The presence of biofilm forming microorganisms on hydrotherapy equipment and facilities

Natalia Jarząb and Maciej Walczak

ABSTRACT

Hydrotherapy equipment provides a perfect environment for the formation and growth of microbial biofilms. Biofilms may reduce the microbiological cleanliness of hydrotherapy equipment and harbour opportunistic pathogens and pathogenic bacteria. The aims of this study were to investigate the ability of microorganisms that colonize hydrotherapy equipment to form biofilms, and to assess the influence of temperature and nutrients on the rate of biofilm formation. Surface swab samples were collected from the whirlpool baths, inhalation equipment and submerged surfaces of a brine pool at the spa center in Ciechocinek, Poland. We isolated and identified microorganisms from the swab samples and measured their ability to form biofilms. Biofilm formation was observed at a range of temperatures, in both nutrient-deficient and nutrient-rich environments. We isolated and identified microorganisms which are known to form biofilms on medical devices (e.g. *Stenotrophomonas maltophilia*). All isolates were classified as opportunistic pathogens, which can cause infections in humans with weakened immunity systems. All isolates showed the ability to form biofilms in the laboratory conditions. The potential for biofilm formation was higher in the presence of added nutrients. In addition, the hydrolytic activity of the biofilm was connected with the presence of nutrients.

Key words | balneology, biofilm formation, brine, hydrotherapy equipment

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INTRODUCTION

In the natural environment, microorganisms are rarely found as planktonic cells (Mnichowska-Polanowska *et al.* 2009; Vu *et al.* 2009; Pantanella *et al.* 2013), but more often will adhere to surfaces and to each other, creating thin films called biological membranes or biofilms (Czaczyk & Myszka 2007; Gajewska & Cieniek 2009; Pietruczuk & Kalicińska 2010; Pantanella *et al.* 2013; Lecuyer *et al.* 2015; Otter *et al.* 2015). A biofilm consists of complex structures in which numerous microbial cells are surrounded by a slime layer (Monds & O'Toole 2009; Pantanella *et al.* 2013; Lambert *et al.* 2014). The function of these clusters is to protect microorganisms from the adverse impact of external environmental factors (Ahimou *et al.* 2007; Lambert *et al.* 2014; Otter *et al.* 2015) and to ensure their better access to nutrients (Singh *et al.* 2006; Vu *et al.* 2009). Additionally, communication between microorganisms enables them to adapt to changing environmental conditions. As a result, they are able to colonize different ecological niches (Gajewska & Cieniek 2009; Otter *et al.* 2015), and survive conditions that would be detrimental to planktonic cells (Furowicz *et al.* 2010).

Pathogens and opportunistic pathogens can colonize biofilms and persist for a long time even under extremely harsh environmental conditions, including high salinity. Whilst embedded in the biofilms, pathogens are protected against stress, chemical agents and disinfectants (Palmer *et al.* 2007; Tamayo *et al.* 2010; Xu *et al.* 2011). Biofilms are difficult to remove and may resist the routine cleaning practices in public facilities such as hotels, hospitals, rehabilitation centers, canteens and other mass caterers as

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well as in private households (Pantanella *et al.* 2013; Otter *et al.* 2015). Structures like biofilm can lead to infections and are therefore considered a threat to the safety of hospital patients (Kokare *et al.* 2009; Pantanella *et al.* 2013; Lambert *et al.* 2014; Otter *et al.* 2015).

Hydrotherapy is the branch of medical science that combines mineral water with modern equipment (for inhalation, underwater and whirlpool massages, etc.) to treat and prevent many diseases and to help patients recover. Due to the relatively high temperature of water (20–38°C) and the availability of nutrients, this equipment provides an ideal environment for the growth and spread of microorganisms, including pathogens (Szczygłowska *et al.* 2012).

Biofilms adversely affect the microbiological cleanliness of the sanitary installations and hydrotherapy equipment and facilities in spas/hospitals (Otter *et al.* 2015). In addition, their multi-species structure promotes the growth of pathogens, such as *Legionella pneumophila* (Leoni *et al.* 2006) and opportunistic pathogens, for example *Pseudomonas aeruginosa* (Rice *et al.* 2012; Leoni *et al.* 2015; Walker & Moore 2015). Therefore, the aim of this study was to evaluate the ability of microorganisms that colonize hydrotherapy equipment and installations to form biofilms.

MATERIALS AND METHODS

Sampling

Sterile swabs were used to take samples from whirlpool baths, inhalation equipment and submerged surfaces of the brine pool at the spa in Ciechocinek, Poland. The swabs were transported to our laboratory immediately after the collection in insulated containers at ≤ 7°C.

Isolation and identification of microorganisms

In order to isolate microorganisms, the tips of the swabs were cut off and placed in tubes containing 10 ml of sterile saline. The tubes were then vortexed (for 2 min) to remove microbial cells from the swab material. Ten-fold serial dilutions were prepared from the obtained suspensions using sterile saline diluent. The diluted suspension was then inoculated on to the following media: nutrient agar (composition [g/l]: peptone – 5.00; yeast extract – 3.00; bacteriological agar – 15.00) for strains with high nutritional requirements; and R2 agar (composition [g/l]: peptone – 0.75, yeast extract – 0.50, tryptone – 0.25, glucose – 0.50, starch – 0.50, sodium pyruvate – 0.30, $MgSO_4$ – 0.024, K_2HPO_4 – 0.30, bacteriological agar – 15.00) for defective strains and strains weakened by physiological shock or with low nutritional requirements. This medium was recommended for the enumeration and cultivation of many bacteria from water. Due to varying nutritional requirements, some strains may grow poorly or fail to grow on this medium (e.g. *Legionella pneumophila*) (Reasoner & Geldreich 1985).

The two media were prepared using the same concentration of brine (instead of distilled water) that was used in the hydrotherapy equipment we examined (brine pool, whirlpool baths, inhalation equipment). The inoculated plates were incubated at 26°C (as in the investigated equipment) for 7 days.

Isolates were identified using the MALDI method (matrix assisted laser desorption and ionization) accompanied by mass measurement of each sample in a mass spectrometer. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid, reliable, and high-throughput diagnostic tool for the identification of microorganisms (Dingle & Butler 2013). It is based on the ionization of co-crystallized sample material by short laser pulses. MALDI/TOF spectra are used for the identification of microorganisms such as bacteria or fungi (Dingle & Butler-Wu 2013). A portion of a colony of the microbe in question is placed onto the sample target and overlaid with matrix. The mass spectra generated are analyzed by dedicated software and compared with stored profiles. MALDI-TOF MS was carried out with a MALDI Microflex LT (Bruker Daltonics, Bremen, Germany).

Biofilm forming ability of the isolated strains

Biofilm formation by individual strains was quantitatively determined according to Fleming *et al.* (2009) and Kim *et al.* (2009).

Start culture

Erlenmeyer flasks, each containing 20 ml of nutrient broth (composition [g/l]: peptone – 5.00, yeast extract – 3.00)

were inoculated with different strains of microorganisms using an inoculation loop, and then incubated for 24 hours at 26°C. Following incubation, the bacterial cultures were diluted 100-fold with saline solution to reach $OD_{600} = 0.5$.

Each strain was tested for biofilm formation in three environments: sterile saline solution (Control); sterile saline solution supplemented with glucose (final concentration 0.25% w/v) (Glucose); and sterile saline solution supplemented with yeast extract (final concentration 0.25% w/v) (Yeast extract).

Subsequently, 200 µl of the prepared suspension (for each variant) was placed in a sterile 24-well microtiter plate. The plates were incubated for 48 hours at 26°C and 30°C. After incubation, all unbound bacteria were removed by rinsing the plates three times using phosphate buffered saline (PBS) buffer (composition [g/l]: NaCl – 8.00, KCl – 0.2; Na2HPO4 – 1.44; KH2PO4 – 0.24) and the biofilm was fixed by drying (1 hour, 60°C). Next, 2 ml of 1% (w/v) crystal violet solution was added to each well and the plates were incubated for 15 min at room temperature. Subsequently, the biofilm was fixed by drying (1 hour, 60°C). The plates were then washed, the dye was solubilized in 0.95% ethanol, and absorbance at 570 nm was determined (Fleming *et al.* 2009; Kim *et al.* 2009). Biofilm was classified using the scheme of Stepanovic *et al.* (2007).

Evaluation of biofilm hydrolytic activity

Hydrolytic activity of biofilms was determined using fluorescein diacetate (FDA) (10 mg FDA/ml acetone) (Peeters *et al.* 2008). Bacterial suspensions were prepared as described above (for the quantitative analysis), and 2 ml of the culture added to each well of sterile 24-well titration plates. The plates were then incubated at 26°C and 30°C for 48 h. After the incubation, the unbound bacteria were rinsed off three times using PBS. Next, each well of the titration plate was filled with 2 ml of sterile PBS and 20 µl of FDA. The plates were incubated in the dark for three hours at 26°C and 30°C. The amount of released fluorescein was measured using a Hitachi spectrofluorimeter F-2500, at an excitation wavelength of 480 nm and an emission wavelength of 505 nm.

Statistical analysis

Statistical analyses of variance (ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference) were performed using program STATISTICA 6.0.

RESULTS

Isolation and identification of bacterial strains

Table 1 shows the results of the identification of the isolated microorganisms. The largest species diversity was recorded among isolates from the whirlpool baths and inhalation equipment, which included several species of gram positive (G+) bacteria, including actinobacteria, gram negative (G−) bacteria, and yeast. The smallest species diversity was observed on the submerged surfaces of the brine pool, which yielded only three species of G+ and G− bacteria and yeast.

Biofilm formation on titration plates

All isolates except *Brachybacterium faecium* were shown to form biofilms in the control environment, and in sterile saline supplemented with glucose and yeast extract (Figure 1).

In sterile saline, the amount of biofilm measured by absorbance (A) at 570 nm ranged from 0 to 0.137 (Figure 1). The lowest amount of biofilm (A = 0.005) was produced by *Acinetobacter junii* isolated from the whirlpool bath (Figure 1(c)), while the highest (A = 0.137), by *Candida parapsilosis* isolated from the brine pool (Figure 1(a)). According to the criteria described by Stepanovic *et al.* (2007) biofilms formed by these strains in the uncontaminated environment were weak.

The amount of biofilm was significantly higher ($p < 0.05$) in saline supplemented with glucose and yeast extract than in the saline solution. The amount of biofilm measured by absorbance (A) at 570 nm ranged from 0 to 0.718 (Figure 1). The smallest amount of biofilm (A = 0.017) was produced by *Microbacterium aurum* isolated from the whirlpool bath (Figure 1(c)). The largest amount was produced by *Stenotrophomonas maltophilia* (A = 0.718) isolated from

Table 1 | Identification of isolated microorganisms

	Microorganism	Gram staining	Pathogenicity	Morphology
Brine pool	*Candida parapsilosis*		Opportunistic pathogen	Yeast
	Pseudomonas mendocina	G−	Opportunistic pathogen	Rods
	Staphylococcus warneri	G+	Opportunistic pathogen	Cocci
Inhalation equipment	*Arthrobacter castelli*	G+	Nonpathogenic	Mycobacteria
	Brachybacterium faecium	G+	Nonpathogenic	Rods
	Brevibacterium casei	G+	Opportunistic pathogen	Mycobacteria
	Kocuria spp.	G+	Opportunistic pathogen	Actinobacteria
	Microbacterium aurum	G+	Opportunistic pathogen	Cocci
	Micrococcus luteus	G+	Opportunistic pathogen	Cocci
	Rhodotorula minuta		Pathogen	Yeast
	Staphylococcus simulans	G+	Opportunistic pathogen	Cocci
	Stenotrophomonas maltophilia	G−	Opportunistic pathogen	Rods
Whirlpool baths	*Acinetobacter junii*	G−	Opportunistic pathogen	Rods
	Agromyces mediolanus	G+	Opportunistic pathogen	
	Delftia acidovorans	G−	Opportunistic pathogen	Mycobacteria
	Microbacterium aurum	G+	Opportunistic pathogen	Cocci
	Microbacterium spp.	G+	Opportunistic pathogen	Mycobacteria
	Pseudomonas mendocina	G−	Opportunistic pathogen	Rods
	Stenotrophomonas acidaminiphila	G−	Opportunistic pathogen	Rods
	Stenotrophomonas maltophilia	G−	Opportunistic pathogen	Rods

the inhalation equipment (Figure 1(b)). According to Stepanovic *et al.* (2007), in this case biofilm formation was moderate.

The amount of biofilm produced in saline solution incubated at 30°C ranged from 0 to 0.153 (Figure 2). The highest amount was produced by *Micrococcus luteus* (A = 0.153) isolated from the inhalation equipment (Figure 2(b)). No biofilm was formed by strains *Kocuria* spp. and *Brachybacterium faecium*, isolated from the same equipment (A = 0) (Figure 2(b)). According to Stepanovic biofilm formation was weak.

Evaluation of biofilm activity

Hydrolytic activity of biofilms formed by the studied strains in pure saline solution, expressed as the amount of released fluorescein, ranged from 0 to 0.257 µg/ml (Figure 3). The highest value (0.257 µg/ml) was recorded for the biofilm formed by *Kocuria* spp. isolated from the inhalation equipment (Figure 3b). No hydrolytic activity was recorded for biofilms formed by *Brachybacterium faecium* and *Micrococcus luteus* isolated from the inhalation equipment (Figure 3b) and *Stenotrophomonas maltophilia* isolated from the whirlpool baths (Figure 3c).

In saline supplemented with glucose and yeast extract the hydrolytic activity of biofilms ranged from 0 to 2.521 µg/ml of released fluorescein (Figure 3). The highest hydrolytic activity was recorded for biofilm produced by *Microbacterium aurum* (2.521 µg/ml), isolated from the whirlpool baths (Figure 3c). No hydrolytic activity was recorded for biofilms formed by *Brachybacterium faecium* and *Micrococcus luteus* isolated from the inhalation equipment (Figure 3b) or for *Stenotrophomonas maltophilia* isolated from the whirlpool baths (Figure 3c).

At 30°C, the hydrolytic activity of biofilms ranged from 0 to 2.446 µg/ml (Figure 4). The highest hydrolytic activity (2.446 µg/ml) was recorded for a biofilm formed by *Stenotrophomonas acidaminiphila*, isolated from the whirlpool baths (Figure 4c). There was no hydrolytic activity of a biofilm formed by *Stenotrophomonas maltophilia*, also isolated from the whirlpool baths (Figure 4c).

Statistical analysis

The results of the biofilm formation were compared using ANOVA with the Tukey post-hoc test.

For the brine pool, the statistical analysis revealed significant differences between the amount of biofilm

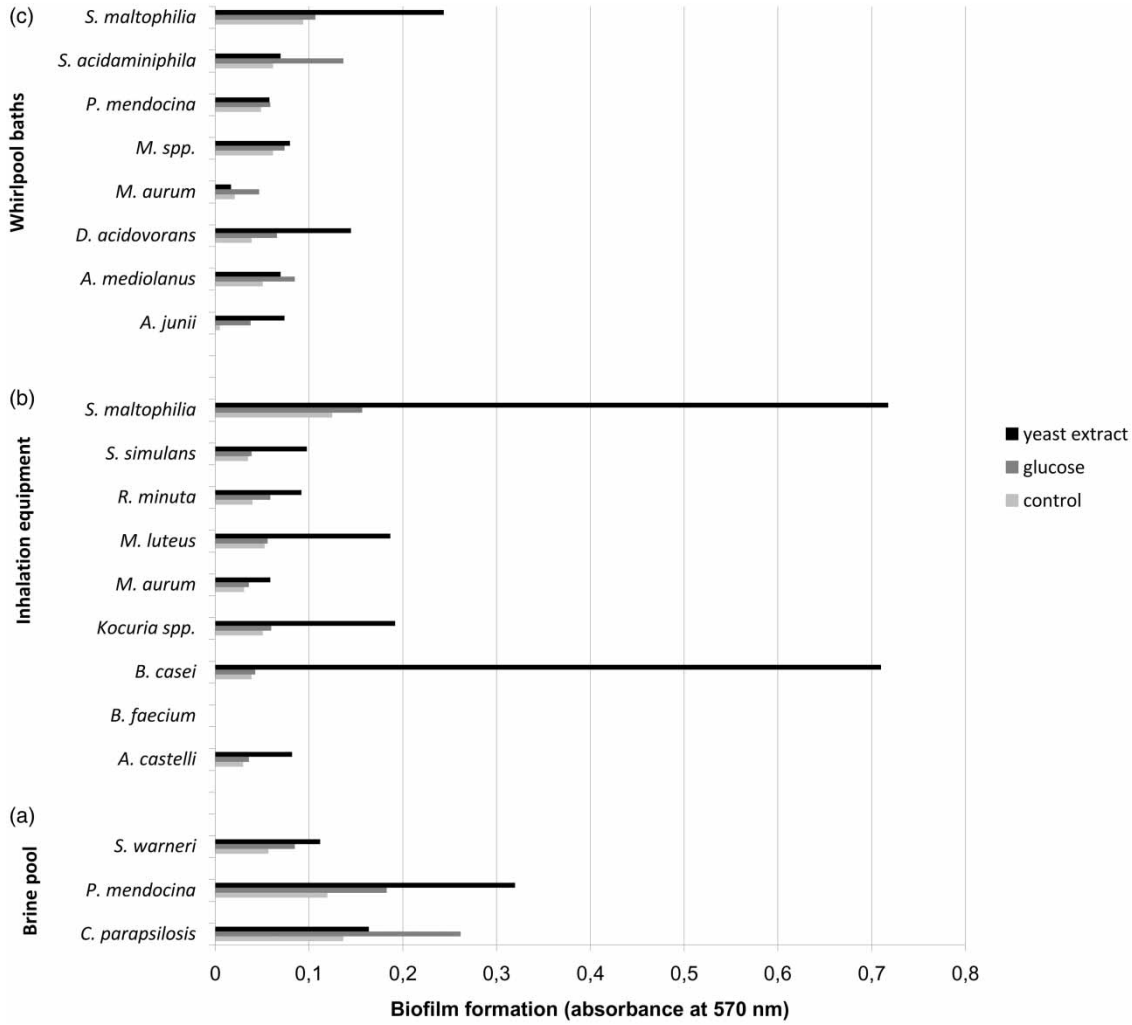

Figure 1 | The amount of biofilm formation on the surface of microtiter plates expressed as absorbance at a wavelength of 570 nm (26°C) for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.

produced in saline with and without the supplements ($p < 0.05$). At the same time, the results for the control and yeast extract samples were homogeneous according to Tukey's HSD test. For the inhalation equipment, ANOVA with the Tukey post-hoc test showed significant statistical differences for all samples (control conditions and the culture with glucose and yeast extract) ($p < 0.05$). The results for the control and culture with glucose were homogeneous according to Tukey's HSD test. And for the whirlpool baths, the statistical analysis revealed significant differences ($p < 0.05$), and no homogeneous groups.

DISCUSSION

Biofilms on the surfaces of hydrotherapy equipment can be a source of pathogens (Pantanella *et al.* 2013; Otter *et al.* 2015). Moreover, they can interfere with proper functioning of the equipment and facilities (Little & Lee 2007; Gajewska & Cieniek 2009; Pantanella *et al.* 2013; Otter *et al.* 2015). This study investigates the presence of biofilm forming strains on a range of hydrotherapy equipment and facilities.

Pathogens, such as *Legionella pneumophila* and *Pseudomonas aeruginosa,* were not identified among the isolated microorganisms; however, opportunistic pathogens

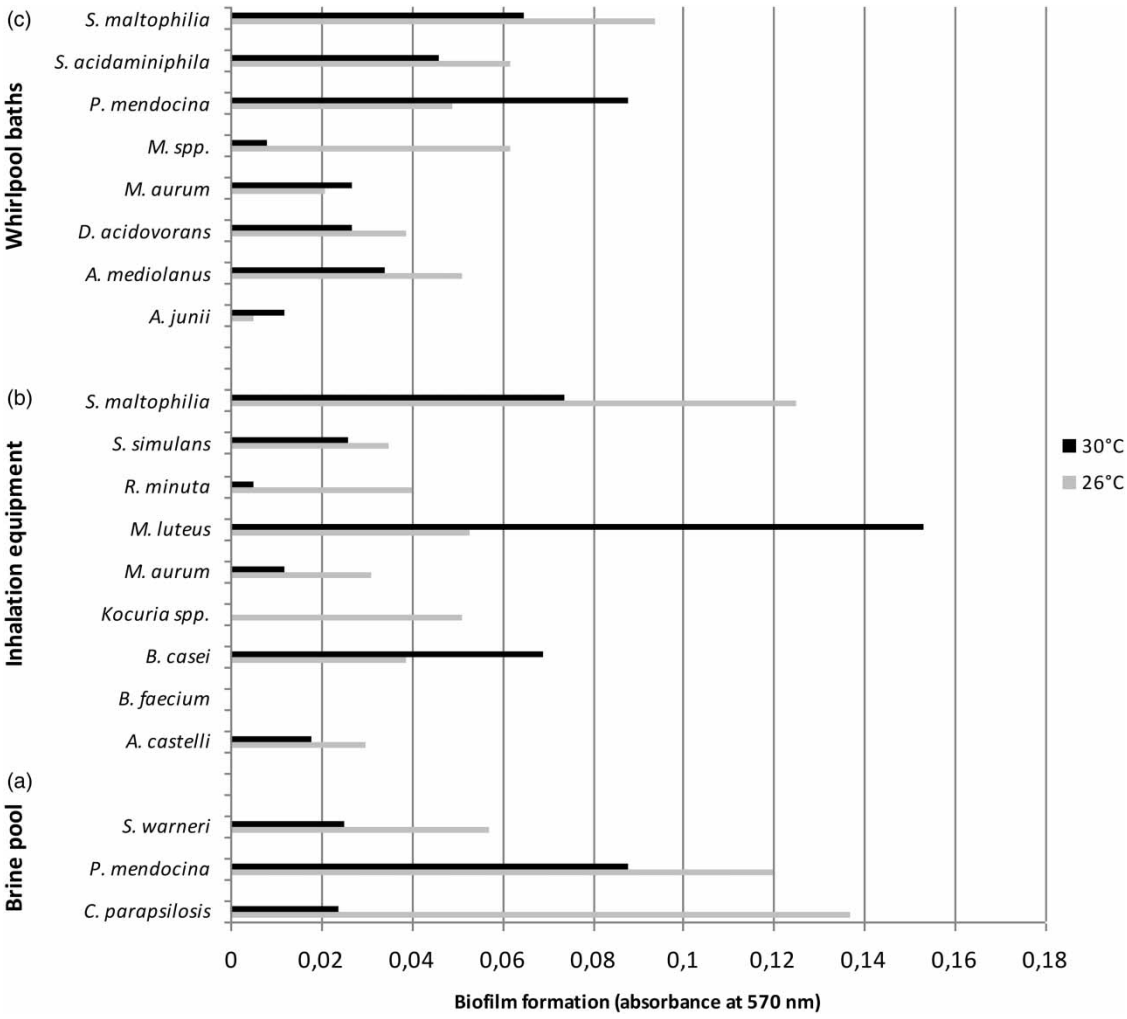

Figure 2 | Biofilm formation on titration plate at 26°C and 30°C for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.

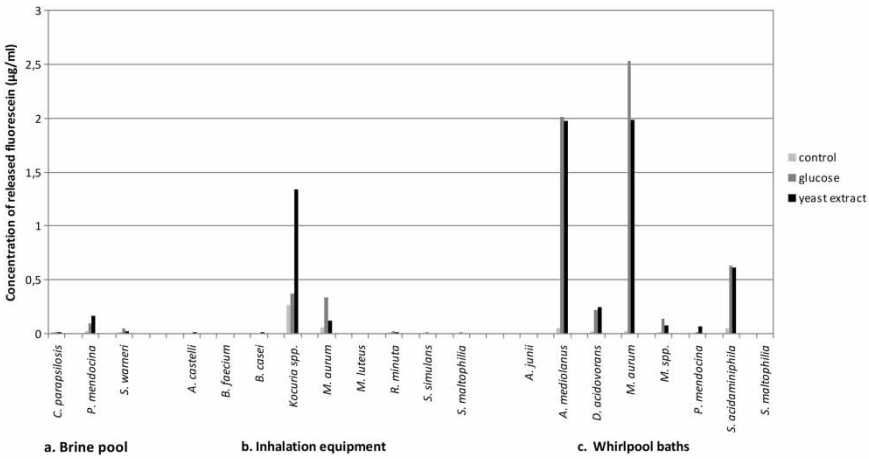

Figure 3 | The hydrolytic activity of biofilm for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.

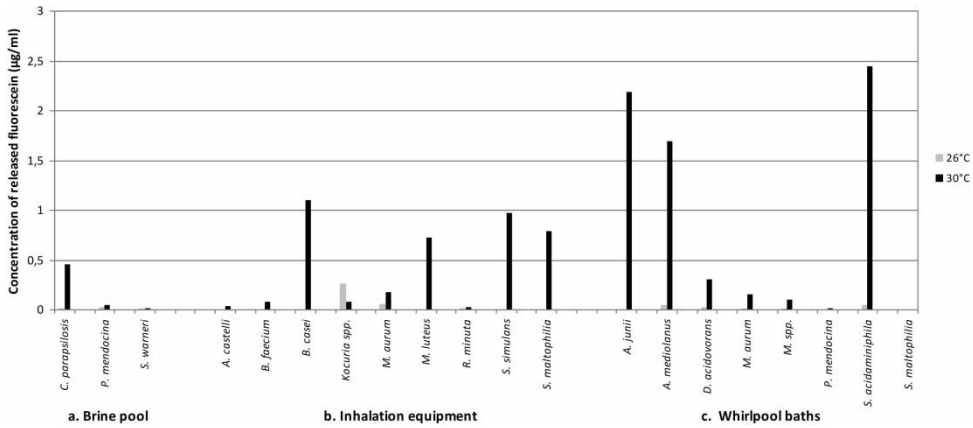

Figure 4 | The hydrolytic activity of biofilm at 26°C and 30°C for (a) brine pool and its infrastructure, (b) inhalation equipment, and (c) whirlpool baths.

were identified that may pose a health hazard to spa patients. The microbiological composition of biofilms formed on the hydrotherapy equipment depends on the maintenance of the equipment, contamination in their vicinity, and the users' health and hygiene standards in the spa (Capelletti & Moraes 2016). The obtained microbiological material was very diverse. Among the isolates were gram-positive and gram-negative bacteria, actinobacteria and yeast. These microorganisms are common not only in aquatic environments, but also in the human biota. The microbial diversity results from a high number of users of hydrotherapy devices. On their surfaces, patients leave microbes, parasites and physiological contaminants (epidermis, hair, sweat, remnants of cosmetics and cleaning products, clothing fibers, and dust) (Pasek 2007). The fact that bacteria indigenous to the human biota were identified among the isolates indicates their adaptability to conditions on the equipment used for spa treatments.

The biofilm formation rate depends on its microbial species composition, physico-chemical parameters, and the amount and type of nutrients (Olańczuk-Neyman *et al.* 2004; Traczewska & Sitarska 2009; van Horn *et al.* 2011; Morimatsu *et al.* 2012). The results of our analysis confirmed this relationship. A higher amount of biofilm was observed in saline enriched with glucose and yeast extract than in pure saline solution. Holà *et al.* (2006) reported that all the tested strains showed a better and richer growth of the biofilm layer at a temperature of 37°C in the nutrient-richer environment. Similarly, Rochex & Lebeault (2007) found that increasing nutrient concentration (from 0.1 to 0.5 g l^{-1} glucose, C/N = 40, C/P = 100) increased the rate and extent of biofilm accumulation. Lim *et al.* (2004) demonstrated that increasing glucose concentration to 2.7% in a given environment leads to an increase in biofilm biomass. This conclusion has been confirmed by other authors (Singh *et al.* 2006; Marić & Vranes 2007). Also Waldrop *et al.* (2014) indicated that biofilm mass was increased at higher glucose concentration. Biofilm mass was increased at higher glucose concentration with a threshold response at 0 to 20 and 160 to 200 mg/dL for *S. epidermidis* and 200 to 240 mg/dL for *S. aureus*. In turn, limitation of nutrients may regulate the onset of biofilm detachment (Holà *et al.* 2006; Morimatsu *et al.* 2012).

Nyenje *et al.* (2013) indicated that temperature is yet another significant factor which influences biofilm formation. The authors demonstrated that *E. cloacae* can adhere to surfaces and form a biofilm at 25°C and at 37°C. Similarly, Zhou *et al.* (2013) observed biofilm formation by *C. werkmanii* at 30°C and at 37°C. Our study confirms this observation. All examined strains except *Brachybacterium faecium* formed biofilms, both at 26°C and 30°C. A small temperature difference did not cause significant differences in the amount of biofilm. Di Bonaventura *et al.* (2007) showed that 32°C was optimal temperature for biofilm formation by *Stenotrophomonas maltophilia,* while Holà *et al.* (2006) found that 37°C is the most favorable for this species. Iversen *et al.* (2004) evaluated microbial adhesion at high temperatures (>40°C) and found that the ability to form a biofilm within this temperature range (26–40°C) is associated with the optimum growth temperature of

different microbial species. At the optimal temperature for growth cell numbers increase rapidly. This facilitates sedimentation, which accelerates the initial adhesion and the formation of microbial biofilm (Marinho *et al.* 2013). Our study was conducted at temperatures similar to those that are selected for spa treatments using the examined hydrotherapy equipment.

Crystal violet staining is widely used for determining the amount of biofilm formed by a wide range of microorganisms (Stepanovic *et al.* 2007); however, the method does not produce consistent results (Peeters *et al.* 2008). Moreover, it is poorly suited for studying the viability of biofilm cells as crystal violet stains both bacterial cells (alive and dead) and the biofilm matrix (Peeters *et al.* 2008). The alternative method using FDA has gained popularity in the evaluation of the viability and the activity of biofilm cells. Simple and efficient, the method guarantees high repeatability of results and is therefore considered highly reliable for the quantification of biofilm (Peeters *et al.* 2008).

CONCLUSIONS

The study confirmed the possibility of biofilm formation on the surfaces of hydrotherapy equipment. The isolated and identified microorganisms were very diverse. No common pathogens were identified among the isolates. All isolates were classified as opportunistic pathogen which can cause infections in humans with weakened immunity systems. All isolates showed the ability to form biofilms in the laboratory conditions. Biofilm formation was observed at both 26°C and 30°C. The introduction of nutrients (to simulate contamination) increased biofilm formation. This indicates that the contamination of natural raw materials (brine) and hydrotherapy equipment enhances biofilm formation and may lead to serious infections. For this reason, it is necessary to regularly monitor equipment, facilities and installations and also water use in spas.

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