RESEARCH ARTICLE

The activity of bacterial peptidylarginine deiminase is important during formation of dual-species biofilm by periodontal pathogen *Porphyromonas gingivalis* and opportunistic fungus *Candida albicans*

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One sentence summary: *Porphyromonas gingivalis* exploits protein citrullination during formation of mixed-species biofilms with *Candida albicans*.
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ABSTRACT

Porphyromonas gingivalis, an anaerobic Gram-negative bacterium critically involved in the development of human periodontitis, belongs to the late colonizers of the oral cavity. The success of this pathogen in the host colonization and infection results from the presence of several virulence factors, including extracellular peptidylarginine deiminase (PPAD), an enzyme that converts protein arginine residues to citrullines. A common opportunistic fungal pathogen of humans, *Candida albicans*, is also frequently identified among microorganisms that reside at subgingival sites. The aim of the current work was to verify if protein citrullination can influence the formation of mixed biofilms by both microorganisms under hypoxic and normoxic conditions. Quantitative estimations of the bacterial adhesion to fungal cells demonstrated the importance of PPAD activity in this process, since the level of binding of *P. gingivalis* mutant strain deprived of PPAD was significantly lower than that observed for the wild-type strain. These results were consistent with mass spectrometric detection of the citrullination of selected surface-exposed *C. albicans* proteins. Furthermore, a viability of *P. gingivalis* cells under normoxia increased in the presence of fungal biofilm compared with the bacteria that formed single-species biofilm.

These findings suggest a possible protection of these strict anaerobes under unfavorable aerobic conditions by *C. albicans* during mixed biofilm formation.

INTRODUCTION

Porphyromonas gingivalis, a Gram-negative rod-shaped bacterium, is an obligatory anaerobe that contributes to the subgingival plaque biofilm in human individuals suffering from periodontitis, a chronic inflammatory disease associated with the degradation of gingival tissues followed by tooth loss (Socransky *et al.* 1998; Darveau, Hajishengallis and Curtis 2012). Numerous virulence factors produced by this pathogen, including adhesive molecules such as hemagglutinins and fimbriae, hemin-binding proteins, secreted proteases and other hydrolytic enzymes, strongly contribute to bacterial ability to modulate the activity of host immune system and enable the tissue colonization (Lamont and Jenkinson 1998).

Recently, an additional potential *P. gingivalis* virulence factor has been identified—an extracellular enzyme, peptidylarginine deiminase (PPAD), that converts positively charged arginine residues in protein molecules to neutral citrulline residues in a process known as deimination or citrullination (McGraw *et al.* 1999; Zhao *et al.* 2017). This type of post-translational modification can significantly change the charge of protein molecule, thus potentially affecting its structure, function and ability to interact with other molecules. The citrullination of human proteins and peptides by PPAD can exert an influence on the functionality of many different systems responsible for the maintenance of homeostasis in the host organism and the response to infectious agents, with notable examples of disturbing the complement system activity due to the inactivation of anaphylatoxin C5a (Bielecka *et al.* 2014) or facilitating bacterial invasion on gingival fibroblasts related to the induction of prostaglandin E2 signaling pathway (Gawron *et al.* 2014). Additionally, the citrullination of C-terminal arginine can modulate the function of a proinflammatory and vasoactive peptide, bradykinin, thus regulating the inflow of plasma to the infected gingival pocket (McGraw *et al.* 1999). Deimination of C-terminal arginine by PPAD also accounts for abolishing the biological activity of the epidermal growth factor and contributes to increased tissue destruction (Pyrc *et al.* 2013). It has also been shown that PPAD is able to modify peptides derived from two human proteins, fibrinogen and α-enolase, and the citrullination of these proteins, primarily performed by human peptidylarginine deiminases, plays an important role in the pathogenesis of rheumatoid arthritis (Wegner *et al.* 2010a,b). To date, *P. gingivalis* PPAD is the only known bacterial peptidylarginine deiminase (Gabarrini *et al.* 2015).

As has recently been reported, PPAD does not exclusively citrullinate molecules of the colonized host. Several proteins located at the surface of *P. gingivalis* cells were also identified to contain the modified arginine residues (Stobernack *et al.* 2016). The citrullinated bacterial proteins can significantly contribute to the virulence, e.g. by a participation to the colonization of the subgingival niche, directly associated with the formation of mixed-species biofilms. Taking into consideration this hypothesis, it seems appropriate to investigate the significance of PPAD

activity to the interactions of *P. gingivalis* with both the human host and members of biofilm heterogeneous microbial community. Apart from a great variety of bacterial organisms that form dental biofilms, one of the most important fungal pathogens of humans—*Candida albicans*—has also often been isolated from different locations in the oral cavity, including root canals and deep periodontal pockets (O'Donnell *et al.* 2015; Gomes *et al.* 2017; Marsh and Zaura 2017).

Candida albicans is primarily a commensal microorganism that can be identified in the mouths of nearly all individuals in human population, and is usually harmless for them as long as the immune system works efficiently (Peters *et al.* 2017). However, when the immunity is compromised owing to various severe diseases, and the delicate balance between the host and the fungus is markedly disturbed, *C. albicans* demonstrates its pathogenic nature, causing various mycoses with variable severity, ranging from oral lesions and mucosal candidiasis to disseminated, systemic infections, often life threatening (Sardi *et al.* 2013; Lewis and Williams 2017). Interestingly, the role of *C. albicans* in complex, polymicrobial biofilms formed in the oral cavity is crucial and indispensable, because *C. albicans* actively contributes to the composition and functions of such biofilms, e.g. reducing oxygen levels and allowing the survival and growth of anaerobic bacteria under aerobic conditions (Fox *et al.* 2014; Janus *et al.* 2017; Lambooij *et al.* 2017). Taking into consideration a possibility that *P. gingivalis* and *C. albicans* can be found at the same infection niche and this cohabitation may result in the increase in the potential for pathogenicity and the ability to infect host tissues (Tamai, Sugamata and Kiyoura 2011), this work aimed to investigate whether one of the recently proposed bacterial virulence factors, i.e. PPAD, could influence the interactions between *C. albicans* and *P. gingivalis* and affect the formation of dual-species biofilm.

As macrophages constitute a significant part of the cells recovered from gingival tissue of chronic periodontitis patients and seem to play an essential role in the development and progression of periodontal diseases by mediating the host immune response (Zhou *et al.* 2005), we used these cells as a model to compare the host responses to the single species- or mixed bacterial–fungal infections.

MATERIALS AND METHODS

Strains and growth conditions

Porphyromonas gingivalis wild-type strain W83 (ATCC® BAA-308™) from American Type Culture Collection (Manassas, VA, USA) and the mutant strain deprived of PPAD (Δ*ppad*), obtained as described previously (Wegner *et al.* 2010b), were grown on agar plates supplemented with 5% v/v sheep blood and in Schaedler broth (BTL, Lodz, Poland) supplemented with 250 mg L^{-1} L-cysteine-HCl (Sigma, St. Louis, MO, USA), 0.5 mg L^{-1} vitamin K (Sigma), 5 mg L^{-1} hemin (Sigma) and, for the mutant strain, with

5 mg L^{-1} erythromycin, at 37°C in an anaerobic chamber (90% N$_2$, 5% CO$_2$, 5% H$_2$). *Candida albicans* strain 3147 (ATCC® 10231™) purchased from American Type Culture Collection was cultured aerobically in the YPD solid or liquid medium (1% yeast extract, 2% soybean peptone and 2% glucose) (Sigma) at 30°C. Cells were counted using optical density measurements after centrifugation of the bacterial (5000 × g, 20 min) or fungal (3000 × g, 5 min) liquid cultures at 4°C, and washing the cell pellet three times with sterile phosphate buffered saline (PBS), pH 7.4 (Biowest, Nuaillé, France).

Biofilm formation in 96-well microtiter plates

Candida albicans cells, 5 × 10^5 in 100 μL of RPMI 1640 medium (Biowest), were placed into the wells of 96-well high-binding black microplate with clear, flat bottom (Corning Incorporated, Kennenbunk, ME, USA) and incubated aerobically for 16 h at 37°C. Before the use in the biofilm formation assay, 2 × 10^9 *P. gingivalis* cells in 1 mL of PBS were mixed with 0.4 μL of Cell-Trace CFSE (5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester) staining solution (Thermo Fisher Scientific, Waltham, MA, USA) and labeled in the dark at 37°C for 40 min. After that, the bacterial cells were harvested by centrifugation (5000 × g, 10 min) and the excess of unbound tracer was removed by the following washing steps with: 1 mL of PBS, 1 mL of Schaedler broth diluted 1:1 with PBS and then 1 mL of PBS. After that, the suspensions of labeled *P. gingivalis* cells were prepared in RPMI 1640 medium (1 × 10^7 or 1 × 10^8 bacterial cells per 50 μL) and added to the wells with previously formed fungal biofilm. Microplates were incubated for 3 or 24 h at 37°C under normoxic or anoxic conditions. The latter were maintained using GENbox jar with GENbox anaer generator (bioMérieux S.A., Marcy l'Etoile, France). According to the manufacturer's instructions, just after 30 min the oxygen concentration in the chamber is close to 0%. In addition, control samples were prepared with only the cells of one species applied to the wells (5 × 10^5 *C. albicans* cells, or 1 × 10^7 or 1 × 10^8 *P. gingivalis* cells per 150 μL of RPMI 1640). After incubation, the supernatant was carefully removed from biofilm and the adsorbed cells were washed three times with 200 μL of sterile PBS. Each sample was prepared in technical triplicate and in at least three biological replicates.

The viability of both cell types was assessed using colony-forming unit (CFU) assay after their mechanical detachment from microplate wells and performing 10-fold serial dilutions. The *C. albicans* cells were cultured aerobically at 30°C for 24 h on YPD agar plates, whereas *P. gingivalis* cells were grown anaerobically at 37°C for 7 days on blood agar plates in GENbox jar with GENbox anaer generator. To control experimental repeatability, four replicates were prepared from each sample. After the incubation under conditions suitable for each microorganism, the numbers of colonies were counted.

To examine the level of the adhesion of CFSE-labeled *P. gingivalis* cells to fungal biofilms, the fluorescence intensity was measured with the excitation and emission wavelengths of 480 and 520 nm, respectively, using Synergy H1 Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Biofilm visualization with confocal scanning laser microscopy

Imaging of single- or dual-species biofilms was performed using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a ×40 oil immersion objective, as a Z-stack of images. For this purpose, 1 × 10^6 *C. albicans* cells in 400 μL of RPMI 1640 were applied into the chamber of Eppendorf Cell Imaging Coverglasses of 170 μm thickness (Eppendorf, Hamburg, Germany), coated with 0.05% poly-L-lysine (Sigma) and cultured under aerobic conditions at 37°C for 16 h to form biofilm. Then, the growth medium was removed and 1 × 10^8 CFSE-labeled *P. gingivalis* cells in 400 μL of RPMI 1640 were added to the chambers and further incubated at 37°C for 24 h under aerobic or anaerobic conditions, as described above. Similarly, the samples of single-species biofilm were also prepared. After growth, biofilms were rinsed three times with 400 μL of PBS to wash out the unbound cells and, prior to imaging, fungal cells were stained with 400 μL of the 0.1% aqueous solution (v/v) of Calcfluor White Stain (CFW) (Sigma) for 5 min. The excess of stain was removed, sterile PBS was pipetted to the chambers with biofilms, and the samples were viewed using excitation wavelength of 405 nm to visualize fungal cells stained with CFW and 488 nm to detect bacterial cells labeled with CFSE. The thickness of biofilm layers was analyzed using ZEN Lite Microscopy Software (Carl Zeiss).

Identification of citrullinated surface-exposed proteins of *C. albicans* with mass spectrometry

The identification of fungal cell wall proteins that may have been modified by the bacterial PPAD enzyme during biofilm formation was performed using liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) with an HCTUltra ETDII ion-trap mass spectrometer equipped with an electrospray ionization ion source (Bruker, Bremen, Germany) and coupled to an ultrahigh-performance liquid chromatograph Dionex Ultimate 3000 system (Carlsbad, CA, USA). *Candida albicans* cells (5 × 10^8) and *P. gingivalis* W83 or Δ*ppad* cells (5 × 10^9) were co-cultured in 5 mL RPMI 1640 medium at 37°C for 24 h with gentle agitation (30 rpm) in an orbital rotary shaker MaxQ 4000 (Thermo Fisher Scientific) under aerobic or anaerobic conditions. Additionally, the monospecies cultures were grown under the same conditions. The cells that formed a biofilm on the bottom of the flasks were detached mechanically and collected, while the culture supernatant was discarded. After harvesting the cells by centrifugation (3000 × g, 5 min) and washing them three times with 1 mL of 25 mM ammonium bicarbonate buffer (NH$_4$HCO$_3$), 100 μL of the same buffer with 5 mM dithiotreitol and 1 μg of sequencing-grade trypsin (Promega, Madison, WI, USA) were added, followed by the incubation for 10 min at 37°C. After this time, peptides from fungal surface-exposed proteins were released by trypsin to the solution and then subjected to further identification. Briefly, the remaining cells were centrifuged and discarded, while the supernatants were collected, filtered through 0.22-μm pore filter units to remove any remaining cells and further incubated overnight at 37°C. The undigested proteins were removed from the samples by addition of trifluoroacetic acid to the final concentration of 0.1% (v/v) and centrifugation (12 000 × g, 12 min) of any precipitates. Samples were dried in a SpeedVac (Martin Christ, Osterode am Harz, Germany), dissolved in 10% acetonitrile with 0.1% formic acid and the trypsin-generated peptides were subjected to separation on a 100 mm × 2.1 mm Accucore C18 column (particle size of 2.6 μm) (Thermo Fisher Scientific), with a gradient of 10%–55% of 0.1% formic acid in 80% acetonitrile for 60 min, with a flow rate of 0.1 mL min^{-1}. The resulting lists of peaks were used to search against the SwissProt protein database with an in-house Mascot server (v.2.3.0; Matrix Science, London, UK) and a taxonomy restriction to Fungi (548 872 sequences for all entries, 31 582 sequences for fungal proteins)

with following parameters: peptide mass tolerance $\pm$ 0.3 Da, fragment mass tolerance $\pm$ 0.5 Da, allowing two missed cleavages. Citrullination of arginine residues and deamidation of asparagine and glutamine residues, both resulting in the mass increase by 1 Da, have been chosen as variable modifications. After automatic search, the MS/MS spectra for peptides indicated as modified were manually reviewed by the verification of sequence-specific fragment ions to confirm the identification of citrullination. The experiments were performed in triplicates and only those peptides that were identified as having particular arginine residue modified to citrulline in all three replicates have been considered in this work.

THP-1 cell culture and macrophage differentiation

The human acute monocytic leukemia cell line (THP-1) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (all compounds purchased from Cytogen) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. THP-1 cells were seeded in 12-well tissue culture plates at a density of 1×10^6 cells/well in the complete medium, and treated with a final concentration of 10 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 h to induce maturation toward adherent macrophage-like cells. The number and extent of differentiation were determined using the Scepter Handheld Automated Cell Counter (Millipore). After cell differentiation, the fresh culture medium without antibiotics was added but the PMA content was preserved for the entire duration of experiments. Inoculation with selected microorganisms was performed after 24 h of cell incubation under the above-described conditions.

THP-1 co-culture with mixed microbial cells

In the study of host responses to formation of mixed biofilm, THP-1 macrophages were incubated with microbial cells at a multiplicity of infection (THP-1:yeast:bacteria ratio) of 1:1:100 for 3 or 24 h, at 37°C. After centrifugation of the cell suspensions (200 $\times$ g, 5 min), the supernatants were removed and the remaining cell pellets were washed three times with PBS and used for RNA isolation. Each sample was analyzed in triplicate in three independent experiments.

Gene expression analyses

Total RNA was extracted from human cells using TRIzol reagent (Invitrogen) according to the protocol provided by the manufacturer. Total RNA was purified with the GenJET RNA purification kit (Thermo Scientific) and On-Column DNase I Digestion Set (Sigma-Aldrich) in order to remove all genomic DNA before performing reverse transcription reactions with the SuperScript III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Expression of individual genes was analyzed using quantitative real-time PCR (qPCR) with primers listed in Table 1. PCR amplification was performed by the SYBR green-based detection assay and the StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA) in 10-μL reaction mixture composed of 2 μL of cDNA, 0.2 μL of 10 μM forward (FW) and reverse (RV) primers, and 5 μL of SYBR KAPA master mix (Kapa Biosystems, Wilmington, MA) under the following conditions: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 20 s, primer annealing at 58°C (for IL-8 encoding

Table 1. List of primers used in this study.

target	primer	sequence (5′ → 3′)
IL1β	FW	CTTTGAAGCTGATGGCCCTAAA
	RV	AGTGGTGGTCGGAGATTCGT
IL6	FW	GGCACTGGCAGAAAACAACC
	RV	GGCAAGTCTCCTCATTGAATCC
IL8	FW	CACCGGAAGGAACCATCTCACT
	RV	TCAGCCCTCTTCAAAAACTTCTCC
IL10	FW	TGAGAACCAAGACCCAGACA
	RV	AAGGCATTCTTCACCTGCTC
MCP-1	FW	CAGCCAGATGCAATCAATGC
	RV	TGCTGCTGGTGATTCTTCTATAGCT
TNFα	FW	TCCTCCAGACACCCTCAACC
	RV	AGGCCCCAGTTTGAATTCTT
GAPDH	FW	GGGAAGCTTGTCATCAATGG
	RV	CATCGCCCCACTTGATTTTG

gene—at 53°C) for 15 s, and extension at 72°C for 20 s. Data analyses were performed with the real-time PCR system Sequence Detection software (version 1.4; Applied Biosystems). The average threshold cycle (C_T) value was obtained and normalized to the average C_T value of the gene encoding human glyceraldehyde-3-phosphate dehydrogenase. The comparative C_T method was used to quantify gene expression, and relative expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

Data were presented as means $\pm$ SD (standard deviation) and analyzed using GraphPad Prism software (GraphPad, LaJolla, CA, USA) with an one-way ANOVA test. The differences were considered statistically significant at $P < 0.05$ (unless otherwise stated).

RESULTS

Formation of dual-species biofilms by *P. gingivalis* and *C. albicans* under anaerobic and aerobic conditions

To investigate a possibility that the activity of bacterial PPAD might be induced during the interactions between *C. albicans* and *P. gingivalis* upon forming dual-species biofilms, two bacterial strains have been used, the reference wild-type strain *P. gingivalis* W83 and *P. gingivalis* W83 knockout strain ($\Delta ppad$), that does not express the PPAD-encoding gene and demonstrates inability to perform protein citrullination (Wegner *et al.* 2010b). In the current study, a model of co-infection was selected, based on the assumption that a formation of a biofilm by aerobic fungi might affect the colonization of the same infection site by anaerobic bacteria. Under both aerobic and anaerobic conditions, the formation of dual-species biofilms by *C. albicans* and *P. gingivalis* strains W83 or $\Delta ppad$ was confirmed by microscopic observations (Fig. 1). Bacterial cells, green fluorescently labeled prior to the addition to pre-formed fungal biofilms, were shown to be located in a close proximity to *C. albicans* hyphal forms, suggesting mutual interactions between these two microorganisms.

To qualitatively evaluate these interactions in a microplate model, the measurements of the fluorescence intensity representing the number of bacterial cells adhered to fungi were carried out after 3 or 24 h of mutual contact of both microorganisms (Fig. 2). The results were presented as relative adhesion (assuming the level of bacterial adhesion to microplate wells to
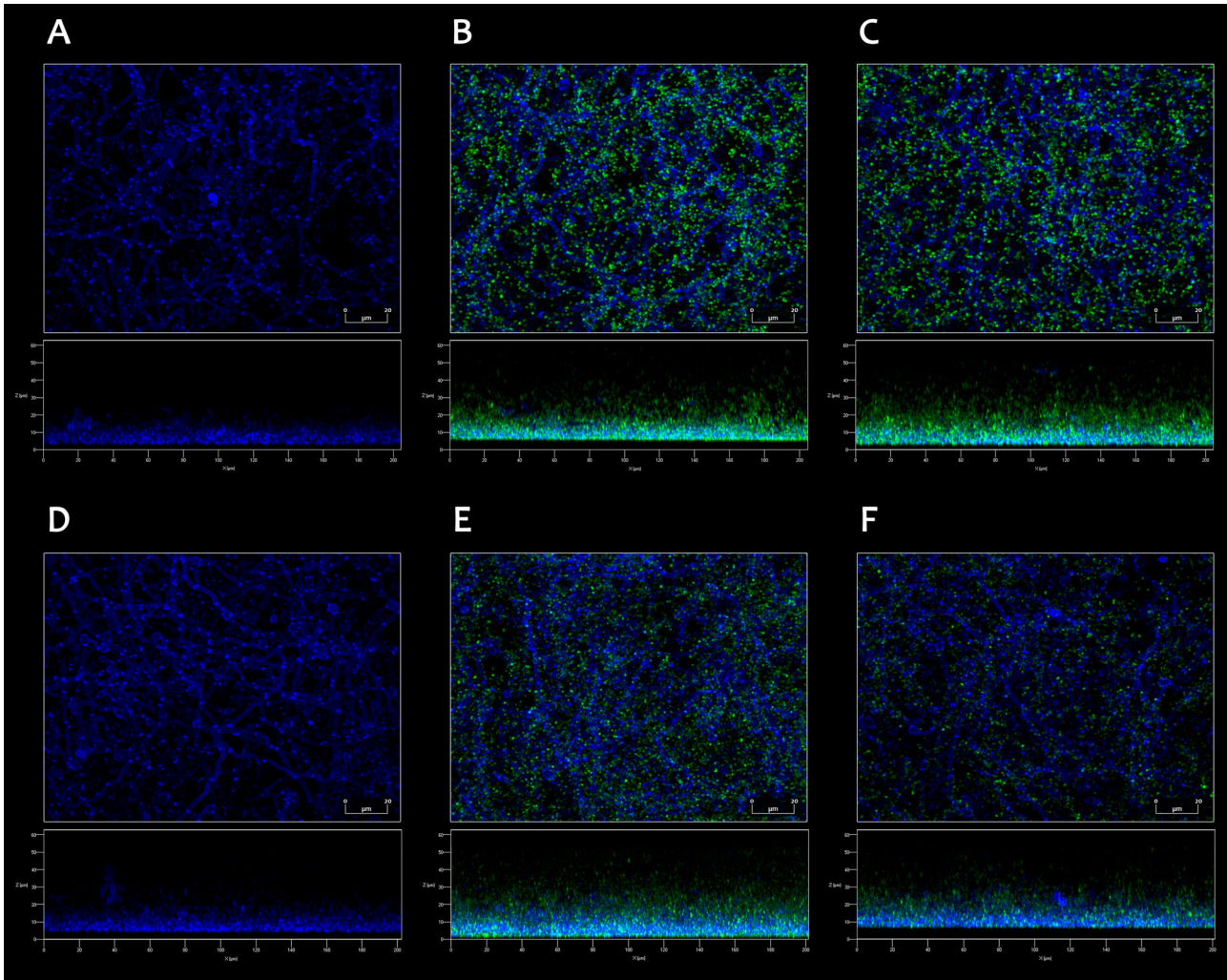
Figure 1. Formation of mixed biofilms by *C. albicans* and *P. gingivalis* wild-type W83 strain (**B**, **E**) and *P. gingivalis* Δ*ppad* strain (**C**, **F**), observed by confocal laser scanning microscopy. Dual-species biofilms were prepared using 1×10^6 *C. albicans* cells and 1×10^8 *P. gingivalis* cells of each used strain and incubated in RPMI 1640 medium without phenol red for 24 h at 37°C under anaerobic (**A–C**) or aerobic (**D–F**) conditions. (**A**, **D**) Monospecies fungal biofilms are presented as a reference. Fungal cells (blue) were stained just before imaging with 0.1% Calcofluor White Stain, and bacterial cells (green) were labeled with CFSE prior to addition to the chambers. The images were analyzed with ZEN Lite Microscopy Software.

represent 100%), in order to avoid the effect of general differences in the adhesion ability between tested strains, occurring after a shorter incubation time. In the initial phase of biofilm formation, the differences in the relative adhesion of *P. gingivalis* W83 strain compared to Δ*ppad* strain were rather slight. However, after prolonged time of contact between microorganisms, the relative adhesion was reduced in the case of Δ*ppad* strain. This difference, in favor of the wild strain, was up to 100% under anaerobic conditions, while under aerobic conditions the level of adhesion of W83 cells to fungal cells was several fold higher compared to Δ*ppad* strain. This observation confirms the important role of PPAD activity for the adherence of bacterial cells to *C. albicans*. It was also important to consider a dependence of the bacterial cells' viability during biofilm formation on the presence or absence of already formed fungal biofilm (Fig. 3). *Porphyromonas gingivalis* CFU assay was performed after co-culture of fungi and bacteria under optimal anoxic growth conditions or in the presence of atmospheric oxygen at a concentration of about 20.9% that is lethal to bacteria after longer incubation. Interestingly, under aerobic conditions in each of the experimental variants (lower vs higher number of bacterial cells used), both bacterial strains exhibited an increased viability in the presence of fungi than when grown alone for 24 h (Fig. 3).

Considering the dependence of bacterial viability on the contact with fungal biofilm under anaerobic conditions, it can be noticed that the presence of fungi might also play a protective or supportive role for *P. gingivalis* Δ*ppad* strain, increasing the number of live bacterial cells collected from the dual-species biofilm (Fig. 3). In the case of wild-type W83 strain, such protection was observed only for samples with a lower number of bacterial cells.

Another interesting issue was the increased viability of *C. albicans* cells, confirmed with CFU assay, in the presence of 1×10^8 *P. gingivalis* cells of Δ*ppad* strain, but not wild-type W83 strain (Fig. 4). Under both anaerobic and aerobic conditions, the fungal cell adhesion to the microplate wells, regardless of the presence of bacteria, did not differ (data not presented), similarly as did the number of the live *C. albicans* cells after 24 h of growth in the single-species biofilm or in the mixed-species biofilm with *P. gingivalis* cells of W83 strain.
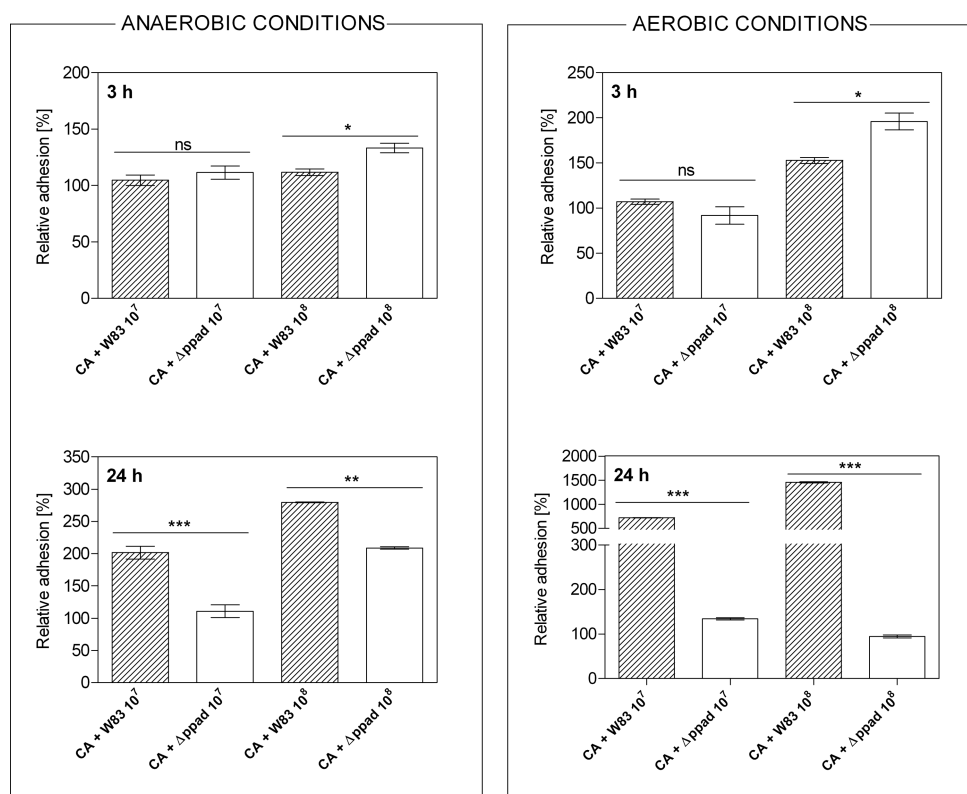
Figure 2. The relative adhesion of *P. gingivalis* cells to *C. albicans* biofilm after 3 or 24 h of co-culture under anaerobic or aerobic conditions. Fungal biofilm was prepared by culturing 5×10^5 cells (CA) in 100 μL RPMI 1640 medium per microplate well for 24 h at 37°C under aerobic conditions. 1×10^7 or 1×10^8 cells of *P. gingivalis* W83 strain or $\Delta ppad$ strain, both labeled with CFSE, were added to be further incubated for 3 or 24 h. The fluorescence intensity of CFSE-labeled cells was measured with the excitation/emission wavelengths of 480/520 nm. The adhesion of bacteria to the microplate wells was assumed to represent 100%. Bars on the graph represent the mean values of three determinations ± SD. Statistical significance levels were determined with one-way ANOVA test with Tukey's multiple comparison test and marked with **for $P < 0.01$, ***for $P < 0.001$ and 'ns' for 'not significant'.

Further important question was whether the molecules exposed at the surface of *C. albicans* cells, including various virulence factors and adhesins, might be modified by PPAD during the contact of both pathogens within the biofilm. Thus, the mass spectrometric identification of modified fungal proteins after cell surface shaving with trypsin (Karkowska-Kuleta *et al.* 2015) was performed and used to indicate particular peptides, in which the arginine residue was converted to citrulline residue, resulting in a peptide mass increase by 1 Da per residue (Table 2). It was important to accurately analyze the spectra in order to exclude incorrect attributions of that mass change to deamidation of asparagine or glutamine residues. Nine different proteins were identified as potentially modified by PPAD, of which one only after co-culture under anaerobic conditions (pyruvate decarboxylase, Pdc1), whereas two only after *C. albicans* growth with *P. gingivalis* under aerobic conditions (heat shock protein, Ssb1 and pH-regulated antigen, Pra1). For each of the proteins listed in Table 2, at least one particular arginine residue was found to be modified to citrulline. The modifications of *C. albicans* proteins were identified only after exposure of fungal cells to bacterial PPAD produced during biofilm formation with *P. gingivalis* wild-type W83 strain. In the control samples, where dual-species biofilm was formed by *C. albicans* and *P. gingivalis* $\Delta ppad$ strain, as well as for the fungal monospecies biofilms, no protein was identified as having arginine modified to citrulline, even though the method of analysis was identical for all samples.

Host responses to mixed *C. albicans* and *P. gingivalis* biofilm formation

To determine the influence of mixed biofilm formation on the host cell response, we used the model of THP-1 cells differentiated into macrophages by PMA treatment (Qin 2012) and analyzed the expression of genes encoding selected cytokines to monitor the progress of infection. The responses of the human cells, contacting with only *P. gingivalis* or only *C. albicans*, served as controls (Fig. 5). Both *P. gingivalis* strains, W83 and $\Delta ppad$, activated the responses of cytokine-coding genes, although the defect in PPAD production by *P. gingivalis* cells resulted in the generally lower expression level of genes that encode TNFα, IL-1β, IL-6, IL-10 and monocyte chemoattractant protein-1 (MCP-1). The opposite effect was observed for the expression of IL-8-encoding gene. The presence of hyphal form of *C. albicans* cells caused changes in the gene expression to the significantly lower level (ca. 10-fold) compared to bacterial responses. More meaningful changes were detected during formation of mixed biofilm, where the protection of *P. gingivalis* cells from the host recognition system was clearly visible. Both short (3 h) and long (24 h) responses of THP-1 cells to mixed infection, represented by the expression of selected genes, were significantly reduced—about 3-fold to 5-fold compared to unprotected bacterial cells. The protection effect was also observed during the biofilm formation between *C. albicans* cells and *P. gingivalis* mutant strain $\Delta ppad$. The results confirmed previous observations and pointed at PPAD as an important virulence factor,
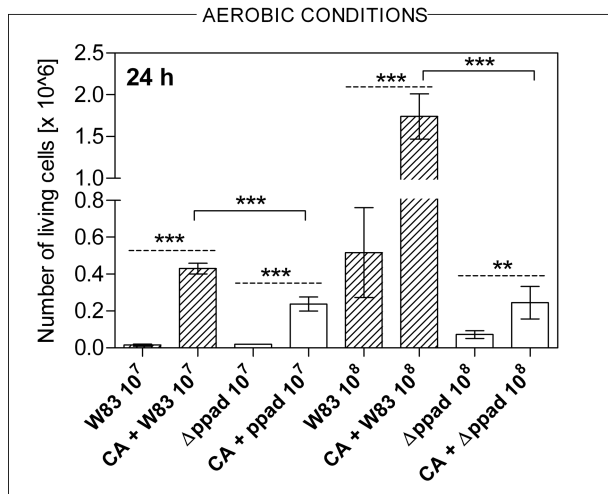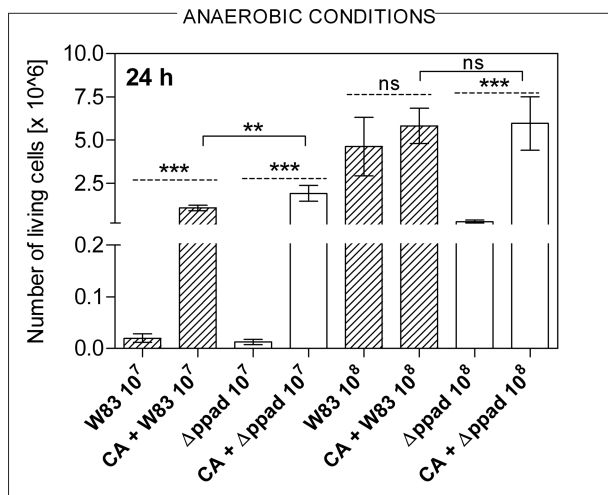
Figure 3. The viability of *P. gingivalis* cells after growth in mixed biofilm with *C. albicans* under anaerobic and aerobic conditions. After formation of dual-species biofilms by *C. albicans* (CA) and *P. gingivalis* strains W83 and Δ*ppad*, cells were mechanically detached from microplate wells and after dilutions transferred to blood agar plates. CFUs were counted after 7 days of anaerobic growth. Bars presented on the graph correspond to the mean values of three determinations ± SD. Statistical significance levels marked with **for $P < 0.01$, ***for $P < 0.001$ and 'ns' for 'not significant' were determined with one-way ANOVA test with Tukey's multiple comparison test. The solid line shows the comparison between two bacterial strains and the dashed line between the number of live bacterial cells in mono- or dual-species biofilm.

Figure 4. The number of live *C. albicans* cells (CA) counted after formation of biofilm with *P. gingivalis* strains W83 and Δ*ppad* under anaerobic and aerobic conditions. The CFU assay was carried out after the mechanical removal of adsorbed cells from the microplate wells and their further aerobic growth on agar plates for 24 h at 30°C. The mean values of three determinations ± SD are presented. One-way ANOVA test with Tukey's multiple comparison test was used to determine the statistical significance levels, which are marked with **for $P < 0.01$, ***for $P < 0.001$ and 'ns' for 'not significant'.

recognized by the host and influencing the interaction with fungal cells.

DISCUSSION

The maintenance of an ecological balance and an appropriate, physiological cooperation between all microorganisms that form the microbiota in the oral cavity contribute to the general health of the human host (Marsh 2010; Lof, Janus and Krom 2017). Several hundreds of different species of bacteria, fungi, viruses and protozoa create a complex community in the oral cavity during the formation of mixed-species biofilms on soft and hard tissues in the mouth (Dewhirst *et al.* 2010; Wright *et al.* 2013; Samaranayake and Matsubara 2017). Disturbing this complicated network of relationships between bacteria that are 'early colonizers' and 'late colonizers' in the oral
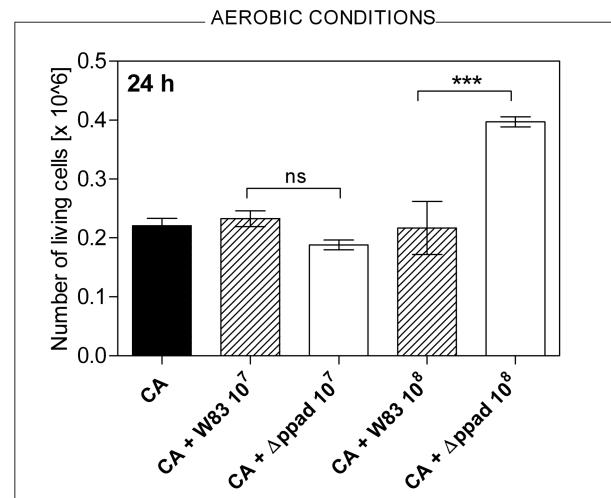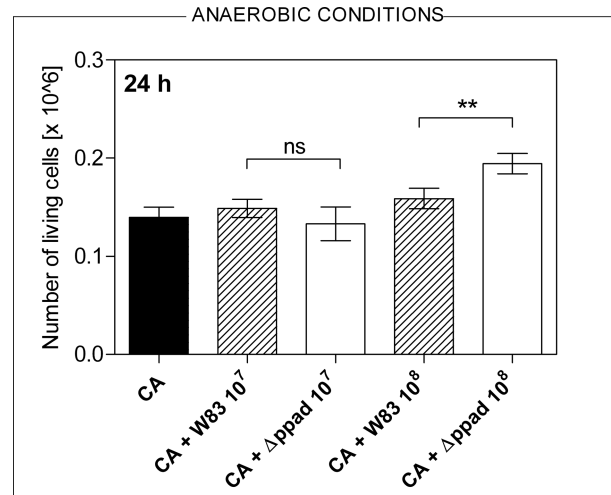
cavity or microorganisms that 'bridge' the representatives of those two groups or occasionally contact with them during accidental infection might lead to the development of such common and onerous diseases such as caries or periodontitis (van Palenstein Helderman 1981; van Winkelhoff *et al.* 2002; Filoche, Soma and Sissons 2007; He *et al.* 2012).

One of the main infectious agents in periodontal disease is *P. gingivalis*, considered as a 'keystone' pathogen in the subgingival biofilm (Honda 2011; Bostanci and Belibasakis 2012). The changes in environmental conditions combined with the initial adhesion of *P. gingivalis* cells to other bacteria, including aerobic streptococci, might shift the microbiological balance in the oral cavity and facilitate further development of the pathogenic state related to gingivitis and periodontitis (Lamont, Hersey and Rosan 1992; Marsh 2010; Lof, Janus and Krom 2017). In addition to different bacterial species found within oral biofilms, other pathogenic microorganism, *C. albicans*, might also be involved in the interactions with *P. gingivalis* (Socransky *et al.* 1998;

Table 2. Mass spectrometry identification of *C. albicans* surface-exposed proteins after formation of dual-species biofilm. *C. albicans* cells (5×10^8) and *P. gingivalis* cells of wild-type W83 strain or Δppad strain (5×10^9) were mixed together and incubated in RPMI 1640 medium for 24 hours at 37°C under aerobic or anaerobic conditions to form mixed-species biofilm. Mono-species biofilm formed only by *C. albicans* cells, without bacteria, served as a control. Fungal surface-exposed proteins were identified with cell surface shaving with trypsin followed by analysis with the Dionex Ultimate 3000 UHPLC system coupled to an HCTultra ETDII mass spectrometer. The representative results from three independent experiments are presented, provided that particular peptide have been identified in each of them (m/z – mass to charge).

Accession number	C. albicans protein name	Peptide	P. gingivalis strain	Observed m/z ratio (charge)	Calculated mass [Da]	Ion score aerobic conditions	Ion score anaerobic conditions
P30575 (ENO1_CANAL)	Enolase 1	319IQIVGDDLTVTNPTR333	—	821.4640 (+2)	1640.9134	108	
			W83	821.9680 (+2)	1641.9214	65	76
			Δppad	821.4160 (+2)	1640.8174	83	
P83776 (HXKB_CANAL)	Hexokinase-2	350IEEDPFENLSDVADLFR366	—	1004.9900 (+2)	2007.9654	31	
			W83	1005.4630 (+2)	2008.9114	75	81
			Δppad	1005.0610 (+2)	2007.9425	103	
P46273 (PGK_CANAL)	Phosphoglycerate kinase	112DGEIFLLENLR122	—	659.9270 (+2)	1317.8394	34	
			W83	660.4390 (+2)	1318.8634	66	59
			Δppad	659.8570 (+2)	1317.6994	53	
P43067 (ADH1_CANAX)	Alcohol dehydrogenase 1	303DTAEAIDFFSR313	—	636.3830 (+2)	1270.7514	67	
			W83	636.8750 (+2)	1271.7354	60	49
			Δppad	636.3180 (+2)	1270.6214	54	
P83779 (PDC1_CANAL)	Pyruvate decarboxylase	37TYEVEGMR44	—	498.7190 (+2)	995.4234	29	
			W83	499.2500 (+2)	996.4854		
			Δppad	498.8180 (+2)	995.6214	19	47
Q59XX2 (MP65_CANAL)	Cell surface mannoprotein MP65	142SEFSQIASEIAQLSGFDVIR160	—	684.0800 (+3)	2049.2182	78	78
			W83	1026.0470 (+2)	2050.0794	73	109
			Δppad	684.0370 (+3)	2049.0892	61	
Q5AIR7 (ENG1_CANAL)	Endo-1,3(4)-beta-glucanase 1	932DASNPSADDTYFPVSR948	—	871.3610 (+2)	1740.7591	54	
			W83	871.8550 (+2)	1741.7554	57	23
			Δppad	871.4090 (+2)	1740.7870	26	
		1082DQJAGFIDNVDSGWTGILR1100	—	n.o.	2076.0276*		
			W83	1039.5030 (+2)	2076.9914	101	72
P87222 (HSP75_CANAW)	Heat shock protein Ssb1	332SQVDEVVLVGGSTR345	—	723.4650 (+2)	1444.9154	59	
			W83	723.9760 (+2)	1445.9374	30	
			Δppad	723.4040 (+2)	1444.7934	76	
P87020 (PRA1_CANAL)	pH-regulated antigen	168TNIFWAGDLLHR179	—	n.o.	1441.7466*		
			W83	481.9460 (+3)	1442.8162	51	

n.o. not observed

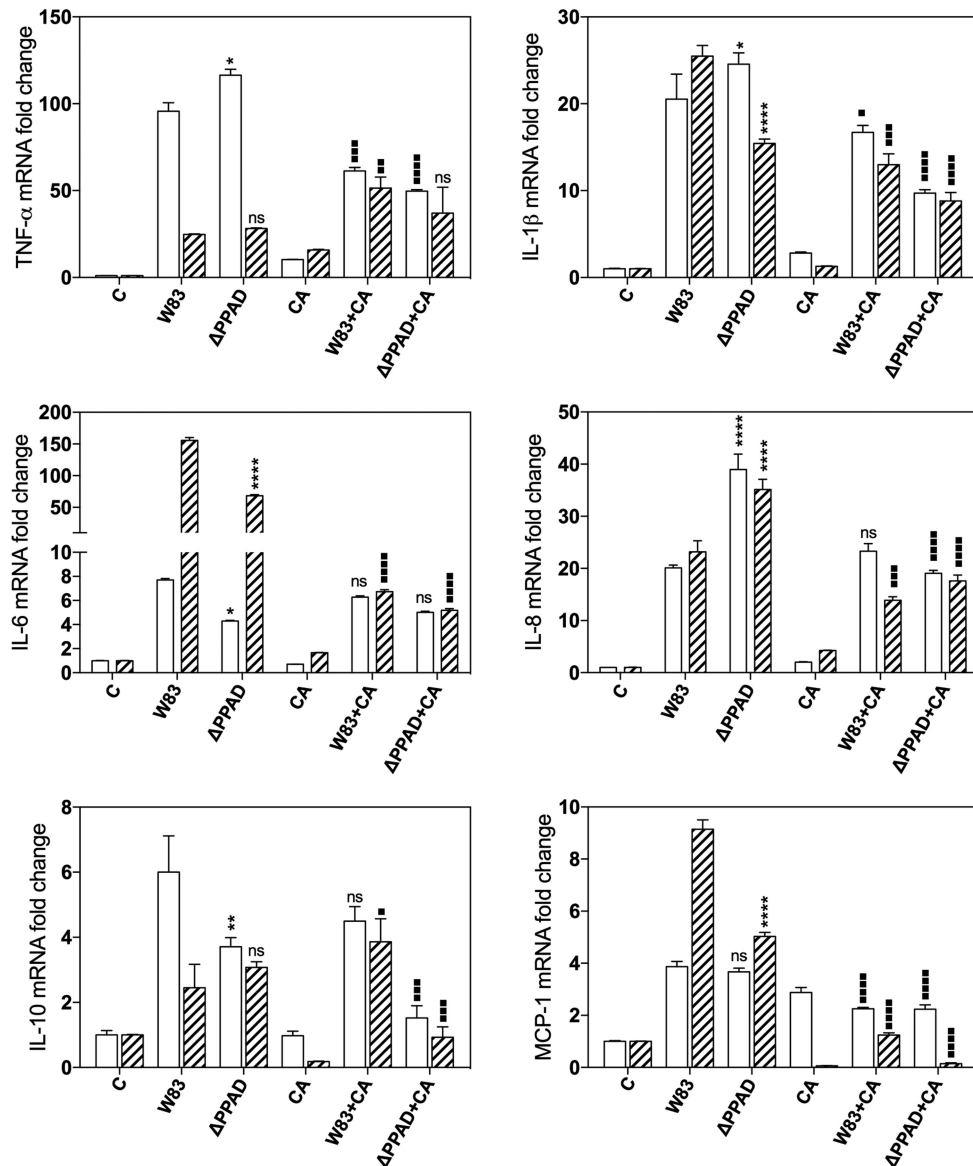* theoretical mass for the specified peptide (unmodified)

Figure 5. Expression profiles of genes that encode IL-1β, TNF-α, IL-6, IL8, IL-10 and MCP-1 in THP-1 monocyte-derived macrophages after contact with *C. albicans* and *P. gingivalis* cells for 3 h (open bars) or 24 h (crosshatch bars) at a multiplicity of infection of 1:1:100 measured by qRT-PCR. Data are expressed as the mean ± SD (three independent experiments). One-way ANOVA test with Tukey's multiple comparison test was used to determine the statistical significance levels. The significance levels were marked with */■ for $P < 0.05$; **/■■ for $P < 0.005$, ***/■■■ for $P < 0.001$, ****/■■■■ for $P < 0.0001$ and 'ns' for 'not significant'. Asterisks were used to compare cell responses to wild and mutant bacterial strains, and squares to compare cell responses to mixed biofilm versus bacteria.

Rautemaa and Ramage 2011; Camargo *et al.* 2016). Since there are data presenting the importance of the interactions between these two pathogens during the development of certain oral infections (Tamai, Sugamata and Kiyoura 2011; Haverman *et al.* 2017), it is important to investigate in detail the character of these interactions and the contribution of particular virulence factors to this contact.

Besides the fact that *C. albicans* can grow either under aerobic and anaerobic conditions (Webster and Odds 1987), it also possesses an ability to decrease the oxygen concentration in the immediate vicinity, thus facilitating the development of the microenvironment that protects and supports the growth of anaerobic bacteria (Fox *et al.* 2014). In the current work, such an important capability was demonstrated with respect to *P. gingivalis* cells. We observed that under experimental conditions at

the atmospheric oxygen concentration, the relative viability of bacteria was considerably higher, when they were co-incubated with *C. albicans* in a dual-species biofilm rather than in the monospecies biofilm. *Porphyromonas gingivalis* demonstrates the ability to adapt its metabolism to microaerophilic conditions at oxygen concentration about 6% (Lewis, Iyer and Anaya-Bergman 2009); thus, the localization of bacterial cells within the complex and heterogeneous structure of fungal biofilm might result in the successful growth and proliferation of bacteria. On the other hand, we cannot currently provide an explanation for the observed increased viability of *C. albicans* cells in biofilms formed with *P. gingivalis* cells deprived of PPAD. Probably, this might be caused by some compensative processes in bacterial cell metabolism, caused by the lack of this important protein, and undoubtedly this issue requires further, detailed studies.

Furthermore, we noticed the significant reduction in the level of adhesion to fungal biofilms after prolonged mutual contact, demonstrated in bacterial cells lacking the ability to citrullinate proteins. This phenomenon might be related to both autocitrullination of *P. gingivalis* surface-exposed proteins and/or modification of proteins derived from the cells of interacting partners. Recently, the extracellular citrullinome of *P. gingivalis* was investigated and several proteins, including important bacterial virulence factors, were found to be modified by PPAD, including arginine-specific cysteine proteinase (RgpA) and adhesive Mfa1 fimbrilin (Stobernack *et al.* 2016). Such modifications of the bacterial cell surface proteins could also play a role during the formation of dual-species biofilm with *C. albicans*; however, the possibility of citrullination of fungal proteins should not be ignored. In the current work, a relatively novel proteomics-like approach—'cell surface shaving' with trypsin (Karkowska-Kuleta *et al.* 2015)—was used after dual-species biofilm formation to identify fungal surface-exposed proteins. As both microorganisms were co-cultured for quite a long time, these fungal proteins could be first processed by *P. gingivalis* arginine-specific gingipains, resulting in the exposition of the C-terminal arginine residues prone to citrullination by PPAD (Wegner *et al.* 2010b).

Enolase was found among *C. albicans* surface-exposed proteins, for which the potential citrullination could be suggested. This is an important 'moonlighting' protein, abundant glycolytic enzyme and well-known dominant antigen detected during disseminated candidiasis, and a binding protein for human plasminogen and components of the plasma kinin-generating system (Sundstrom and Aliaga 1992; Jong *et al.* 2003; Karkowska-Kuleta *et al.* 2011; Seweryn *et al.* 2015). *Candida albicans* enolase is also responsible for the adhesion to polyvinyl chloride-made medical devices (Núñez-Beltrán, López-Romero and Cuéllar-Cruz 2017). A suggested ability of *P. gingivalis* PPAD to citrulllinate human enolase provides an interesting link between the inflammation during periodontal disease and the development of serious autoimmune disorder, rheumatoid arthritis (Kinloch *et al.* 2005; Wegner *et al.* 2010a).

In addition, two other *C. albicans* cytoplasm-derived enzymes, also found at the surface of fungal cells, were identified to be modified by bacterial PPAD. These included phosphoglycerate kinase (Pgk1) and alcohol dehydrogenase (Adh1), known as antigens involved also in binding of human plasminogen (Pitarch *et al.* 2001; Crowe *et al.* 2003). Moreover, the possible citrullination of the pH regulated antigen (Pra1) might be important for the interactions of fungi with the host during infection, as this protein participates in the regulation of host immune system activation through the interactions with immune cells and complement receptors and regulators (Luo *et al.* 2009, 2010; Losse *et al.* 2011; Soloviev, Jawhara and Fonzi 2011). Additional roles of Pra1 such as the binding to plasminogen and zinc sequestration during invasion on endothelial cells (Luo *et al.* 2009; Citiulo *et al.* 2012) might also be altered by potential citrullination of this protein by PPAD. Two other candidal enzymes, endo-1,3-β-glucanase, Eng1, and cell surface mannoprotein, MP65, play a role in maintaining the proper function of the fungal cell wall (Esteban *et al.* 2005; Sandini *et al.* 2007), and the latter protein is also involved in the interactions with the host, and in the biofilm formation (Pietrella *et al.* 2006; Sandini *et al.* 2011). Thus, the potential changes in the functionality of all of the abovementioned proteins caused by PPAD-dependent modification might significantly affect the interactions of *C. albicans* with bacteria or host cells during biofilm formation.

Numerous studies have demonstrated that during the periodontal infection *P. gingivalis* is able to induce the production of many inflammatory mediators from different types of host cells such as epithelial cells, fibroblasts, endothelial cells and macrophages (Gemmel and Seymour 1993; Quinchia-Rios *et al.* 2008; Damgaard *et al.* 2016). Results obtained in our current study fit to previously published data on the immune response of macrophages to infection with *P. gingivalis* wild-type cells (Gmiterek *et al.* 2016; Lam *et al.* 2016), including the reports of the stimulation of monocyte-derived macrophages to the secretion of proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-8 and MCP-1, that subsequently recruit next immune cells to infiltrate infected tissues (Zhou and Amar 2006). We presented here, for the first time, markedly changed responses in macrophages that contacted the *P. gingivalis* mutant strain deprived of PPAD, thus providing a direct evidence for the significance of this enzyme as an important virulence factor. We found that the expression of the gene, encoding IL-1β, known to inhibit macrophage apoptosis, resulting in an impaired elimination of *P. gingivalis* from the infected host cells, was decreased in the host response to mutant strain. Similar effects were noticed for gene encoding IL-6 that is responsible for the differentiation of activated B cells into immunoglobulin-secreting plasma cells and may play an important role in regulating the immune response of patients with chronic periodontitis (Zhou and Amar 2006; Trindade *et al.* 2012; Gmiterek *et al.* 2016). On the other hand, the expression of the gene encoding TNF-α, the protein that coordinates many processes of macrophage activation in response to *P. gingivalis*, including the production of proinflammatory cytokines and surface expression of TLR2 (Papadopoulos *et al.* 2013) seemed not to be influenced significantly by PPAD presence, as both strains generated similar responses of macrophages. Also, the expression of the gene encoding anti-inflammatory cytokine IL-10 in macrophages infected with mutant strain was decreased compared to parent strain. These results corroborate other study that presented IL-10 production by THP-1 cells upon the contact with wild-type strain 7436 and a strain defective in other *P. gingivalis* virulence factor, HmuY (Gmiterek *et al.* 2016). We also identified changes in the expression of the gene that encodes the chemokine MCP-1, the most potent chemoattractant for monocytes. Its significantly higher levels were detected in gingival crevicular fluid, saliva and serum in subjects with chronic periodontitis compared to healthy controls (Gupta, Chaturvedi and Jain 2013; Zhu *et al.* 2015). In our model, the responses of THP-1 to *P. gingivalis* $\Delta ppad$ cells were significantly decreased compared to wild-type *P. gingivalis* cells. Similarly, decreased responses of human cells were observed for $\Delta ppad$ cells that contact fibroblasts, where *P. gingivalis* was found to be involved in the modulation of the prostaglandin-dependent pathway. The host cells infected with wild-type *P. gingivalis* strain showed the upregulated expression of cyclooxygenase-2 and microsomal PGE synthase-1, two key enzymes that participate in PGE2 synthesis. But these effects were strongly reduced in cells infected with the $\Delta ppad$ strain as well as with the strain that expresses a catalytically inactive enzyme (Gawron *et al.* 2014).

The opposite effect was observed in our study for the expression of IL-8- encoding gene, which seemed to increase in contact with $\Delta ppad$ mutant strain. This effect is confusing in line of data that both wild and mutant strains express comparable amount of gingipains, the enzyme involved in IL-8 degradation, and that PPAD exerted only a minor IL-8-citrullinating activity (Moelants *et al.* 2014).

However, the most important results of our current study concerned the attenuated macrophage responses during the mixed infection with bacteria and *C. albicans*, and in particular, the decreased expression of genes that encode the analyzed cytokine and chemokine. Similar type of bacteria protection from host responses was observed previously for host cells upon contacts with mixed biofilms. However, depending on the type of contacting microorganisms, the responses can differ significantly (Peleg *et al.* 2010). The interpretation of results presented hereby is challenging and needs more research, as we can expect many possible way of mutual pathogen interactions. One could concern antagonistic interactions where the virulence of bacteria is reduced by the contact with fungi, as was presented for mixed biofilm with *P. aeruginosa* (Lopez-Medina *et al.* 2015). Other possibility is that the fungal cells may suppress local host immune response to allow bacteria to survive under unfavorable conditions (Roux *et al.* 2009). Alternatively, fungal biofilm formation can serve as a camouflage for bacterial cells which hide in it against host recognition. However, further work is required to understand the relevance of *C. albicans* and *P. gingivalis* mixed biofilms for the predisposition to invasive infection and their susceptibility to host immune defense.

The association of the periodontitis with different severe systemic diseases, including cardiovascular diseases, Alzheimer's disease, diabetes or rheumatoid arthritis (Otomo-Corgel *et al.* 2012), urges to thoroughly investigate any conditions that may contribute to the development of *P. gingivalis*-related infections associated with the formation of complex microbial communities within the dental plaque. It is likely that the citrullination of proteins by bacterial PPAD might be an important phenomenon during the formation of mixed-species biofilm together with *C. albicans*. This complex structure built by the fungal cells present in various morphological forms and containing the extracellular matrix might provide a protective environment for anaerobic bacteria in the oral cavity, thus facilitating and accelerating the development of the serious gingival diseases.

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