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Influence of Various Factors on Adhesion of Yeast *Candida* Spp. and *Pichia* Spp. to Abiotic Surfaces

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Abstract

The aim of this study was to assess the potential of *Candida* species and *Pichia* species to adhere to stainless steel (AISI 304) material with different degrees of surface roughness and polystyrene as most frequently used contact materials. Cell surface hydrophobicity (CSH) of *Candida* and *Pichia* strains was determined in order to assess correlation between the cell surface hydrophobicity and yeast adhesion to polystyrene. *Candida albicans* showed a higher ability to adhere to both surfaces compared with non-*albicans Candida* species. Regarding *Pichia* species, *P. membranifaciens* strains were less adherent to stainless steel than *P. pijperi*. Surface roughness of stainless steel was found to affect the adhesion of *Candida* and *Pichia* strains, whereas cell surface hydrophobicity was not correlated with adhesion. We also investigated the antimicrobial and antibiofilm activity of plant extracts such as *Humulus lupulus*, *Alpinia katsumadai* and *Evodia rutaecarpa* against *C. albicans*, *C. glabrata* and *P. membranifaciens*. According to the MIC values, all plant extracts were effective in inhibiting yeast strains. It was observed that biofilms of *C. glabrata* were more resistance to plant extracts as compared to *C. albicans*. However, extracts of *A. katsumadai* and *E. rutaecarpa* promoted the growth and development of a preformed biofilm of *P. membranifaciens*.

Keywords: adhesion, yeast, stainless steel, cell surface hydrophobicity, plant extracts

Резюме

Щамове *Candida albicans* показват по-висока способност да се прилепват към двете повърхности в сравнение с не-*albicans* видове *Candida*. Що се отнася до видовете *Pichia*, щамовете на *P. membranifaciens* са по-слабо прикрепени към неръждаемата стомана, отколкото *P. pijperi*. Установено е, че повърхностната грапавост на неръждаема стомана оказва влияние върху адхезията на щамовете *Candida* и *Pichia*, докато хидрофобността на клетъчната повърхност не е свързана с адхезията. Изследвана е и антимикробната и антибиофилмната активност на растителни екстракти като *Humulus lupulus*, *Alpinia katsumadai* и *Evodia rutaecarpa* срещу *C. albicans*, *C. glabrata* и *P. membranifaciens*. Според стойностите на MIC, всички растителни екстракти са ефективни за инхибиране на щамове дрожди. Биофилмите на *C. glabrata* са по-устойчиви на растителни екстракти в сравнение с тези на *C. albicans*. Въпреки това, екстрактите на *A. katsumadai* и *E. rutaecarpa* насърчават растежа и развитието на предварително оформен биофилм от *P. membranifaciens*.

Introduction

Yeasts are used in food and drink fermentation, but they can also cause spoilage in a wide range of fermented and non-fermented foods. Food spoilage is a serious sensorial and economic problem for the food industry as microbial contamination can occur during processing, storage, and consumption of the end products (Bokulich and Bamforth, 2013). *Pichia*, *Candida*, *Saccharomyces* and *Rhodotorula* are the genera mainly involved in spoilage of products in the beverage industry (Loureiro and Malfeito-Ferreira, 2003). These con-

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taminating microorganisms can form biofilms on food contact surfaces, being difficult to eradicate, increasing the probability of microbial survival and further dissemination during food processing (Brugnoni *et al*., 2012). It is well known that biofilms are more resistant to antimicrobial agents compared to planktonic cells (Simões *et al*., 2010; Tomičić *et al.*, 2016) and this makes them difficult to eliminate.

Among all of the strategies that have been exploited to overcome resistance to antifungal drugs and preservatives, the use of natural substances has shown particular promise, and many natural substances have been detected to show antifungal properties. Natural products, such as plant extract, provide unlimited opportunities for control of microbial growth owing to their chemical diversity (Negi, 2012; Nawrot *et al*., 2013). Plants such as hop (*Humulus lupulus* Linneus; Cannabinaceae), *Alpinia katsumadai* Hayata (syn. *A. katsumadae* Hayata; Zingiberaceae) and E*vodia rutaecarpa* Benth. (Chinese name, Wu zhu yu) have been used since ancient times for flavouring foods and beverages as well as for medicinal purposes with varying success to cure and prevent diseases or spoilage (Gröblacher *et al*., 2012; Wang *et al*., 2013; Kramer *et al*., 2014). Data on the antifungal effect of these plants on *Candida* spp. and *Pichia* spp. is still scarce.

Microbial adhesion to surfaces is considered a multifactorial process. Many studies suggest that microbial cell surface hydrophobicity (CSH) and surface properties of materials such as surface roughness (Ra) significantly influence microbial adhesion (Li, 2003; Brugnoni *et al*., 2007; Tomičić and Raspor, 2017). However, the relationship between these factors and microbial adhesion still remains controversial.

The aim of this study was to assess the potential of *Candida* and *Pichia* species to adhere to stainless steel (AISI 304) material with different degrees of surface roughness (Ra = 25.20 – 961.90 nm) and polystyrene, typical materials for the food processing industry. Cell surface hydrophobicity (CSH) of the tested strains was determined in order to test for a possible correlation between this physicochemical property and the ability to adhere to the polystyrene surface. In this study, we also evaluated the antimicrobial activity of plant extracts such as *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* against *Candida albicans*, *Candida glabrata* and *Pichia membranifaciens* and investigated whether these plant extracts can interfere with biofilm formation as well as acting on preformed biofilms. Thus, the knowledge of how these microorganisms adhere and which factors affect this phenomenon proves to be of great importance in order to avoid their colonization.

Materials and methods

Strains and growth conditions

A total of eight *Candida* strains and three *Pichia* strains were used to study the cell surface hydrophobicity and adhesion to polystyrene and stainless steel surfaces. *Candida* and *Pichia* strains were obtained from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, Slovenia (Table 1).

The strains were preserved in Yeast Peptone Dextrose medium (Sigma-Aldrich, St. Louis, USA) (YPD) supplemented with 40% glycerol at −80°C and revitalized from frozen stocks by cultivation on Malt Extract agar for microbiology (MEA) (Merck-KGaA, Darmstadt, Germany) for 24 h at 37°C (*Candida* strains) or 27°C (*Pichia* strains).

Stailess steel surfaces

Five types of stainless steel (type AISI 304) discs (5.0 mm in diameter and 1.0 mm in thickness) with different degrees of surface roughness (Ra = 25.20 – 961.90 nm) were obtained from Iskra Pio (Ljubljana, Slovenia) (Table 2).

Atomic force microscopy (AFM, VEECO Dimension 3100, Town of Oyster Bay, NY, USA) was used for the characterization of the surface topography of the material on a sub-micrometer scale, and a parameter such as roughness (Ra) was measured. All the stainless steel discs were cleaned with 96% ethanol, rinsed with distilled water and autoclaved at 121°C for 15 min before use.

Extracts

The plant extracts used in this study, *A. katsumadai* seeds (Cat. no. 680381, Plantasia, Oberndorf/ Salzburg, Austria) and *E. rutaecarpa* dried unripe fruits, that is, follicles including seeds (Plantasia; Cat. No. 040377, Oberndorf, Austria) were purchased from a commercial source and extracted with 96% ethanol for 24 h at room temperature to obtain extracts rich in phenolic compounds which were dried by gradual pressure decrease at 45°C using a rotavapor (Kovač *et al*., 2014). *H. lupulus* extract was derived from the brewery Laško, Ljubljana, Slovenia.

Determination of minimal inhibitory concentrations (MICs).

The minimal inhibitory concentrations (MICs) of plant extracts were determined using a broth microdilution method in accordance with the

Table 1 Yeast strains used in the study and their origin

Species (strain)	Origin
C. albicans (ATCC 10261)	Man, nail, of case of paronychia
C. glabrata (ZIM 2367)	Tracheal aspiration
C. glabrata (ZIM 2369)	Bronchoalveolar wash (BAL)
C. glabrata (ZIM 2382)	Urine from indwelling catheter
C. parapsilosis (ATCC 22019)	Man, case of sprue
C. parapsilosis (ZIM 2014)	Sputum
C. parapsilosis (ZIM 2234)	Fruit juice concentrate
C. krusei (ATCC 6258)	Man, sputum of bronchitic convict
P. pijperi (ZIM 1368)	Must of Refošk
P. membranifaciens (ZIM 2302)	Spoiled wine
P. membranifaciens (ZIM 2417)	White cheese of cow's milk

Table 2 Surface roughness of each type of stainless steel disc

Disc type	3D polished	brushed	3C	electropolished	brushed (240)
Average roughness, Ra (nm)	25.20	71.90	160.88	592	961.90

guidelines of the Clinical and Laboratory Standards Institute (CLSI), standard M27-A3 (CLSI, 2008). Briefly, the extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) before dilution in the Malt Extract Broth for microbiology (MEB) medium (Merck, KgaA, Darmstadt, Germany). Serial dilutions were then performed and the extracts were tested at ten concentrations that varied from 800 to 1.5625 µg/mL. The final concentration of DMSO in microtiter wells did not exceed 1%. After inoculation of yeast strains, plates were incubated for 48 h at 37°C for *Candida* strains or 27°C for *Pichia* strain and the absorbance (A) was measured at 650 nm using a microplate reader (Tecan, Mannedorf/Zurich, Switzerland). The MIC was defined as the lowest extract concentration with 50% reduction in opacity compared with the extract-free control. The negative control, growth control and DMSO control were included.

Cell surface hydrophobicity (CSH)

The relative cell surface hydrophobicity of yeast strains was determined using the Microbial Adhesion To Hydrocarbon (MATH) test of Rosenberg (Rosenberg, 1984) with modifications. The yeasts were cultivated in 6 mL of the MEB medium for 48 h at 37°C (*Candida* strains) or 27°C (*Pichia* strains). After cultivation, cells were centrifuged at 1500 × g for 3 min and washed twice with phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK). Yeasts were then resuspended in 6 mL of 4 M an Ammonium sulfate (Merck KGaA, Darmstadt, Germany) in PBS, which increased the hydrophilicity of the aqueous phase and adjusted to an absorbance of 0.7-0.8 at 650 nm (A_0). Subsequently, a volume of 0.2 mL of xylene (Merck KGaA, Darmstadt, Germany) was added to assay tubes containing 1.4 mL of yeast suspension. A tube without the addition of xylene was used as a control. The tubes were vortexed for 1 min and left for 15 min at room temperature in order to obtain separation of the phases. After incubation, a volume of 300 µL of the lower aqueous layer was gently removed and the absorbance of samples (A) and control (A_0) was measured at 650 nm. The CSH was assessed using the formula: $CSH (\%) = (1 – A/A_0) \times 100$. The assays were performed in triplicates.

Adhesion assay

Adhesion assay was performed as previously described with a few modifications (Tomičić and Raspor, 2017). Prior to testing, strains were grown on MEA plates at 37°C (*Candida* strains) or 27°C (*Pichia* strains) for 24 h. After the incubation, a loopful of actively growing cells was suspended in the MEB medium and the concentration of cells was determined and adjusted to 1×10^7 cells/mL by using the Bürker-Türk counting chamber (Brand, Wertheim, Germany). The assay to polystyrene surface was initiated by the addition of 200 µL cell suspensions into a flat-bottomed 96-well polysty-

rene microtiter plate (Non-treated, catalog number: 266120, Nunc, Roskilde, Germany). For adhesion assay to stainless steel, five types of discs (5.0 mm in diameter and 1.0 mm in thickness) with different degrees of surface roughness were used. The discs of each type stainless steel were placed on the bottom of Petri plates (30 mm in diameter) and 2 mL of cell suspensions prepared as above were pipetted into each plate, covering the discs. The plates were incubated for 24 h at 37°C (*Candida* strains) or 27°C (*Pichia* strains). In all experiments, a positive (assay medium with yeast strains) and a negative control (growth medium without yeast strains) were included. The experiments were performed with twelve replicates.

For adhesion assay with plant extracts, the three most adhesive yeast strains were used. Biofilm was formed on stainless steel (Ra=592 nm) discs which were placed on the bottom of Petri plates. The assay was initiated by the addition of 2 mL standardized cell suspensions with or without the presence of plant extracts into selected plates, which were then incubated for 24 h at 37°C (*Candida* strains) or 27°C (*Pichia* strain). In the first part of the experiment, in order to investigate the presence of inhibitory activity on the first steps of biofilm formation, yeast cells were exposed to different extract concentrations (1/2 × MIC and 1 × MIC). In the second part of the experiment, following the biofilm formation after 24 h of incubation, the medium was aspirated and non-adherent cells were removed by washing three times with sterile the PBS. Extracts in MEB at different concentrations (1/2 × MIC and 1 × MIC) were added to the adherent cells and the plates were incubated for 3 h. A positive control (assay medium without extracts and with yeast strains) and a negative control (growth medium without yeast strains) were included in all experiments.

An amount of yeast cells adhered to the polystyrene and stainless steel surfaces was measured using the crystal violet (CV) staining method. After the incubation period, the cell suspensions were aspirated from a 96-well polystyrene microtiter plate and Petri plates (30 mm in diameter) with the stainless steel discs. The plates were then gently washed three times with sterile distilled water to remove non-adherent cells and dried with a hair drier for 10 min. Subsequently, 200 µL of a crystal violet (CV, Merck KGaA, Darmstadt, Germany) solution (0.5%) was added to all wells of polystyrene microtiter plate, whereas discs in Petri plates were stained with 1 mL of 0.5% CV. After 20 min, the CV solution was removed and the plates were gently washed three times with sterile distilled water and dried for another 10 min with a hair drier. The discs from Petri plates were then transferred to a clean 96-well microtitre plate and 100 µL of 33% acetic acid added into each well to release the dye. The plates were shaken for 3 min and the contents of the walls without stainless steel discs were transferred into a new 96-well microtiter plate. The amount of adhered cells, that is, the concentration of the released crystal violet was determined by measuring the absorbance (A) at 584 nm using a microplate reader (Tecan, Mannedorf/Zurich, Switzerland).

Statistical analysis

Descriptive statistical analyses for calculating the means and the standard error of the mean were performed using Microsoft Excel software (Microsoft Office 2013). All obtained results were expressed as the mean ± standard deviation (±SD). Statistical analysis was performed using StatSoft Statistica, ver. 10 (IBM, Armonk, NY, USA). The effect of various factors on yeast adhesion was analysed and the statistical significance determined using Analysis of variance (ANOVA) and the post hoc Tukey‘s HSD test. For correlations between hydrophobicity and adhesion to polystyrene surfaces, the regression model was used. A P-value of <0.05 was considered statistically significant.

Results and Discussion

In the present study, we strictly followed the aim to assess the potential of *Candida* species and *Picha* species to adhere to polystyrene and stainless steel surfaces and examined the influence of factors such as the cell surface hydrophobicity and surface roughness of stainless steel, as well as plant extracts on the adhesion of selected yeasts.

Candida spp. and Pichia spp. hydrophobicity and its relation with adhesion to polystyrene

Many studies have shown that cell surface properties, such as hydrophobicity, play an important role in the initial phase of microbial adhesion (Li, 2003; Brugnoni *et al*., 2007). The cell surface hydrophobicity (CSH) of *Candida* and *Pichia* strains was determined by the water-hydrocarbon (xylene) biphasic assay (Rosenberg, 1984) in order to understand the connection between adhesion and hydrophobicity.

The results showed a wide distribution of the *Candida* and *Pichia* strains over the range of hydrophobicity from 10 to 90% as presented in Fig. 1 ($P < 0.05$).

According to the classification suggested by

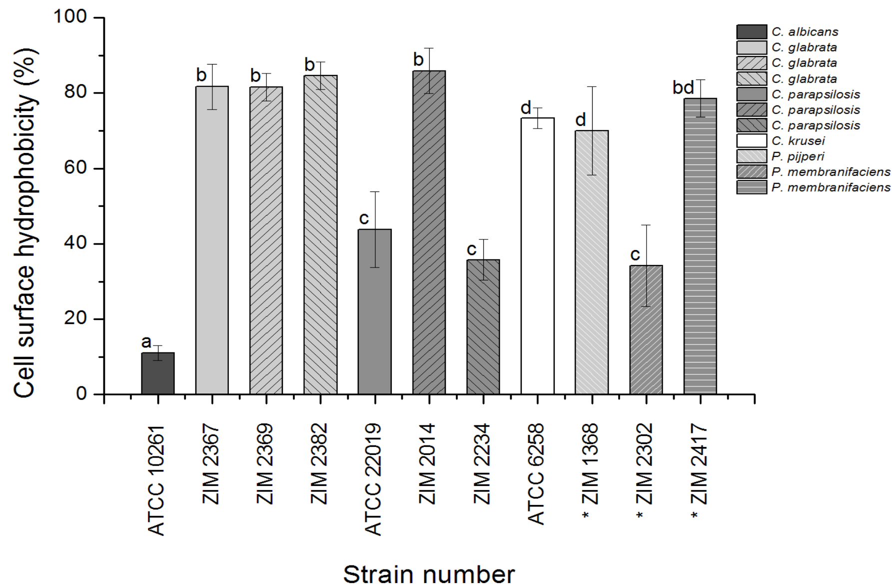

Fig. 1. The cell surface hydrophobicity (CSH) of *Candida* spp. and *Pichia* spp. (*) was measured according to the microbial adhesion to hydrocarbon (MATH) test. Data represent means ± standard deviation of three replicates. Different letters (a, b, c, d) mark significant differences among strains (P < 0.05).

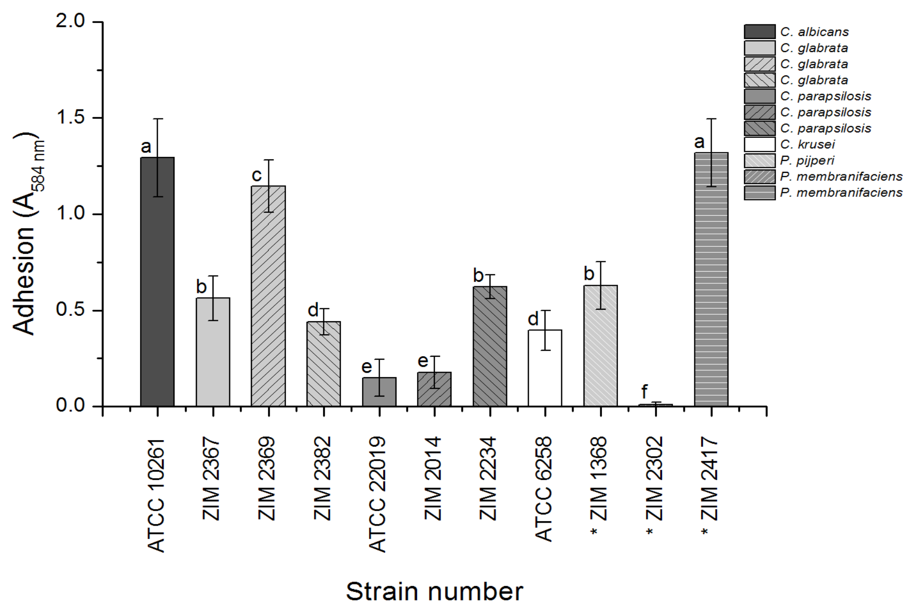

Fig. 2. Adhesion of *Candida* spp. and *Pichia* spp. (*) to polystyrene was measured using the crystal violet test. The experiments were performed with twelve replicates and the arithmetic mean of the absorbance (A) values are presented. Different letters (a, b, c, d...) mark significant differences among strains (P < 0.05).

Li and McLandsborough (1999), the tested strains were classified into three groups. The majority of strains were strongly hydrophobic (CSH values higher than 55%), while *C. parapsilosis* (ATCC 22019 and ZIM 2234) and *P. membranifaciens* ZIM 2302 were moderately hydrophobic showing CSH values between 30% and 54%. On the other hand, only the reference strain *C. albicans* ATCC 10261 was moderately hydrophilic. Interestingly, the highest number of adhered cells was found for *C. albicans* (Fig. 2), which actually had the lowest value of CSH, these opening the question ofwhether CSH is primarily responsible for the potential of adhesion.

In the present study, CSH is not significantly correlated with the amount of cells adhered to polystyrene, highlighting that CSH cannot be considered as a single predictor for adhesion. Regarding the correlation between CSH and adhesion to

polystyrene, the findings of other authors are inconsistent (Li, 2003; Silva-Dias *et al*., 2015). Different rankings of hydrophobicity among *Candida* species were already influenced by different quantification methodologies, different growth conditions, or different temperatures (Gallardo-Moreno *et al*., 2003; Silva-Dias *et al*., 2015). In our research, cell surface hydrophobicity (CSH) was demonstrated using microbial adhesion to hydrocarbon (MATH) test, which is today considered as the method of choice for this type of measurements.

In this part of the study, we also evaluated the potential of adhesion of *Candida* and *Pichia* strains to a polystyrene surface. The results of the extent of adhesion of *Candida* and *Pichia* strains to polystyrene obtained by using the crystal violet staining method are presented in Fig. 2. It was evident that all *Candida* and the majority of *Pichia* strains were able to adhere to polystyrene, although differences were observed according to species and strains. We verified that *C. albicans* showed the highest extent of adhesion to polystyrene and stainless steel surfaces followed by the other tested species: *C. glabrata*, *C. krusei* and *C. parapsilosis*. Comparatively to non-*albicans Candida* strains, *C. albicans* was already characterized as displaying higher adhesion ability and efficiency of infection (Hawser and Douglas, 1994; Kuhn *et al*., 2002), which has been connected to adhesins like Als1, Als3, and Hwp1 (Nobile and Mitchell, 2006; Tronchin *et al*., 2008). Regarding *Pichia* species, only strain *P. membranifaciens* ZIM 2302 was completely non-adhesive. It has also been observed that strains of *C. glabrata*, *C. parapsilosis* and *P. membranifaciens* showed a high degree of heterogeneity within the species in their ability to adhere to polystyrene. With this, we confirmed intraspecies variation by *C. glabrata* and *C. parapsilosis* regarding adherence to different biomaterials (Luo and Samaranayake, 2002; Silva *et al*., 2010).

Adhesion of Candida spp. and Pichia spp. to stainless steel is influenced by surface roughness

Figure 3 presents the results of the adhesion of *Candida* spp. and *Picha* spp. to stainless steel (type AISI 304) with different degrees of surface roughness (Ra = 25.20 – 961.90 nm), obtained by using the crystal violet staining method. It was observed that different species showed variation in their adhesion ability. The strongest adhesion ability showed *C. albicans* followed by *C. glabrata*, *C. krusei* and *C. parapsilosis* (P < 0.05), which is in agreement with the results of adhesion on the polystyrene surface shown in Fig. 2. Regarding *Pichia* species, *P. membranifaciens* were less adherent to stainless steel in comparison with *P. pijperi* (P < 0.05). High intraspecies variability was also indicated.

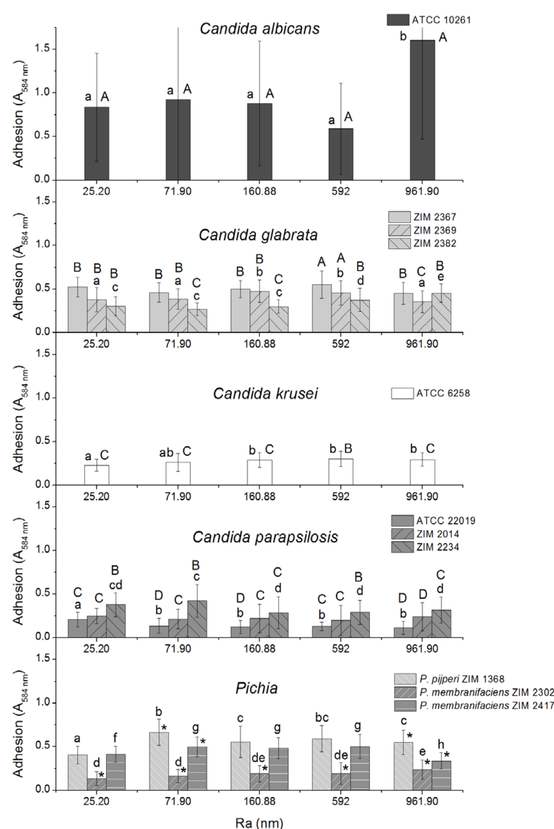

Fig. 3. Adhesion of *Candida* spp. and *Pichia* spp. to stainless steel with different degrees of surface roughness (Ra) was measured using the crystal violet test. The experiments were performed with twelve replicates and the arithmetic mean of the absorbance (A) values are presented. Different letters (a, b, c, d...) mark significant differences among Ra for each strain, while different letters (A, B, C, D…) indicate that there is a significant difference among *Candida* species at the same Ra. The asterisks (*) mark significant differences among *Pichia* strains at the same Ra (P < 0.05). Unmarked terms are not significant.

One of the important factors for adhesion is surface roughness as shown in this study (Fig. 3), which is also observed by others (Ortega *et al*., 2008; Bohinc *et al*., 2014). The results showed that the surface roughness of stainless steel influenced the adhesion of the majority of strains, which was especially evident for *C. albicans* ATCC 10261, *C. glabrata* ZIM 2382 and all strains of the genus *Pichia* (P < 0.05), while on the strains ZIM 2367 and ZIM 2014 did not have any impact (P > 0.05). This indicates that some irregularities on the surface can provide protection to the cells, and may also be associated with problems in surface cleaning (Korber

et al., 1997), resulting in rapid re-growth of a biofilm.

Antimicrobial activity of plant extracts

The abundance of unwanted yeast growth in food processing plants can lead to problems in quality and safety with significant economic losses. Contamination of food and spoilage by yeast is a problem that needs to be controlled adequately (Loureiro, 2003; Querol and Fleet, 2006), therefore searching for new agents that can inhibit the growth and development of yeast biofilms are needed. The use of plant extracts with known antimicrobial properties can be of great significance in food preservation. Taking this into account, this part of the study was carried out to assess the antifungal properties of plant extracts such as *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* against *C. albicans*, *C. glabtata* and *P. membranifaciens* and its biofilm.

The *in vitro* antimicrobial activity of plant extracts against yeasts was determined according to the CLSI method. The Minimal inhibitory concentration (MIC) was defined as the lowest concentration of plant extract that inhibited the visible growth of test yeast by 50% compared to positive control. As presented in Fig. 4., the MIC values varied from 100 to 400 µg/mL.

The potential for antimicrobial activity of *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* extracts has hardly been investigated, with studies reported only for bacteria (Bezek *et al*., 2016; Klančnik *et al*., 2012; Kramer *et al*., 2014). With specific reference to *C. albicans*, *C. glabrata* and *P. membranifaciens*, no antimicrobial activities have been previously reported for these extracts. According to the MIC values (Fig. 4), *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* extracts were effective in the inhibition of tested yeasts, which indicates that *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* are promising plants with antifungal activities against *C. albicans*, *C. glabrata* and *P. membranifaciens*.

The effect of plant extracts on yeasts C. albicans, C. glabrata and P. membranifaciens biofilm

Based on the results of the antimicrobial activity of plant extracts, concentrations corresponding to 1/2 × MIC and 1 × MIC were selected in order to examine their effect on the initial phase of biofilm formation and preformed biofilms.

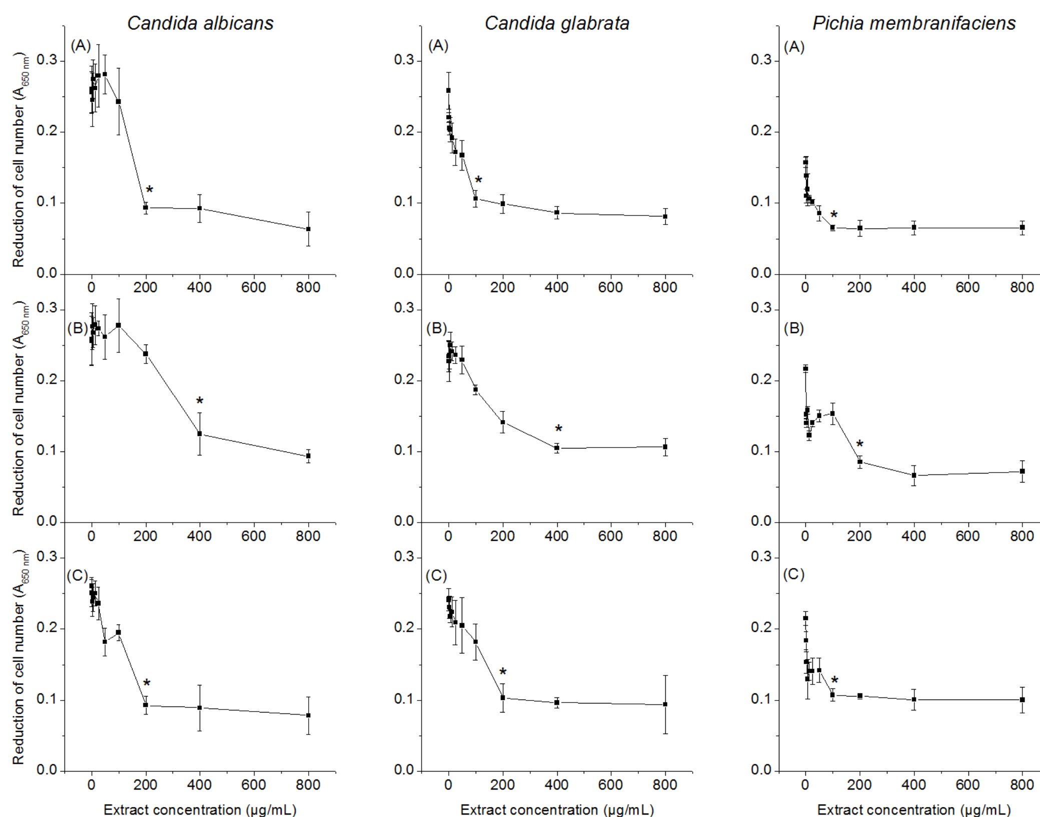

Fig. 4. Antimicrobial activity of the extracts *H. lupulus* (A), *A. katsumadai* (B) and *E. rutaecarpa* (C) against *C. albicans* ATCC 10261, *C. glabrata* ZIM 2369 and *P. membranifaciens* ZIM 2417. The experiments were performed with eight replicates and the arithmetic mean of the absorbance (A) values were used. The asterisks (*) mark the value of the minimal inhibitory concentration (MIC).

According to our knowledge, no study has been performed so far on the extracts of *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* that may inhibit biofilm formation of *C. albicans*, *C. glabrata* and *P. membranifaciens* to stainless steel surfaces. Therefore, this part of the study was initiated to evaluate the effect of these extracts on biofilm formation by adding different concentrations (1/2 × MIC and 1 × MIC) of the extracts immediately at the start of the experiment or after 24 h of biofilm formation for 3 h. The effect of extracts on the initial phase of biofilm formation and preformed biofilms was determined using the crystal violet staining method.

Based on the findings in this part of the study, the extracts of *H. lupulus*, *A. katsumadai* and *E. rutaecarpa* exhibited the highest antibiofilm activity against *C. albicans* (Fig. 5). This suggests that active components of these extracts have strong potential to affect *C. albicans* biofilm formation. As reported before by Bezek *et al.* (2016) (Bezek *et al.*, 2016), *E. ruticarpa* fruit ethanol extract (EREE) and its fractions had the ability to inhibit *Campylobacter jejuni* adhesion and biofilm formation with the most visible effect of the quinolinone alkaloid fraction. Pogačar *et al*. (2015) showed that a chemically characterized extract (seed ethanol extract, SEE) and its residual material of hydrodistillation (hdSEE-R) from *A. katsumadai* seeds had the most significant antiadhesion activity against *C. jejuni* to the PSI cl 1 cells. In our contribution, *A. katsumadai* was also effective in the initial phase of biofilm formation of *C. glabrata* (Fig. 5), which is not the case with the preformed biofilm.

This highlights that the yeast cells in a biofilm are more resistant to antimicrobial agents compared to planktonic cells. The inability of antimicrobial agents to remove established biofilms has been observed previously (Sandasi *et al*., 2010; Adukwu *et al*., 2012). In general, our results clearly demonstrate that plant extracts were the most effective against *C. albicans* in comparison to *C. glabrata*. Regarding *P. membranifaciens*, it was found that *A. katsumadai* and *E. rutaecarpa* extracts significantly increased biofilm formation at a concentration of 1/2 × MIC. *A. katsumadai* extract also had a slight stimulative effect on the preformed 24 h biofilms after 3 h exposure. These results are supported by the study of Sandasi *et al*. (2010), in which some extracts promote the growth and development of a preformed *L. monocytogenes* biofilm *in vitro*. The enhanced biofilm development observed upon exposure to some extracts in this study may be because of the presence of certain compounds within the extracts that favor the development of these biofilms. Some natural compounds have been reported to promote microbial adhesion (Ofek *et al.,* 2003; Sandasi *et al*., 2008).

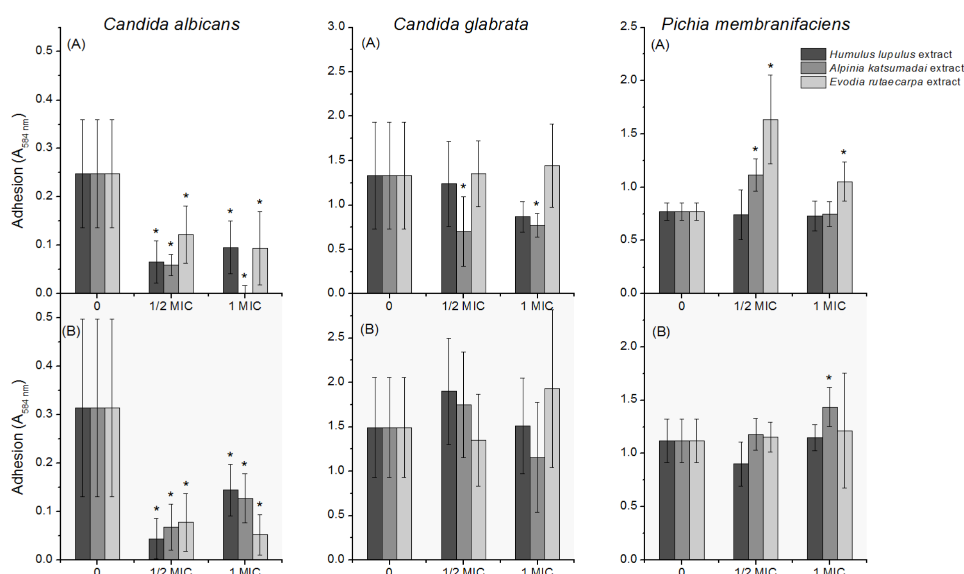

Fig. 5. The antibiofilm activity of plant extracts against *C. albicans* ATCC 10261, *C. glabrata* ZIM 2369 and *P. membranifaciens* ZIM 2417 to the surface of stainless steel. The effect of plant extracts at different concentrations (1/2 × MIC and 1 × MIC) on the initial phase of biofilm formation (A) and preformed 24 h biofilms with a 3 h exposure (B). The extent of biofilm formation was measured by the Crystal violet assay. The experiments were performed with ten replicates and the arithmetic mean of the absorbance (A) values were used. Each bar represents the mean ± standard deviation (SD). * $P < 0.05$ versus control.

Conclusions

In conclusion, this work demonstrates that the tested *Candida* and *Pichia* strains were able to adhere to the polystyrene and stainless steel surfaces (type AISI 304). This is of significance with respect to food quality and safety because it indicates that the attachment of *Candida* spp. and *Pichia* spp. to food-contact surfaces may play an important role in their enhanced persistence through adequate machinery and devices in food and medical environment. Our results also emphasize the significant potential of *A. katsumadai*, *E. rutaecarpa* and *H. lupulus* extracts as antifungal agents. The results show that plant extracts were efficient to inhibit the initial stage of biofilm formation as well as already preformed biofilm of *C. albicans*. Although *A. katsumadai* was able to inhibit cell attachment of *C. glabrata*, the inhibition of growth in a preformed biofilm was not as efficient as expected. We found that *P. membranifaciens* tolerates well extracts in biofilm formation, and although from the MIC profile we expected biofilm reduction, to our surprise the extract even stimulated the biofilm formation and growth. The reason why *P. membranifaciens* tolerates much better the same extracts which inhibit *C. albicans* may be connected to the natural surroundings of *P. membranifaciens,* which is still in the nature exposed to those compounds. Although a preventive effect of the tested plant extracts has been shown, further studies would be needed for the purpose of isolation and identification of the constituents which exhibit antibiofilm properties that might be essential to include as alternatives in the control of biofilms.

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References

Adukwu, E. C., S. C. H. Allen, C. A. Phillips (2012). The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *J. Appl. Microbiol*. **113**: 1217-1227.

Bezek, K., M. Kurinčič, E. Knauder, A. Klančnik, P. Raspor, F. Bucar, S. Smole Možina (2016). Attenuation of adhesion, biofilm formation and quorum sensing of *Campylobacter jejuni* by *Euodia ruticarpa*. *Phytother. Res.* **30**: 1527-1532.

Bohinc, K., G. Dražić, R. Fink, M. Oder, M. Jevšnik, D. Nipič, P. Raspor (2014). Available surface dictates microbial adhesion capacity. *Int. J. Adhes. Adhes*. **50**: 265-272.

Bokulich, N. A., C. W. Bamforth (2013). The microbiology of malting and brewing. *Microbiol. Mol. Biol. Rev*. **77**: 157-172.

Brugnoni, L. I., M. A. Cubitto, J. E. Lozano (2012). *Candida krusei* development on turbulent flow regimes: Biofilm formation and efficiency of cleaning and disinfection program. *J. Food Engin*. **111**: 546-552.

Brugnoni, L. I., J. E. Lozano, M. A. Cubitto (2007). Potential of yeast isolated from apple juice to adhere to stainless steel surfaces in the apple juice processing industry. *Food Res. Inter.* **40**: 332-340.

CLSI (2008). Reference method for broth dilution antifungal susceptibility testing of yeasts. 3rd ed. Wayne: Clinical and Laboratory Standards Institute. Approved standard M27-A3.

Gallardo-Moreno, A. M., E. Garduño, M. L. González-Martín, C. Pérez-Giraldo, J. M. Bruque, A. C. Gómez-García (2003). Analysis of the hydrophobic behaviour of different strains of *Candida parapsilosis* under two growth temperatures. *Colloids Surf. B Biointerfaces* **28**: 119-126.

Gröblacher, B., O. Kunert, F. Bucar (2012). Compounds of *Alpinia katsumadai* as potential efflux inhibitors in *Mycobacterium smegmatis*. *Bioorg. Med. Chem*. **20**: 2701-2706.

Hawser, S. P., L. J. Douglas (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect. Immun*. **62**: 915-921.

Klančnik, A., B. Gröblacher, J. Kovač, F. Bucar, S. Smole Možina (2012). Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts. *J. Appl. Microbiol*. **113**: 1249-1262.

Korber, D. R., A. Choi, G. M. Wolfaardt, S. C. Ingham, D. E. Caldwell (1997). Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to trisodium phosphate. *Appl. Environ. Microbiol*. **63**: 3352-3358.

Kovač, J., N. Gavarić, F. Bucar, S. Smole Možina (2014). Antimicrobial and resistance modulatory activity of *Alpinia katsumadai* seed phenolic extract, essential oil and post-distillation extract. *Food Technol. Biotechnol*. **52**: 248–254.

Kramer, B., J. Thielmann, A. Hickisch, P. Muranyi, J. Wunderlich, C. Hauser (2014). Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. *J. Appl. Microbiol*. **118**: 648-657.

Kuhn, D. M., J. Chandra, P. K. Mukherjee, M. A. Ghannoum (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun*. **70**: 878-888.

Li, J., L. A. McLandsborough (1999). The effect of the surface charge and hydrophobicity in *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* **53**: 185-193.

Li, X. (2003). Quantitative variation of biofilms among strains

in natural populations of *Candida albicans*. *Microbiology* **149**: 353-362.

Loureiro, V., M. Malfeito-Ferreira (2003). Spoilage yeasts in the wine industry. *Int. J. Food Microbiol*. **86**: 23-50.

Luo, G., L. P. Samaranayake (2002). *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *APMIS* **110**: 601-610.

Nawrot, R., J. Barylski, G. Nowicki, J. Broniarczyk, W. Buchwald, A. Goździcka-Józefiak (2013). Plant antimicrobial peptides. *Folia Microbiol*. **59**: 181-196.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *Int. J. Food Microbiol*. **156**: 7-17.

Nobile, C. J., A. P. Mitchell (2006). Genetics and genomics of *Candida albicans* biofilm formation. *Cell Microbiol*. **8**: 1382-1391.

Ofek, I., D. L. Hasty, N. Sharon (2003). Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunol. Med. Microbiol.* **38**: 181-191.

Ortega, M. P., T. Hagiwara, H. Watanabe, T. Sakiyama (2008). Factors affecting adhesion of *Staphylococcus epidermidis* to stainless steel surface. *Jap. J. Food Engin.* **9**: 251-259.

Pogačar, M. Š., A.Klančnik, F. Bucar, T. Langerholc, S. Smole Možina (2015). *Alpinia katsumadai* extracts inhibit adhesion and invasion of *Campylobacter jejuniin* animal and human foetal small intestine cell lines. *Phytother. Res*. **29**: 1585-1589.

Querol, A., G. H. Fleet (2006). Yeasts in food and beverages. Berlin, Germany: Springer-Verlag Berlin Heidelberg, pp. 445.

Rosenberg, M. (1984). Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene. *FEMS Microbiol. Lett.* **25**: 41-45.

Sandasi, M., C. M. Leonard, A. M. Viljoen (2008). The effect of five common essential oil components on *Listeria monocytogenes* biofilms. *Food Control* **19**: 1070-1075.

Sandasi, M., C. M. Leonard, A. M. Viljoen (2010). The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **50**: 30-35.

Silva-Dias, A., I. M. Miranda, J. Branco, M. Monteiro-Soares, C. Pina-Vaz, A. G. Rodrigues (2015). Adhesion, biofilm formation, cell surface hydrophobicity, and antifungal planktonic susceptibility: relationship among *Candida* spp. *Front Microbiol.* **6**: 1-8.

Silva, S., M. Negri, M. Henriques, R. Oliveira, D. Williams, J. Azeredo (2010). Silicone colonization by non-*Candida albicans Candida* species in the presence of urine. *J. Med. Microbiol.* **59**: 747-754.

Simões, M., L. C. Simões, M. J. Vieira (2010). A review of current and emergent biofilm control strategies. *LWT - Food Sci. Technol*. **43**: 573-583.

Tomičić, R., P. Raspor (2017). Influence of growth conditions on adhesion of yeast *Candida* spp. and *Pichia* spp. to stainless steel surfaces. *Food Microbiol.* **65**: 179-184.

Tomičić, Z., J. Zupan, T. Matos, P. Raspor (2016). Probiotic yeast *Saccharomyces boulardii* (nom. nud.) modulates adhesive properties of *Candida glabrata*. *Med. Mycol.* **54**: 835–845.

Tronchin, G., M. Pihet, L. M. Lopes-Bezerra, J.-P. Bouchara (2008). Adherence mechanisms in human pathogenic fungi. *Med. Mycol.* **46**: 749-772.

Wang, X. X., K. Zan, S. P. Shi, K. W. Zeng, Y. Jiang, Y. Guan (2013). Quinolone alkaloids with antibacterial and cytotoxic activities from the fruits of *Evodia rutaecarpa*. *Fitoterapia* **89**: 1-7.