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Influence of Acetylsalicylic Acid (Aspirin) on Biofilm Production by *Candida* Species

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Summary

***Candida* spp. are important causative agents of infections associated with biofilm formation. Management of biofilm-related infections is extremely difficult and therefore new therapeutic solutions are needed. This study for the first time explored the possible effect of aspirin on *Candida* spp. Biofilm-producing capacity. Two strains of *C. guilliermondii*, and one strain per species of *C. kefyr*, *C. glabrata*, *C. albicans*, and *C. parapsilosis* were included in the study. The antifungal property of aspirin was tested by the broth microdilution method, while effect of aspirin on biofilm formation was determined by the microtiter-plate test. The minimal inhibitory concentrations of aspirin obtained ranged from 2.17 to 8.67 mM and minimal fungicidal concentrations were from 4.33 to 8.67 mM. The concentrations of aspirin which induced statistically significant decrease in biofilm formation ranged from 0.43 mM to 1.73 mM of aspirin, depending on the tested yeast strain. Therefore, the significant effects of aspirin on growth and biofilm formation of *Candida* spp. were achieved only with suprapharmacological concentrations of the drug. The influence of the inoculum size on the effect of aspirin on biofilm formation was determined for *C. albicans* only and a significant decrease was observed also at suprapharmacological concentrations of aspirin, irrespective of the inoculum size. The results obtained in the present study show aspirin to be a drug with the potential to affect and suppress biofilm formation by *Candida* spp., and provide support for further investigation.**

Key words: **Acetylsalicylic acid, aspirin, biofilm, *Candida* spp.**

INTRODUCTION

Candida spp. are causative agents of infections such as native valve endocarditis, central venous catheter, contact lens and intrauterine device infections [1]. Biofilm is a common trait for all these infections, as the microorganisms that cause device-related and chronic infections preferentially grow in biofilms [1]. Management of biofilm-related infections is extremely difficult, since cells in biofilms show increased resistance to antibiotics, antiseptics and disinfectants [1]. Thus, new therapeutic solutions are needed, particularly those with the ability to prevent biofilm formation.

Acetylsalicylic acid (aspirin), a synthetically made drug, was introduced for human treatment more than 100 years ago [2]. Aspirin has a short half-life in blood, approximately 20 minutes, because it is rapidly converted to salicylate [2]. The action of aspirin and salicylate in the human body is mediated by inhibition of cyclooxygenase (COX), the main enzyme in the biosynthesis of prostaglandins from arachidonic acid [2]. It has been previously shown that salicylic acid applied at suprapharmacological doses (5 mM) decreases biofilm production by staphylococci [3,4]. A recent study has shown that aspirin *in vitro* suppresses the growth, formation of hyphae and other filamentous structures of *Candida albicans* [5].

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It was proposed that the mechanism of *C. albicans* growth reduction involves inhibition of biosynthesis of 3(R)-hydroxyoxylipins, which belongs to the arachidonic acid metabolites [5]. However, the influence of aspirin on biofilm formation by *Candida* spp. has not been investigated yet and, therefore, the present study aimed to explore whether aspirin would display a suppressive effect on *Candida* spp. biofilm-producing capacity.

MATERIALS AND METHODS

Candida isolates

Fifty clinical isolates of *Candida* spp. were tested for biofilm formation and six most prominent biofilm producers, with optical density (OD) values of measured biofilm ranging from 0.39 to 1.84, were selected for the experiment. The isolates were identified by API 20 C AUX (bioMérieux, Marcy-l'Etoile, France), as: two strains of *C. guilliermondii* (the second strain is marked with *), and one strain per species of *C. kefyr*, *C. glabrata*, *C. albicans*, and *C. parapsilosis*. Stock cultures were maintained on Sabouraud dextrose agar (bioMérieux) (SDA) at 4°C. Prior to inoculation, all strains were transferred from the stock cultures to SDA and incubated aerobically at 35°C for 24 hours. The cultures were then used for preparation of appropriate suspensions in yeast nitrogen base (Biolife S.r.l., Milano, Italy) supplemented with 50 mM glucose (Merck, Darmstadt, Germany) (YNBG). The number of cells was checked with a Neubauer's counting chamber (hemocytometer).

Acetylsalicylic acid (aspirin)

Aspirin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (100 mg) was dissolved in 95% ethanol (1 ml), which resulted in 554.94 mM stock solution of the drug. The subsequent dilutions were made in YNBG.

Antifungal susceptibility testing

Antifungal testing by broth microdilution method based on the NCCLS M-27A method [6], was performed to determine the minimal inhibitory concentrations (MICs) of aspirin for planktonic cells. The procedure we applied differed in that we used YNBG medium instead of RPMI 1640. The MIC has been defined as the lowest dilution of aspirin for which no growth was detected visually (clear well) or 80% reduction in optical density as compared to control growth without aspirin. Minimal fungicidal concentrations (MFCs) were determined by subsequent plating of 20 µl from each well to SDA. After incubation at 35°C for 48 h, MFC was defined as the lowest dilution of aspirin that killed the yeast. The tests were repeated three times.

Effect of aspirin on biofilm formation

Quantification of biofilm production by microtiter-plate test was based on the previously described method [7]. In short, each well of a sterile 96-well flat-bottomed polystyrene microplate (Spektar, Čačak, Serbia; not prepared by the manufacturer for tissue culture work) contained 150 µL of YNBG, 50 µL of aspirin solution and 50 µl of yeast suspension. The final concentration of aspirin ranged from 13.87 mM to 6.78 µM, and the final number of cells in each well was 0.5×10^5 to 2.5×10^5 cells/ml. The positive control wells, which allowed maximal biofilm formation, contained 200 µL of YNBG and 50 µl of yeast suspension only. The negative control wells contained 250 µL of YNBG only. The first step was evaluation of cell growth. Following the incubation at 35°C for 24 h, it was determined spectrophotometrically by measuring the OD (without previous shaking) at 492 nm using a Multiskan EX reader (Labsystems, Helsinki, Finland). Thereafter, the amounts of biofilm produced were examined. The content of each microplate was poured off, and each well was washed three times with 300 µl of sterile phosphate buffered saline. The remaining attached cells were fixed with 160 µl of methanol per well and stained for 5 min with 160 µl per well of crystal violet used for Gram staining (Gram-color staining set for microscopy; Merck). Excess stain was rinsed off by placing the microplate under running tap water. After the microplates were air dried, the dye bound to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well. After the glacial acetic acid was added, plates were left at room temperature for at least 4 h without shaking (to enable complete resolubilization of the dye), and thereafter, the OD of each well was measured at 570 nm using Multiskan EX reader. The strain was considered as a biofilm producer if the measured OD was higher than cut-off OD value (cut-off OD = mean OD of negative control + 3 SD of negative control). All strains were tested in triplicate and the test was repeated three times.

Influence of the inoculum size on the effect of aspirin on biofilm formation

The influence of the inoculum size on the effect of aspirin on biofilm formation was determined for *C. albicans* only. The test was performed as described above, apart from the final number of yeast cells which ranged from ~25 to ~2.5×10^6 cells/ml.

Statistics

The Wilcoxon signed ranks test was used to analyze the differences in growth and biofilm production between yeasts exposed to different concentrations of aspirin and yeasts grown in absence of this drug. P values of <0.05 were considered significant.

RESULTS

We performed antifungal susceptibility testing in order to determine MICs and MFCs of aspirin for tested yeast strains. The MICs on the basis of a visual endpoint / MICs on the basis of an 80% reduction in growth / MFCs were as follows: *C. guilliermondii* 4.33/2.17/4.33, *C. kefyr* 4.33/4.33/4.33, *C. glabrata* 8.67/4.33/8.67, *C. albicans* 8.67/8.67/8.67, *C. guilliermondii** 4.33/2.17/8.67 and *C. parapsilosis* 4.33/2.17/8.67 mM of aspirin.

The averaged results of growth and biofilm formation in the presence of different concentrations of aspirin are presented in *Figure 1*. The decrease in *Candida* spp. growth and biofilm production was drug dose dependent. The cut-off concentrations of aspirin which induced statistically significant decrease in biofilm production were: 0.43 mM for *C. kefyr*, 0.87 mM for *C. guilliermondii*, *C. glabrata*, *C. albicans*, and *C. parapsilosis*, and 1.73 mM for *C. guilliermondii**. The concentrations of aspirin needed for statistically significant decrease in *Candida* spp. growth were even higher (*Figure 1*).

The results of the investigation of the influence of aspirin on growth and biofilm formation by *C. albicans* in YNBG inoculated with various numbers of yeast cells are shown in *Figure 2*. It is apparent that size of the inoculum significantly affected the amount of the biofilm produced. The cut-off concentrations of aspirin which caused a statistically significant decline in biofilm formation were: 0.43 mM for ~2.5 x 10^6 cells/ml, 0.87 mM for ~2.5 x 10^5 cells/ml, 0.87 mM for ~2.5 x 10^4 cells/ml, 1.73 mM for ~2.5 x 10^3 cells/ml, 1.73 mM for ~2.5 x 10^2 cells/ml, 1.73 mM for ~25 cells/ml.

DISCUSSION

Candida biofilm formation is a highly complex phenomenon which involves initial adhesion to surfaces followed by cell growth and aggregation, and production of extracellular matrix [8]. The present study investigated the ability of aspirin to prevent biofilm production by different species of *Candida*. In addition, the possible inhibitory and/or fungicidal effects of aspirin on *Candida* spp. were also examined. The values of MICs of aspirin for tested yeast strains ranged from 2.17 to 8.67 mM, depending on the method applied and the strain tested. These values are similar to the MICs obtained with sodium

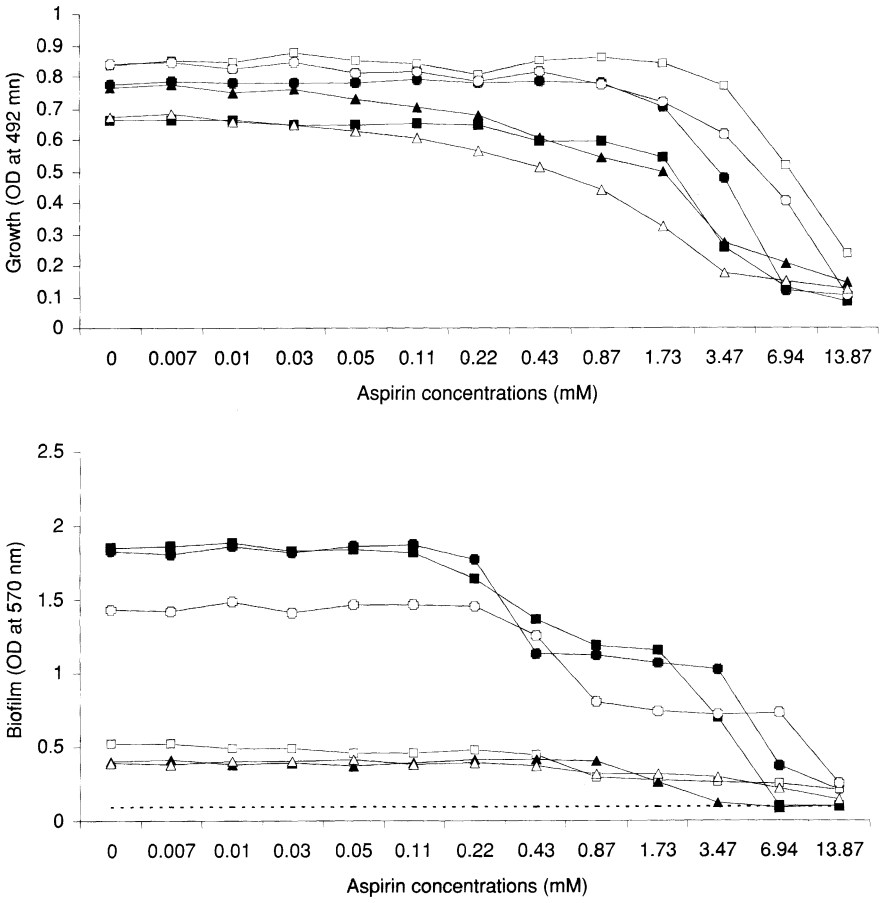

FIGURE 1 - Influence of aspirin on the growth and biofilm formation of six *Candida* spp. with initial inoculum of 10^5 cells/ml (filled squares = *C. guilliermondii*, filled circles = *C. kefyr*, open squares = *C. glabrata*, open circles = *C. albicans*, filled triangles = *C. guilliermondii**, open triangles = *C. parapsilosis*, dash line = biofilm cut-off OD).

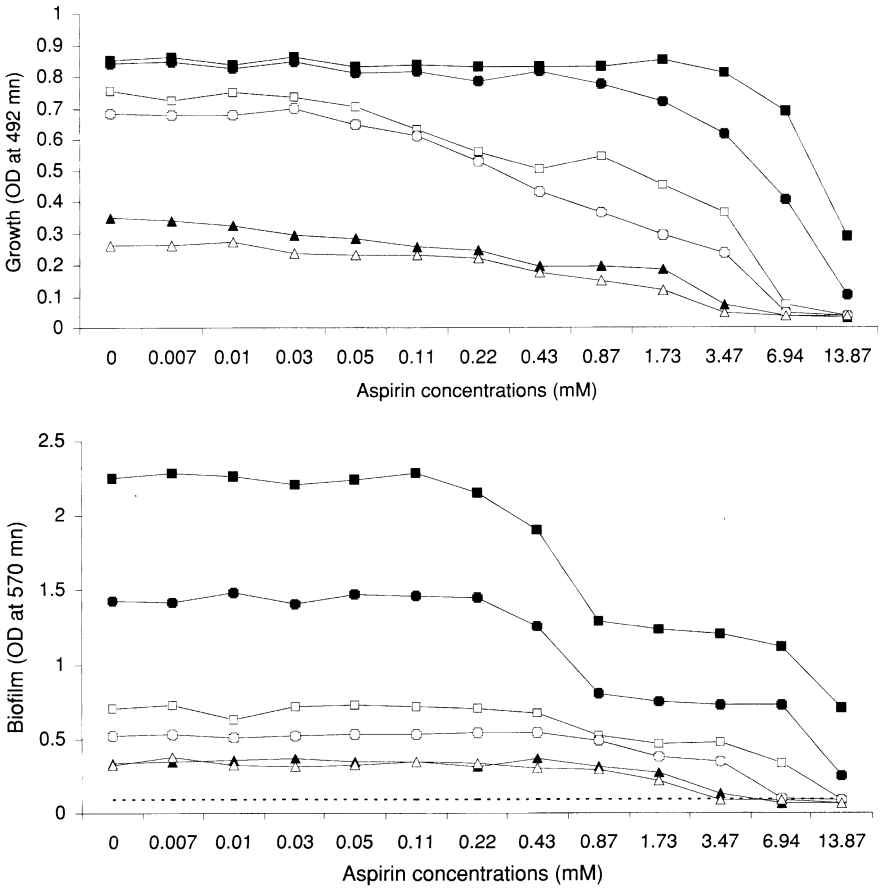

FIGURE 2 - Influence of aspirin on the growth and biofilm formation of *C. albicans* with different initial inoculums (filled squares = ~2.5 x 10^6 cells/ml, filled circles = ~2.5 x 10^5 cells/ml, open squares = ~2.5 x 10^4 cells/ml, open circles = ~2.5 x 10^3 cells/ml, filled triangles = ~2.5 x 10^2 cells/ml, open triangles = ~25 cells/ml, dash line = biofilm cut-off OD)

salicylate for *C. albicans* in the previous study by Scott *et al.* [9].

There is no standardized method for investigation of *Candida* biofilm production [10], and previously used protocols vary widely. We chose YNBG as a medium to support growth and biofilm formation by the yeasts since it has been shown that biofilm formed by *C. albicans in vivo*, on infected intravenous catheters, was similar in structure to the biofilm grown in YNBG *in vitro* [8]. The high inoculum of 10^5 cells/ml was used because the same or even higher inocula were used in other experiments investigating biofilm production by yeasts, irrespective to the method applied [8,10,11,12,13,14].

The cut-off concentrations of aspirin which induced a statistically significant decrease in biofilm production varied from 0.43 to 1.73 mM in different species of tested yeast strains, while even higher concentrations were required for a statistically significant decrease in *Candida* spp. growth. It must be noted that the concentration of salicylate achieved by therapeutic doses of aspirin ranges from 10 µM to 100 µM in humans [2]. However, studies investigating the influence of salicylate on biofilm formation by staphylococci also showed its effectiveness only at a concentration as high as 5 mM [3,4]. Therefore, the significant effects of aspirin on growth and biofilm formation of *Candida* spp., as well as effects on biofilm production by staphylococci obtained by other investigators, were achieved only with suprapharmacological concentrations of the drug.

In addition to the aspirin concentration, the other factor of possible interest is the size of the yeast inoculum. It is not likely that an inoculum as high as the one used in our and previous studies [8,10,11,12,13,14], corresponds to the situation found *in vivo*. Therefore, we investigated the influence of aspirin on growth and biofilm formation by *C. albicans* in YNBG inoculated with various numbers of yeast cells. A statistically significant decrease in growth and biofilm formation was observed also at suprapharmacological concentrations of aspirin, irrespective of the inoculum size.

This is the first report to date on the aspirin effect on biofilm formation by *Candida* spp. and the results presented demonstrate a suppressive effect of the drug and its ability to prevent biofilm production. This was achieved only with suprapharmacological concentrations of aspirin, which limits the applicability of the data we obtained. As noted above, this has also been shown in studies evaluating effects of aspirin on *S. aureus* biofilm production *in vitro*.

However, *in vivo* studies revealed different results. It has been reported that aspirin applied at doses feasible in human therapy, either alone or in combination with vancomycin, caused a reduction in *S. aureus* aortic vegetations in a rabbit endocarditis model [15,16]. Although the limitation of the present study is the small number of strains tested, the results we obtained show aspirin to be a drug with the potential to affect and suppress biofilm formation by *Candida* spp., and provide support for further *in vivo* investigation. One possible direction for future studies is evaluation of the effects of aspirin in combination with some antifungal agents on *Candida* spp. biofilm production, since the synergism between fluconazole and sodium salicylate against *C. albicans in vitro* has already been reported [9].

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