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Antibiofilm activity of mushroom extracts against *Staphylococcus aureus* F. J. Rosen.

Introduction

In recent years, a growing interest has developed in the mechanisms of the action of natural products, because they are a major source of chemical diversity and have provided important therapeutic agents for many bacterial diseases (Payne et al., 2007). Mushrooms have long been appreciated for their taste, flavour, desirable aroma, texture, and nutraceutical and medicinal attributes (Strapáč et al., 2016). Moreover, they are a renowned source of products with an array of bioactivities, from antibacterial to antiviral, cytotoxic, anti-inflammatory, anti-feeding, antifungal or antioxidant and might be a valuable resource in the search of new bioactive extracts to inhibit biofilm production (Martín-Rodríguez et al., 2014; Alves et al., 2014). In this context, flavonoids and phenolic compounds have been revealed as potential inhibitors of biofilm formation and the production of virulence factors in the pathogenic bacteria by interfering with quorum sensing mechanisms (Nazzaro et al., 2013).

The main factors associated with biofilm formation are the iron uptake system and adhesive matrix proteins. Adhesion is favoured by the presence of virulence factors known as adhesins, which are grouped in a family known as the microbial surface components recognising adhesive matrix molecules (MSCRAMM). Major proteins adhesins in this group include fibronectin binding proteins A and B (FnBpA, FnBpB), bone sialoprotein binding protein (Bbp), iron regulated surface determinants A and B (IsdA, IsdB), and serine aspartate repeat gene proteins D and E (SdrD, SdrE) (Rasmussen et al., 2013; Cucarella et al., 2001). *Staphylococcus aureus* F. J. Rosen also produces cytotoxins and hemolysins (α, β, γ and δ), which possess the ability to form pores in host cells enabling lysis. In staphylococci, the expression of a series of toxins and virulence factors are controlled by the accessory gene regulator (Agr) system (Jarraud et al., 2002).

In the present study, a water extract obtained from a sample of *Macrolepiota procera* (Scop.) Singer, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Auricularia auricula-judae* (Bull.) Quél., *Armillaria mellea* (Vahl) P. Kumm. and *Laetiporus sulphureus* (Bull.) Murrill was explored for its antibiofilm activity against *Staphylococcus aureus* strains.

Material and methods

Bacterial strains

The following staphylococci strains were used in this study from our own laboratory: *Staphylococcus aureus* No. 5 and *S. aureus* No. 51, isolated from ixodid ticks (Acari); *S. aureus* No. 12, and *S. aureus* No. 14, isolated from ewe´s milk. All cultures were identified by matrix-assisted laser desorption/ ionization (MALDI) biotyper (Bruker Daltonik, Leipzig, Germany). All staphylococci strains were cultured at 37°C on blood agar (Blood agar base No. 2, Oxoid, Basingstoke, United Kingdom and with 5% defibrinated sheep blood).

Mushroom extracts

The preparation of mushrooms water extracts were determined as described previously (Strapáč et al., 2016). For our analysis, we used 1 kg of freshly harvested fruiting bodies of *Macrolepiota procera*, *Armillaria mellea* and *Laetiporus sulphureus*, collected in the autumn of 2014 in an area of Dargov, Bankov near Košice and Ižkovice, respectively, in the Slovak Republic. The last two are commercially available mushrooms, *Pleurotus ostreatus* and *Auricularia auricula-judae*. Water extracts were prepared by the extraction of 100 mg samples in 2 cm^3 of water for 24 h with occasional vigorous stirring at 8°C in a refrigerator. Then, the extracts were filtered and stored at 4°C.

Biofilm production assay

For the detection of biofilm formation, the crystal violet method with Nunc Maxisorp plates were used (Nunc, Roskilde, Denmark) by a previously published method (O´Toole, 2011) with some modifications. Overnight cultures of *Staphylococcus aureus* were removed from each well and 3 times washed with saline, fixed with methanol, and stained with 0.1% crystal violet. The bound dye was released with 33% acetic acid, and the optical density (OD) at 570 nm was measured by using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, Vermont, USA).

Anti-biofilm activity of mushroom extracts

To detect the effects of mushrooms water extracts of *Macrolepiota procera, Pleurotus ostreatus*, *Auricularia auricula-judae*, *Armillaria mellea*, and *Laetiporus sulphureus* on staphylococcal biofilm formation, 10 µl of extracts were added to the each well and

the plates were incubated 24 h at 37°C. The biofilm quantification has been described above. Wells containing medium were used as blank controls. The percentage of the inhibition of biofilm formation was calculated according to the following equation: (1 – OD590 of test / OD590 of untreated control) × 100%.

Polymerase chain reaction

The presence of virulence genes of staphylococci was carried out by polymerase chain reaction (PCR). Strains were screened for the following genes: α-hemolysin gene (*hla*) as described by (Jarraud et al., 2002), serine-aspartate repeat proteins E gene (*sdrE*) (Sabat et al., 2006), iron-regulated surface determinants A gene (*isdA*) (Verkaik et al., 2010) and B gene (*isdB*) (Waryah et al., 2016), bone sialoprotein – binding protein gene (*bbp*) (Tristan et al., 2003), fibronectin- binding protein A gene (*fnbpA*) (Booth et al., 2001), iron-siderophore transporter gene (*sirB*) (Dale et al., 2004), and accessory gene regulator (*agr II*) (Shopsin et al., 2003).

Statistical analysis

All assays were performed in eight replicates and the means as well as the standard deviations were calculated. A one-way analysis of variance (ANOVA) and Tukey's test were used to compare data utilising Statistica 9.0 software (StatSoft, Tulsa, Oklahoma, USA).

Results

In present study, genes *hla* and *isdA* were found to occur in all Staphylococci. The presence of *sdrE* gene was detected in three out of four strains. Genes *agrII*, *isdB*, *bbp* and *sirB* were only detected in strains isolated from ixodid ticks. Gene *fnbpA* was detected in *Staphylococcus aureus* No. 51. The biofilm formation of *S. aureus* strains was reduced by all mushrooms extracts without affecting the bacterial growth. The best results were observed for the *Armillaria mellea* (70.87%), *Pleurotus ostreatus* (67.00%), *Laetiporus sulphureus* (64.14%) and *Auricularia auricula-judae* (62.77%), while *Macrolepiota procera* showed the lowest reduction of biofilm formation (47.72%). The extracts reduced biofilm formation in the range of 47.72–70.87%, which means that the biofilm was formed in the presence of extracts in the range of 29.13–52.28%. We showed that a more significant anti-biofilm effect of the extracts, except for *M. procera*, was of *Staphylococcus aureus* isolated from ixodid ticks (82.00%) in comparison to *S. aureus* isolated from ewe's milk (50.00%) (Tab. 1). The extract from *M. procera* had a similar effect on strains isolated from ixodid ticks (46.50%) and ewe's milk (49.00%).

Tab. 1. The effect of mushrooms extracts on biofilm formation of *Staphylococcus aureus* F. J. Rosen. 5 isolated from ixodid ticks; significant differences are indicated with asterisks (* – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$, Tukey's test)

Name of mushrooms	Mean value n = 8	Standard deviation (±SD)
Control	0.216	0.100
Armillaria mellea	0.037**	0.006
Laetiporus sulphureus	0.042**	0.014
Pleurotus ostreatus	0.025***	0.009
Auricularia auricula-judae	0.039**	0.010
Macrolepiota procera	0.150	0.048

Discussion

We investigated the main factors associated with biofilm formation. The results of our study are in agreement with Kateete et al. (2011) who reported that 100% isolates had *hla* gene and with Verkaik et al. (2010) who reported that 100% isolates had *isdA* gene. Tristan et al. (2003) reported positivity rates of *fnbA* and *bbp* among *Staphylococcus aureus* isolates 28.00% and 22.00%. Liu et al. (2015) showed that 68.10% of isolates contained the *sdrE* gene.

Our study focused on the antibiofilm activity of mushrooms extracts. We have confirmed here the great potential of mushrooms to produce antibiofilm compounds, and we showed good antibiofilm effects of aqueous extracts in terms of the reduction of biofilm formation. Similar observations have been made previously by others with different bacteria (Kostić et al., 2017), and showed the antibiofilm activity of *Armillaria mellea* extract against *Pseudomonas aeruginosa*. Antibiofilm activity was associated with content of phenolic compounds and organic acids. Another study that worked on the organic extracts of *Macrolepiota procera* and *Laetiporus sulphureus* showed antibiofilm activity against *S. aureus* (Carvalho et al., 2015). Li and Dong (2010) reported the inhibition of *Escherichia coli* T. Escher. biofilm formation (73.00%) by *Auricularia auricula-judae* extract. This is a pioneer study since, as far as we know, there are no reports on the antibiofilm activity by the mushroom extracts of *Pleurotus ostreatus*, against *S. aureus*; nevertheless, other studies are required to elucidate the mechanism of action.

Conclusion

Extracts from mushrooms are a complex of different chemical compounds. An identification and understanding of the mechanisms of mushrooms extracts action will enable their further application to new innovative strategies for the control of microbial contamination and infection via the food chain.

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Abstract

Mushrooms are a renowned source of products with an array of bioactivities, from antibacterial to antiviral, cytotoxic, anti-feeding, antifungal, or antioxidant and might be a valuable resource in the search of new bioactive extracts to inhibit biofilm production. We demonstrate the effect of five mushroom water extracts, *Macrolepiota procera, Pleurotus ostreatus, Auricularia auricula-judae, Armillaria mellea,* and *Laetiporus sulphurous* on biofilm formation of four *Staphylococcus aureus* strains isolated from ixodid ticks (Acari) and ewe´s milk. The PCR was used for the detection of virulence genes (*hla*, *isdA, B*, *bbp*, *sirB*, *fnbpA*, *sdrE, agr II*). The ability of biofilm formation and anti-biofilm activity of mushrooms extracts was assessed in a quantitative crystal violet assay. The biofilm formation of *S. aureus* strains was significantly reduced by all mushrooms extracts ($p < 0.001$). We showed that a more significant anti-biofilm effect of the extracts was of *Staphylococcus aureus* isolated from ixodid ticks in comparison to *Staphylococcus aureus* isolated from ewe´s milk. In the present study, *A. mellea*, *P. ostreatus*, *L. sulphurous*, *A. auricula-judae,* and *M. procera* extracts inhibited biofilm formation by 70.87%, 67.00%, 64.14%, 62.77% and 47.71%, respectively. The results suggest that compounds in mushrooms extracts might be useful to control and handle detrimental infections caused by animal and human pathogens.

Key words: biofilm, fungi, ixodid ticks (Acari), milk, *Staphylococcus aureus*

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Aktywność antybiofilmowa ekstraktów grzybowych przeciw *Staphylococcus aureus* F. J. Rosen.

Streszczenie

Grzyby są znanym źródłem produktów bioaktywnych – począwszy od środków przeciwbakteryjnych po przeciwwirusowe, cytotoksyczne, przeciwlękowe, przeciwgrzybicze lub przeciwutleniające. Mogą być cennym źródłem nowych bioaktywnych ekstraktów poszukiwanych w celu zahamowania produkcji biofilmu bakteryjnego. W pracy pokazano wpływ pięciu wodnych ekstraktów z *Macrolepiota procera*, *Pleurotus ostreatus*, *Auricularia auricula-judae*, *Armillaria mellea* i *Laetiporus sulphurous* na tworzenie się biofilmu czterech szczepów *Staphylococcus aureus*, izolowanych z kleszczy (Acari) i mleka owczego. Do wykrywania genów wirulencji (*hla*, *isdA*, *B*, *bbp*, *sirB*, *fnbpA*, *sdrE*, *agr II*) zastosowano metodę PCR. Zdolność tworzenia biofilmów i aktywność anty-biofilmową ekstraktów grzybów oceniano w analizie ilościowej fioletem krystalicznym. Tworzenie biofilmu szczepów *S. aureus* było znacznie mniejsze w środowisku ekstraktów z grzybów ($p < 0{,}001$). Wykazaliśmy, że bardziej wrażliwy na działanie anty-biofilmowe ekstraktów grzybów był *S. aureus* wyizolowany z kleszczy niż wyizolowany z mleka owczego. W niniejszych badaniach, *A. mellea*, *P. ostreatus*, *L. sulphurous*, *A. auricula-judae* i *M. procera*, hamowały tworzenie się biofilmu (o odpowiednio 70,87%, 67,00%, 64,14%, 62,77% i 47,71%). Wyniki sugerują, że związki zawarte w wodnych ekstraktach z grzybów mogą być przydatne do kontrolowania i zwalczania szkodliwych infekcji powodowanych przez patogeny zwierzęce i ludzkie.

Słowa kluczowe: biofilm, grzyby, kleszcze (Acari), mleko, *Staphylococcus aureus*

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