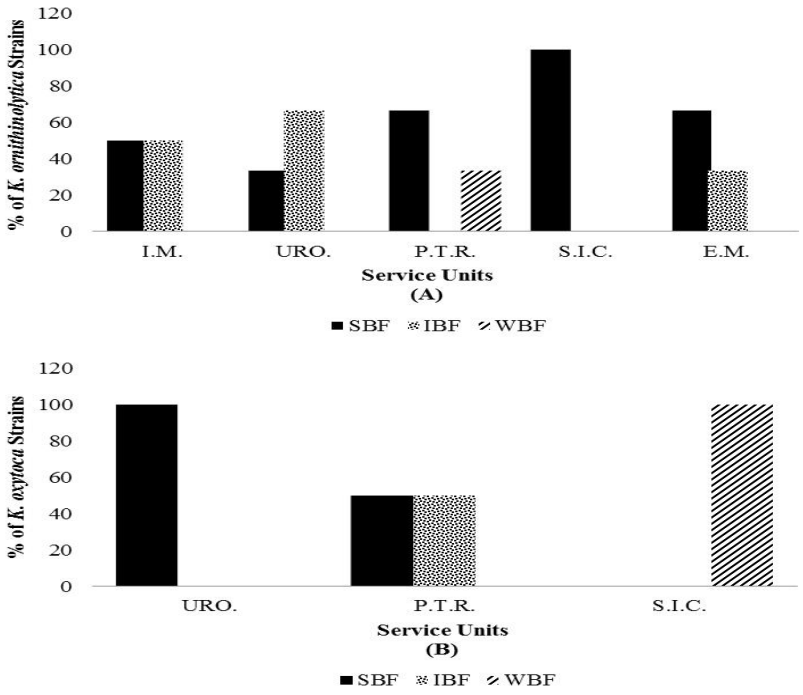

Figure 3: Percentage of WBF, IBF and SBF *Klebsiella* species in different service units **(A)** *K. ornithinolytica* **(B)** *K. oxytoca*.

Essential oils of *Citrus limonum* and *Zingiber officinale* were applied and the inhibitory effects of different concentrations of these essential oils on biofilm formation of *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* strains investigated. Moreover, according to our results, whereas undiluted essential oil of *Zingiber officinale* caused a decrease on all highest biofilm forming

Klebsiella species, the 10^{-1} dilution of this oil did not cause any significant effect on the biofilm formation amount. Also, different concentrations of *Citrus limonum* essential oil caused an inhibition on biofilm formation of all *Klebsiella* species except for *K. oxytoca* strain which was treated with 10^{-1} diluted amount of *Citrus limonum* (Fig. 4A, Fig. 4B and Fig. 4C).

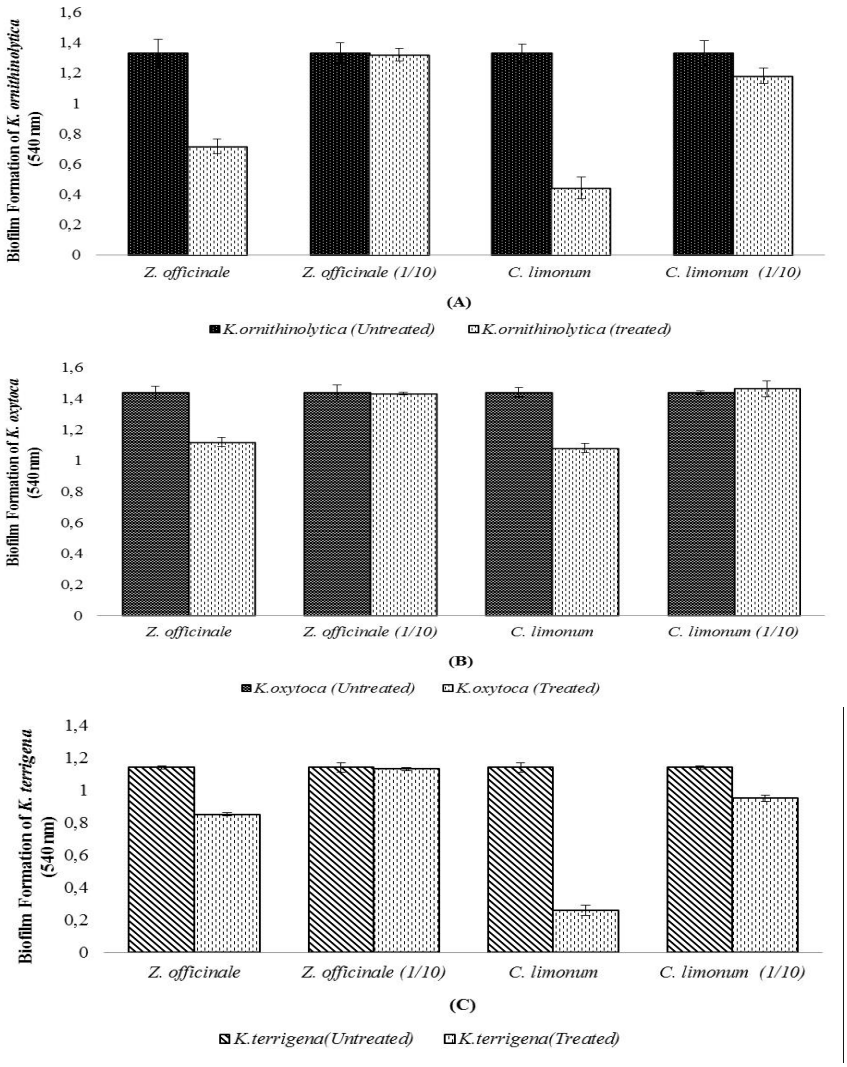

Figure 4: Anti-biofilm effects of *Citrus limonum* and *Zingiber officinale* essential oils against **(A)** *K. ornithinolytica*, **(B)** *K. oxytoca* and **(C)** *K. terrigena* strains.

In the last part of this study, when the effects of co-occurrences of highest biofilm forming *K. ornithinolytica, K. oxytoca* and *K. terrigena* strains on biofilm formation were examined, the amount of biofilm formed by co-occurrences of *K.ornithinolytica-K.oxytoca* and *K. oxytoca–K. terrigena* strains was more than the amount of biofilm formed by each separately, whereas biofilm formation amount of co-occurrence of *K. ornitholytica-K. terrigena* strains was observed to be lower than the biofilm formation amount of *K. ornithinolytica* but higher than biofilm formation amount of *K. terrigena* (Fig. 5).

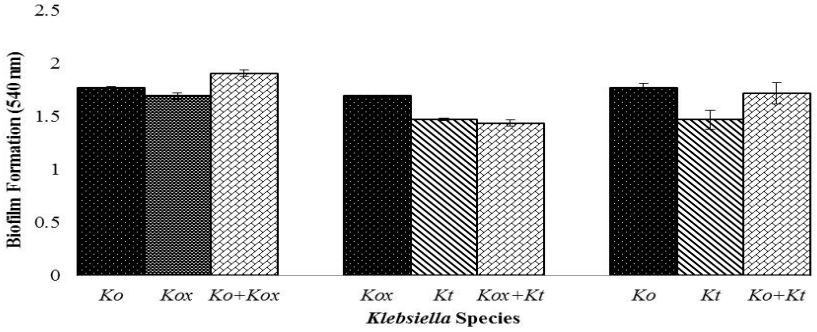

Figure 5: Biofilm formation by co-occurrences of highest biofilm forming *Klebsiella* species

(Ko: K. ornithinolytica; Kox.: K. oxytoca; Kt: K. terrigena; Ko+Kox: co-occurences of K. ornithinolytica and K. oxytoca; Kox+Kt: co-occurences of K. oxytoca and K. terrigena; Ko+Kt: co-occurences of K. ornithinolytica and K. terrigena)

Discussion

Various microorganisms are able to form biofilms and with the help of this layer, they are able to prevent themselves from both the immune response of host cells and various antimicrobial agents (Donlan, 2002; Abdel-Aziz and Aeron, 2014). Among biofilm producing pathogens, it is observed that biofilm formation remains an important stage in the pathogenesis of *Klebsiella* species by virtue of having fimrial adhesis that enhances its attachment to the host cell thereby simplifying the biofilm formation (Langstraat et al., 2001; Martino et al., 2003). In order to prevent biofilm formation by clinical strains and biofilm acquired nosocomial infections, the investigation of biofilm formation levels of different microorganisms becomes necessary. Therefore, the determination of biofilm formation levels of three different species of *Klebsiella* genus (*K. ornithinolytica*, *K. oxytoca* and *K. terrigena*); evaluation of their prevalence in different service units and clinical materials according to their biofilm formation levels were aimed at in this study. Aside that, the effect of the strongest biofilm forming strains' co-occcurrences on biofilm formation amount and anti-biofilm effect of two different essential oils on biofilm formation of strongest biofilm forming *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* strains were also investigated. In view of all these results; this study aims at shedding more light on most studies carried out on the prevention of *Klebsiella* related nosocomial biofilm infections. Furthermore, investigation of the effects of *Citrus limonum* and *Zingiber officinale* essential oils on biofilm formation of strongest biofilm forming *Klebsiella* strains will provide a better understanding of usage avability of these natural substance in therapeutic procedures.

In this study, when *Klebsiella* strains were investigated according to their biofilm formation levels, it was observed that the highest percentage of SBF strains belong to *K.ornithinolytica* species (57%) whereas, all *K. terrigena* strains were either IBF (Intermediate Biofilm Forming) or WBF (Weak Biofilm Forming) (Fig. 1). Being a natural inhabitant of soil and water, *K. terrigena* is not as prevalant as *K. ornithinolytica* and *K. oxytoca* strains. Therefore, *K. terrigena* was not found to be investigated as biofilm related infections agent as the other *Klebsiella* species (Stock and Wiedemann, 2001; Shaikh and Morgan, 2011).

According to recent studies, nosocomial infections usually acquired from various implants such as heart valves, artificial veins, joint protheses and urinary tract catheters (Dohnt et al., 2011). Besides, nowadays, urinary tract infections, are known to be the most common bacterial infections in human. This has become a serious health challenge with lots of disorders and increased usage of urinary catheters resulting into increase in catheter acquired urinary tract infections (Gales et al., 2000; Lina et al., 2007; Niveditha et al., 2012). As a reusult, 40% of nosocomial infections of UTI are known to be related with catheterization in patients (Gristina 1987). Also, long term usage of catheters provides an opportunity for various gram negative and gram positive bacteria hence they trigger nosocomial infections by means of causing biofilm formation (Gristina et al., 1987; Ohkawa, 1991; Jacobsen et al., 2008). Furthermore, when the insidance of nosocomial UTIs was investigated, it was observed that 6%-17% of these infections were generally acquired from the genus of *Klebsiella sp.* and *K. ornithinolytica* was also indicated as one of the causative agents of UTI infections (Imirzalioglu et al., 2008). According to our results, when all strains were evaluated together, the urine sample was found to be the most SBF isolated clinical material among all *Klebsiella* species. Hence, we suggest that this infection might as well be related with long term catheter usage. Because all of the SBF strains belong to *K. ornithinolytica* were found to be isolated from surgical intensive care unit and *K. oxytoca* were isolated from urology and from hospitalized patients (Fig. 2A and Fig. 2B). Confirming these, when all SBF strains were evaluated together, it was observed that the service of PTR and the clinical material of urine sample was the most SBF isolated material and service unit respectively. Therefore these *Klebsiella* infections might as well be nosocomial. In a similar vein, another study, *K. ornithinolytica* strains with the ability to form biofilms were also isolated from catheter samples of patients (Hol'al et al. 2010). Therefore, the isolated SBF strains from catheters were regarded as lead agents of urinary tract infections. Conversely, catheter acquired UTIs are thought to be acquired from gram negative and gram positive microorganisms which are found in fecal flora (Tenke et al., 2006).

When the clinical material from which SBF *K. oxytoca* strains isolated, was examined, sperm cells were surprisingly found as the most SBF isolated material. When recent literature were examined within the content of the results obtained, sperm cells were indicated to include lots of commencial bacteria. Therefore, it became plussible seeing some of these opportunistic pathogens in ~~the~~ sperm cells infections especially in immunocompromised patients (Rehewy et al., 1979; Huyser et al., 1991). Furthermore, in this study all the isolated strains from the sperm were SBF and also in literature *Klebsiella* genus was known as one of the most important nosocomial infection factors among all gram negative bacteria after *Escherichia coli.* Hence it became extremely important to take precautions and to find new treatment strategies against *Klebsiella* acquired infections. Because biofilm formation was one of the most important problem avoiding in the treatment of infectious diseases especially in MDR strains (Podschun and Ullmann, 1998). Therefore, new essential oils grouped at phitoalexines with high bacteriostatic or bacteriocidal activity, were being used in the treatment of many infectious diseases. Being produced to protect against microbial attacks, it was not surprising to see their inhibition effects on microbial growth or antibiofilm effects on different microorganisms hence creates new treatment strategy (Gibbons, 2008; Gursoy et al., 2009). Moreso, to prevent ototoxic and neuphrotoxic side effets of synthetic drugs, different researches have been investigated with various essential oils' such as lavender, lemon and tea, citronella, *Satureja hortensis L.* (summer savory), *B. papyrifera*, *B. rivae*, oregano and lemongrass oils and it has been observed that these oils have antibiofilm effects on various bacterial and fungal species (Nostro et al., 2007; Schillaci et al., 2008; Gursoy et al., 2009; Lang et al., 2012; Millezi et al., 2012; Taweechaisupapong et al., 2012). Thus, in this study, two different essential oils (*Citrus limonum* and *Zingiber officinale*) were used in order to investigate their antibiofilm effects on three different *Klebsiella* species (*K. ornithinolytica*, *K. oxytoca* and *K. terrigena*). Results obtained, show that, undiluted *Citrus limonum* and *Zingiber officinale* oils inhibit the biofilm formation amounts in all species (Fig. 4A, Fig. 4B and Fig. 4C). Because, these oils identified in the literature as monoterpens and indicated to affect the cell membrane as a toxic agent and cause deformation of the structure decrease the function of microbial cell membrane (Sikkema et al., 1995). Thus, the findings of this research with essential oils will advance the inhibition effects of *Citrus limonum* and *Zingiber officinale* oils and show the thereapeatic avability of these for *K. ornithinolytica*, *K. oxytoca* and *K. terrigena* strains' treatment.

Although many researchers support the benefits of interactions between interspecies, there have been lots of microbial interactions defined as antagonistic interactions resulting from synthesis of some antagonistic compounds by different species as important ingredients for the formation of microbial communities in nature (Taweechaisupapong et al., 2012). In other studies, it was noted that, when compared with mono species forms, dual species cause an increase in antimicrobial resistance (Van der Veen and Abee, 2011). Also, other researchers show the possibility of synergetic and antagonistic interactions between different bacterial species and opined that these interactions were related with certain intrinsic characters which are specific for different species (Liu et al., 2014). To this end, 3 different co-occurrences were prepared (*K. ornithinolytica*, *K. oxytoca* and *K. terrigena*) in our study and effect of co-occurrences of highest biofilm forming strains on biofilm formation amount was investigated. Confirming other researches, co-occurrences of *K. ornithinolytica-K.oxytoca* and *K. oxytoca-K. terrigena* caused more biofilm formation than the amount of biofilm formed by each separately. The other co-occurences was also prepared by the co-cultivation of *K. ornithinolytica-K. terrigena* species and compared to *K. terrigena* monospecies' biofilm formation amount to show an increase owing to these strains' co-cultivation. Again, biofilm formation amount of *K. ornithinolytica* was higher than biofilm formation amount of co-cultivated highest biofilm forming *K. ornithinolytica - K. terrigena* species. As such, we suggest that this antagonistic interaction between *K. ornithinolytica* and *K. terrigena* species might result from the synthesis of some antagonistic compounds such as bacteriocins. It is literally known that, bacteriocins are antimicrobial proteins formed by interspecies in order to affect the permeability and instability of cell membrane and eliminate the bacterial strain in the culture or to mediate the dynamics of microbial population (Hetz et al., 2002; Al-Mathkhury et al., 2011). Confirming all these, in our study biofilm formation in *K. ornithinolytica*'s might have been affected by the antagonistic compounds such as bacteriocins, synthesized by *K. terrigena* (Fig. 5).

In brief, according to our results, most SBF isolated species of *Klebsiella* genus were found to be as *K. ornithinolytica* and *K. oxytoca* and none of *K. terrigena* species was found as SBF because its clinical strains was not widespread as others. Also, the sample of urine and the service of surgical intensive care unit are the most SBF strains isolated material and service respectively. Therefore, these infections were seen as nosocomial and it is important to take precautions against *Klebsiella* acquired infections. Furthermore, co-occurences effect of *Klebsella* species was also investigated in this study and our findings revealed that co-occurences of *K. ornithinolytica- K. oxytoca* and *K. oxytoca-K. terrigena* increased the biofilm formation abilities by using their intrinsic factors and very important in clinical mean especially in long term catheter usage, and treatment strategies. Conversely, co-occurences of *K. ornithinolytica - K. terrigena* decreased the biofilm formation level of mono-species of *K. ornithinolytica* and can be investigated in subsequent studies to ascertain whether it was derived from bacteriocin effect or not.

In all, essential oils of *Citrus limonum* and *Zingiber officinale* were investigated in order to examine their anti-biofilm effects and it was found that they can be used in medical cases as alternative treatment agents against bacterial infections.

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