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Environmental characteristics and taxonomy of microscopic fungi isolated from washing machines

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ABSTRACT

Washing machines (WMs) are convenient places for fungal colonization. This study is focused on fungal diversity of WMs, and investigates relationships between habits of WM users and colonising species. Housekeeping conditions and habits were assessed in Hungary with a questionnaire. Several fungal species were identified by microscopy and sequence analysis of diagnostic loci. Based on the results, 32 % of the sampled WMs were highly polluted with various species of fungi. Forty six percent of them were colonised also by opportunistically human pathogenic species. In total, 32 yeast and 39 filamentous fungal strains were isolated. Growth tests were conducted with five selected taxa (*Cutaneotrichosporon dermatis*, *Cystobasidium slooffiae*, *Meyerozyma guilliermondii*, *Candida parapsilosis* and the *Fusarium oxysporum* species complex (FOSC)) to ascertain their tolerance ranges. None of the examined isolates were able to grow >50 °C, 4.10 < pH < 10.88. FOSC could grow at high salinity. More species were detected in WMs operated in rooms without heating systems ($p = 0.0025$). The number of species was higher in WMs located in the kitchen than the ones kept in bathroom or in other rooms ($p = 0.0205$). WMs may serve as a reservoir of pathogenic fungi, the presence of which may depend on the usage of these devices.

1. Introduction

Several different microscopic fungal species colonize indoor environments including walls, furniture or household equipment, where they produce spores and microbial volatile organic compounds (MVOCs) including terpenes and terpene-derivates, ketones, alcohols and sulphur compounds (Sahlberg et al., 2013). MVOCs and spores are responsible for the typical mouldy odour causing irritation, respiratory illnesses and cytotoxicity (Ammann, 1999; Wålinder et al., 2005), tiredness, depression and psychosomatic effects (Brewer et al., 2013), allergic reactions, asthma (Kim et al., 2007), as well as infections in immunocompromised patients (De Hoog and Guarro, 1995).

Fungi can colonize visible and hidden places where the microclimatic conditions are favourable for them. Rooms and household equipment connected with water systems provide suitable environments for microorganisms. The organic deposits in these environments are good sources of nutrients for both moulds and yeasts (Gattlen et al., 2010). In spite of the fact that these devices can be found in almost every household, relatively few mycological studies have set a focus on them. Water-connected devices subjected to mycological observations included showerheads (Feazel et al., 2009), drains (Short et al., 2011), toilet bowls (Pitts et al., 1998), dishwashers (Zalar et al., 2011) and washing machines (Terpstra, 1998; Gattlen et al., 2010; Babič et al., 2015).

Most of the pro- and eukaryotic microorganisms are well adapted to environmental changes. Among them, fungi are predominantly successful in the colonization of peripheral environments, particularly species which have low competitive skills but good adaptability thrives. Researchers isolated fungi even from hypersaline environments (*Cladosporium sphaerospermium*) and from ice (*Penicillium crustosum*) (Gostinčar et al., 2010). These fungi have unique adaptive strategies on the molecular level. The washing machines and dishwashers can be considered as extreme

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environments due to the high temperatures and constantly changing pH and humidity levels inside the equipment (Zalar et al., 2011; Babič et al., 2015). These conditions can be favourable or disadvantageous for fungal colonization. Nowadays the excessive use of washing machines, the shift of washing temperatures toward lower values, the high humidity, the usage of different kinds of fabric softeners and liquid detergents, and the component change of these cleaners promote fungal colonization and nutrition (Babič et al., 2015). However, it is not exactly well known, which user habits provide advantages to certain species.

The aim of this study was to investigate the possible relationship between fungal colonisation in washing machines and certain housekeeping conditions or habits of the machine users, as well as to gain more information about the diversity of fungal species occurring in washing machines.

2. Materials and methods

2.1. Study design

A self-designed questionnaire was prepared to estimate the habits of washing machine users, and some related housekeeping conditions of households in Budapest, Hungary. Biological samples were taken from several parts of the devices to assess the diversity of colonizing fungal species. Washing machines of 61 households were sampled during the summer of 2016. The dissemination and collection of the questionnaire, sampling from washing machines, evaluation of the devices based on the extent of pollution and species identification were performed by the authors. The questionnaires were anonymously filled in by participants of the survey.

2.2. Questionnaire

Information about the washing machines, the habits of their users, housekeeping conditions, the occurrence of current and former dark discolorations anywhere in the machines were taken from the questionnaires and analysed in relation to the presence and number of fungal species. Information was collected about 1) the washing machines (brand, type, age, top-loader or front-loader), 2) the location in the house where the washing machine is operated (bathroom, kitchen, cellar, laundry or other), 3) the ventilation of the room (window, fan, functional dehumidifier), 4) the existence (heated or unheated) and type of the heating in the room, 5) the type of the surface material in the room, 6) the habits of washing machine users (frequency of usage, temperature of the washing program, type of the detergent used, allowing the machines to dry after washing), 7) current and previous discolorations detected anywhere in the washing machine and the methods tried for the removal of discolorations, and 8) housekeeping conditions (age and type of the building, type of insulation and ventilation, number of persons living together in the building).

2.3. Evaluation of washing machines

The machines were evaluated by the sampling person based on the extent of discoloration on a three-grade scale (unpolluted, moderately polluted, highly polluted, Fig. 1.)

2.4. Isolation of fungi

To avoid the possible bias deriving from the different sampling methods, all samples were collected by the same author with sterile cotton swabs by rubbing the inside surface of the rubber door seals, as well as the drawers of washing powder and fabric softener. The samples were taken from the most contaminated surface determined by visual inspection. Swabs were transported to the laboratory in sterile tubes and processed within 24 h. Samples were inoculated to malt extract agar medium (MEA; 30 g l^{-1} malt extract, 5 g l^{-1} peptone, 15 g l^{-1} agar) containing 0.1 g l^{-1} chloramphenicol and incubated at 25 °C for five days. Samples were also collected with scalpels from the deposited materials located on the surface of the washing machines. After culturing the samples, pure cultures of fungi were isolated. The composition of deposited materials was studied with a Carl Zeiss Jenaval light microscope at 300× magnification.

2.5. Morphological and molecular characterization

Fungal isolates were identified to the genus level based on their morphological characteristics examined by a Carl Zeiss Jenaval light microscope at 300× magnification. Only non-sporadic fungi, i.e. fungi having at least four CFU/sample were isolated as pure cultures and deposited at the Szeged Microbiological Collection (www.szmc.hu).

Genomic DNA was extracted from the tissues of the filamentous fungal strains cultured on MEA liquid medium by the Omega Bio-tek Fungal DNA Mini Kit according to the manufacturer's instructions. In the case of yeast colonies another protocol was applied. Yeasts were incubated in MEA liquid medium (5 g l^{-1} malt extract, 2.5 g l^{-1} yeast extract, 10 g l^{-1} glucose in distilled water) for 24 h at 37 °C. After centrifugation (Heraeus Fresco 17, Thermo Scientific, USA) of 2 ml culture broth at 6391 g for 5 min, the pellet was resuspended in 500 µl SET lysis buffer (1 % w/v SDS, 50 mM EDTA, 100 mM Tris, pH 8, 7 M ammonium-acetate, pH 7). Eppendorf tubes were filled with glass beads (0.5 mm in diameter) and put into an automatic vortex (Scientific Industries, INC. Bohemia, N.Y., 11716, USA) for three minutes. After adding 275 µl of 7 M ammonium acetate to each tube, they were incubated at 65 °C (VEB MLW Labortechnik Ilmenau incubator, GDR) for 5 min, and then placed on ice for 5 min. Under a fume cupboard, 500 µl chloroform/isoamylalcohol (24:1) was added to each tube and the two phases were separated by centrifugation (16617 g, 10 min). The upper phase was transferred to sterile Eppendorf tubes and overlaid with 500 µl isopropanol. Next, the samples were incubated on ice for 10 min. After centrifugation (16617 g, 10 min) the resulting pellet was washed with 500 µl of ethanol (70 %) and centrifuged again (16617 g, 10 min). The precipitated DNA was dissolved in 100 µl of bidistilled water and RNase enzyme (Viogene RNase A) was added to the samples (37 °C, 30 min). DNA samples were stored at −20 °C before use. The quality of DNA samples was checked by gel electrophoresis (4V/cm^3) in 1 % agarose. PCR reactions were run in an MJ Mini™ Bio-Rad Personal Thermal Cycler. Species identification was performed by amplification of the internal transcribed spacer (ITS1 − 5.8S rDNA − ITS2) region of the ribosomal RNA gene cluster with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) as described by Andersson et al. (2009). For subsets of the isolates a fragment of the translation elongation factor 1α gene was amplified with primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG) and TEF-LLErev (5′-AACTTGCAGGCAATGTGG) according to Hatvani et al. (2007), or a part of the calmodulin gene (*cal1*) was amplified with primers CMD5 (5′-CCGAGTACAAGGARGCCTTC-3′) and CMD6 (5′-CCGATRGAGGTCATRACGTGG-3′) following the protocol described by Hong et al. (2005). Sanger-sequencing of the amplicons was performed on a 3500 Genetic Analyzer (Life Technologies) at BayGen, Szeged. Species identification was performed by nucleotide-nucleotide BLAST analysis (Altschul et al., 1990) at the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov).

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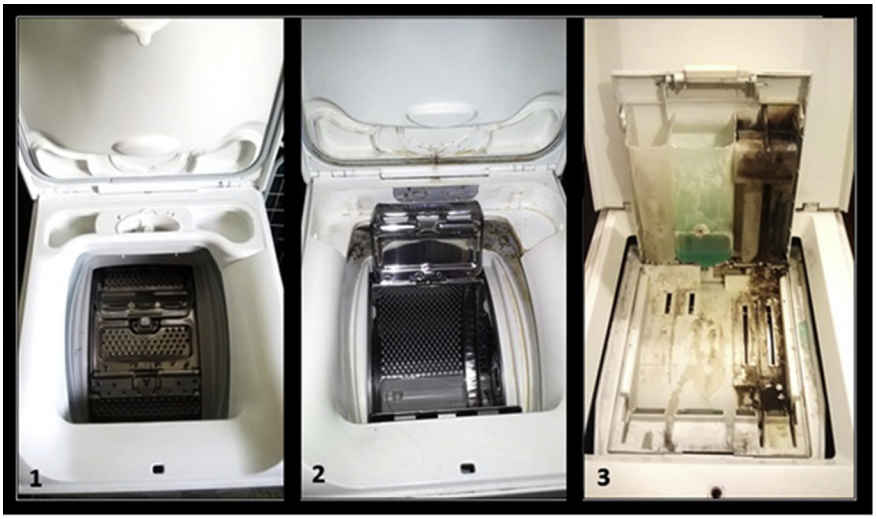

Fig. 1. Unpolluted (1), moderately polluted (2) and highly polluted (3) washing machines.

2.6. Tolerance tests

From the identified species, a filamentous fungal strain belonging to *Fusarium oxysporum* species complex (FOSC) and four yeast isolates (*Candida parapsilosis*, *Meyerozyma guilliermondii*, *Cystobasidium sloffiae* and *Cutaneotrichosporon dermatis*) were selected for testing their growth under different conditions. These species were chosen due to their capability of human pathogenicity and frequency of presence in the samples. The growth of the species was tested independently for temperature-, pH- and halotolerance. The temperature values examined were: 25 °C (control), 37 °C (human core body temperature) and 50 °C (thermophilic fungal preference; Cooney and Emerson, 1964; Crisan, 1964). To simulate the conditions in washing machines, two different temperature profiles were applied: 22 h at 25 °C/2 h at 40 °C (referred to as 40 °C treatment), and 22 h at 25 °C/2 h at 60 °C (referred to as 60 °C treatment). The pH tests were conducted at 25 °C at the pH values of 2.09, 4.1, 7, 8.36, and 10.88 set by Britton-Robinson buffer solutions (Britton and Robinson, 1931). To set up the halotolerance test, sodium-chloride was added to MEA at the concentrations of 0, 30, 60, 90 or 120 g l^{-1}. The tolerance tests lasted for five days, and the cultures were sampled each day. Each of the tests were performed in three replicates.

2.7. Statistical analysis

Due to the complex geometry of the surface of washing machines, the sampling area cannot be determined and quantitative fungal data (i.e. CFU/m^2) cannot be given, consequently presence/absence of taxa were used for statistics. Generalized linear models were used to analyse the relationship between the identified species and the data from the questionnaire. The effect of each variable on the number of species was investigated by Poisson regression. Logistic regression analysis was conducted to examine which factors have impacts on the presence of species. Model selection was performed by using AIC (Akaike Information Criterion). One way ANOVA was used for the analysis of cell numbers and the diameter of the colonies. Dunnett post hoc test was used for the comparison of the treated groups with the control. Bonferroni correction was applied in the case of multiple comparisons. All statistical calculations were performed in R-statistics (www.r-project.org) using the packages multcomp, Rcmdr, RcmdrMisc, MASS and lattice.

3. Results

The deposited materials in the samples contained textile fibers, residuals of detergents, limescale, fungal and bacterial biofilms. Thirty-three percent and 64 % of the sampled washing machines were highly and moderately polluted, respectively. Two of the 61 washing machines had no visible deposition. A total of 71 pure cultures were isolated, 32 belonging to yeasts and 39 to filamentous fungi. After microscopic analysis, 8 genera could be surely separated (*Acremonium*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Mucor*, *Rhizopus*, *Scolecobasidium* and *Trichoderma*). Based on the sequenced DNA samples, 22 different filamentous fungi and 8 different yeast species were identified (Supplement, Table 3.). Most frequently isolated taxa identified by molecular methods are: *Acremonium sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams 1971, *Aspergillus insuetus* (Bainier) Thom and Church 1929, *Aspergillus jensenii* Jurjević, S.W. Peterson & B.W. Horn 2012, *Aspergillus niger* Tiegh. 1867, *Aspergillus* sp., *Candida orthopsilosis* Tavanti, A. Davidson, Gow, M. Maiden and Odds 2005, *C. parapsilosis* (Ashford) Langeron and Talice 1932, *Cladosporium halotolerans* Zalar, de Hoog & Gunde-Cim. 2007, *Cladosporium* sp., *Clavicipitaceae* sp., *CutaneoCutaneotrichosporon dermatis* (Sugita, M. Takash., Nakase & Shinoda) Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout 2015, *Cutaneotrichosporon jirovecii* (Frágner) Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout 2015, *Cystobasidium slooffiae* (E.K. Novák & Vörös-Felkai) Yurkov, Kachalkin, H.M. Daniel, M. Groenew., Libkind, V. de García, Zalar, Gouliam., Boekhout & Begerow 2014, *Exophiala* sp., *F. oxysporum* Schltdl. 1824, *Fusarium solani* (Mart.) Sacc. 1881, *Fusarium fujikuroi* Nirenberg 1976, *Fusarium proliferatum* (Matsush.) Nirenberg 1976, *Fusarium* sp., *Helotiales* sp., *M. guilliermondii* (Wick.) Kurtzman & M. Suzuki 2010, *Mucor spinosus* Schrank 1813, *Penicillium chrysogenum* Thom 1910, *Penicillium citrinum* Thom 1910, *Penicillium terrigenum* Houbraken, Frisvad & Samson 2011, *Penicillium viridicatum* Westling 1911, *Phialemoniopsis curvata* (W. Gams & W.B. Cooke) Perdomo, Dania García, Gené, Cano & Guarro 2013, *Pichia membranifaciens* (E.C. Hansen) E.C. Hansen 1904, *Pleosporales* sp., *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison 1928, *Scolecobasidium* sp., *Sordariomycetes* sp., *Trichoderma orientale* (Samuels & Petrini) Jaklitsch and Samuels 2014.

Apart from fungi, bacteria could also be present in washing machines, especially in biofilms. Despite the use of selective media, in some cases the fungal samples were overgrown by bacteria

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(Table 1.). Although some washing machines were evaluated as moderately or highly polluted based on the dark discolorations seen on their surfaces, no fungi could be isolated from them.

Penicillium, *Cladosporium* and *Rhodotorula* were the most frequent genera in the sampled washing machines. Apart from these several other taxa were present in high frequency (Table 2).

Based on the questionnaire and the collected samples, the brand and the type of the washing machines had no impact on the number of species detected and the presence of certain fungal taxa (Fig. 2). No significant correlation could be identified between the age and type of the building, the storey where the washing machine was placed inside the homes, the number of residents as well as the number of fungal species and the occurrence of certain taxa. Based on the responses, almost half of the buildings were completely insulated. The presence of the genus *Penicillium* showed a significant negative association with the insulation level of the buildings:

Table 1
The frequency of fungal taxa in each sampled washing machines from Hungarian households. The "*" marks samples overgrown by bacteria. ND: no data, F: front-loading, T: top-loading, m: moderately polluted, h: highly polluted, u: unpolluted.

Washing machine	Age	Loading type	Visual inspection	Number of taxa	Frequency of taxa (%)
1	ND	T	m	0	0
2	ND	T	h	1	5.26
3	0–5	F	m	2	10.53
4	11–15	T	m	3	15.79
5	ND	T	h	6	31.58
6	ND	T	h	1	5.26
7	ND	T	h	0	0*
8	ND	T	h	1	5.26
9	6–10	T	m	3	15.79
10	0–5	F	m	1	5.26
11	11–15	F	h	3	15.79
12	0–5	T	m	0	0*
13	16+	T	m	2	10.53
14	6–10	F	m	3	15.79
15	16+	T	h	3	15.79
16	16+	T	m	1	5.26
17	6–10	T	m	2	10.53
18	6–10	T	h	5	26.32
19	0–5	T	m	1	5.26
20	16+	T	h	3	15.79
21	6–10	F	m	4	21.05
22	11–15	T	h	1	5.26
23	16+	T	m	1	5.26
24	11–15	F	h	3	15.79
25	16+	T	m	1	5.26
26	6–10	T	h	0	0*
27	6–10	F	m	3	15.79
28	16+	T	m	2	10.53
29	0–5	T	m	1	5.26
30	0–5	T	m	0	0
31	0–5	F	m	3	15.79
32	0–5	F	m	0	0.00
33	0–5	F	m	4	21.05
34	0–5	T	m	1	5.26
35	ND	T	h	2	10.53
36	6–10	T	m	0	0
37	16+	T	h	4	21.05
38	6–10	T	u	6	31.58
39	6–10	T	h	5	26.32
40	6–10	T	m	0	0
41	0–5	F	m	0	0
42	6–10	T	m	0	0
43	6–10	T	h	4	21.05
44	0–5	F	m	2	10.53
45	6–10	F	h	2	10.53
46	6–10	F	m	3	15.79
47	0–5	F	m	1	5.26
48	16+	T	m	2	10.53
49	0–5	F	m	2	10.53
50	6–10	T	h	2	10.53
51	16+	T	m	3	15.79
52	0–5	F	m	3	15.79
53	0–5	F	m	2	10.53
54	6–10	F	m	0	0
55	0–5	T	h	2	10.53
56	0–5	T	m	0	0
57	0–5	T	h	1	5.26
58	0–5	T	m	2	10.53
59	0–5	F	m	2	10.53
60	0–5	T	m	2	10.53
61	0–5	T	u	0	ND

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Table 2
The prevalence of fungal taxa in the sampled washing machines from Hungarian households.

Taxa	Positive samples (%)
Acremonium spp.	5.1
Aspergillus sect. Nigiri	1.7
Aspergillus sp.1.	1.7
Aspergillus sp.2.	1.7
Aspergillus sp.3.	1.7
Cladosporium sp.1.	1.7
Cladosporium spp.	18.6
Fusarium spp.	13.6
Geotrichum spp.	1.7
hyphomycetes sp.1.	1.7
hyphomycetes sp.2.	6.8
hyphomycetes spp.	33.9
Mucor spp.	5.1
Other yeast spp.	44.1
Penicillium spp.	22.0
Rhizopus spp.	3.4
Rhodotorula spp.	18.6
Scolecobasidium spp.	1.7
Trichoderma spp.	3.4

compared to fully insulated houses, the presence of the genus *Penicillium* was six times higher (OR = 6, CI: 0.9832–49.7003, p = 0.0613) in partially insulated houses and eight times higher in uninsulated houses (OR = 8, CI: 1.5301–62.0262, p = 0.0216). The number of species was higher in washing machines located in the kitchen (avg. 3.33) than those in the bathroom (avg. 1.74) or other rooms (avg. 2.00) (OR = 1.9111, CI: 0.9273–3.5203, p = 0.0544), however, only 6 % of the users placed the washing machine in the kitchen. The number of fungal species was not significantly lower in machines placed in a room with a window, fan or a functional dehumidifier compared to the equipment placed in rooms with no possibility of ventilation.

Thirty percent of the responders keep their washing machines in an unheated room. Significantly higher number of species was detected from washing machines placed in rooms without heating (avg. 2.10) (OR = 2.3906, CI: 1.3700–3.9296, p = 0.0011) than in the ones stored in a heated place (avg. 1.72). The wall surface material was tile in 81 %, concrete in 13 % and dispersion paint in 6 % of the rooms, though any significant associations with the species number and the presence of certain genera could not be identified. In most of the cases the users set up washing programs twice or thrice a week. The washing frequency had no significant effects on the species number and occurrence of the microscopically identified genera.

The most frequently used detergent type was liquid detergent, though most of the responders use more than one type of detergent. Eighty one per cent of the responders use liquid detergent during their washings, but only 53 % use exclusively liquid detergent, furthermore 17 % of the users use only natural cleaners such as washing soda. Seventy six per cent of the users noticed visible contamination on different parts of their machine. Based on the statistical analysis, the species number was significantly higher in those machines where there was dark discoloration in the detergent dispenser ($avg._{dark}$ 2.00, $avg._{no\ dark}$ 1.46) (OR = 1.5798, CI: 1.0512–2.4223, p = 0.0311). In the cases of dark discoloration, 73 % of the users tried to remove it with different kinds of chemicals and mechanical ways. Although the contamination vanished completely or partially in most of the cases, it was found to reappear later in all of the cases. Most of the responders do not apply high temperature disinfectant washing cycles without clothes weekly, and only few of them apply that monthly. Most of the users never or rarely use the 90 °C washing program for washing clothes or disinfect the machine. No significant relationship could be identified between these washing machine user habits and the species number or the presence of most of the taxa. *Penicillium* was significantly less common in washing machines from which the visible contamination had been removed regularly by either mechanical or chemical methods (OR = 0.1384, CI: 0.0226–0.7134, p = 0.0214). Significantly more *Cladosporium* isolates were recovered from front-loader washing machines than from top-loader ones (OR = 3.9039, CI: 1.0031–17.2016, p = 0.0551).

3.1. Thermotolerance

The growth of *C. dermatis* at 37 °C started after three days of incubation (Fig. 3). On the first and second days of incubation, the number of cells was significantly lower in the treated groups than in the control. This fungus was unable to grow at 50 °C. *C. slooffiae* was able to grow at 50 °C, but the number of cells was significantly lower in the treated groups compared with the control. This species was also unable to grow at 50 °C. The growth of the FOSC isolate was slight at 37 °C, while its colonies did not start to grow at 50 °C. Both the 40 °C and 60 °C treatments inhibited the growth of *C. slooffiae*, *M. guilliermondii* and *C. dermatis*. In the case of *C. parapsilosis* and FOSC (Fig. 4.), the 40 °C treatment resulted in a significant growth compared with the control.

3.2. pH tolerance

C. dermatis was unable to grow at pH 2.09, 4.1 and 10.88. The growth of this species at pH 8.36 and the growth of the control (at pH 7) were the same. The growth of *C. slooffiae* and *M. guilliermondii* at different pH values was similar to that of *C. dermatis*. *C. parapsilosis* was unable to grow at pH 2.09, 4.1, 8.36 and 10.88. FOSC was able to grow at pH 8.36 and 10.88. At pH 10.88 the growth of FOSC started three days later than in the control experiment.

3.3. Halotolerance

The growth of *C. dermatis* was delayed at 9 % of salinity, while it was unable to grow at 12 % of salinity. *C. slooffiae* was unable to grow above 9 % of NaCl concentrations. The growth of *C. slooffiae* at 6 % of salinity was already delayed. This group started to grow only on the third day of the experiment. In the treated groups the number of cells was significantly lower compared to the control (at 3 % of salinity: p = 0.00484, at 6–12 % of salinity: p < 0.001). Two fungi, *M. guilliermondii* and *C. parapsilosis* showed some growth in each treatment (3, 6, 9 and 12 %). The number of cells in *M. guilliermondii* was higher but not significantly at 3 % and 6 % of salinity concentrations compared with the control (p = 0.8781 and p = 0.1988, respectively). At 9 % and 12 % of salinity, the growth of each strain was delayed. FOSC was also able to grow at each NaCl concentration, at higher concentrations the fungus started to grow about two days later (Supplement, Tables 4 and 5).

4. Discussion

In some cases – according to the visual observation – washing machines had been categorized as moderately or highly polluted, though no fungi have been culturable. The reason is that these devices were dry inside and there were only unviable fungal elements on the surface of these machines.

The microclimatic conditions of washing machines may depend on several factors, including the habits of users, the type of the building where the washing machine is kept, and its location within the house. Most of the sampled washing machines were polluted with fungi, though it is yet unknown whether they are reservoirs of the Hyphomycetes contaminating the indoor air, thus, this

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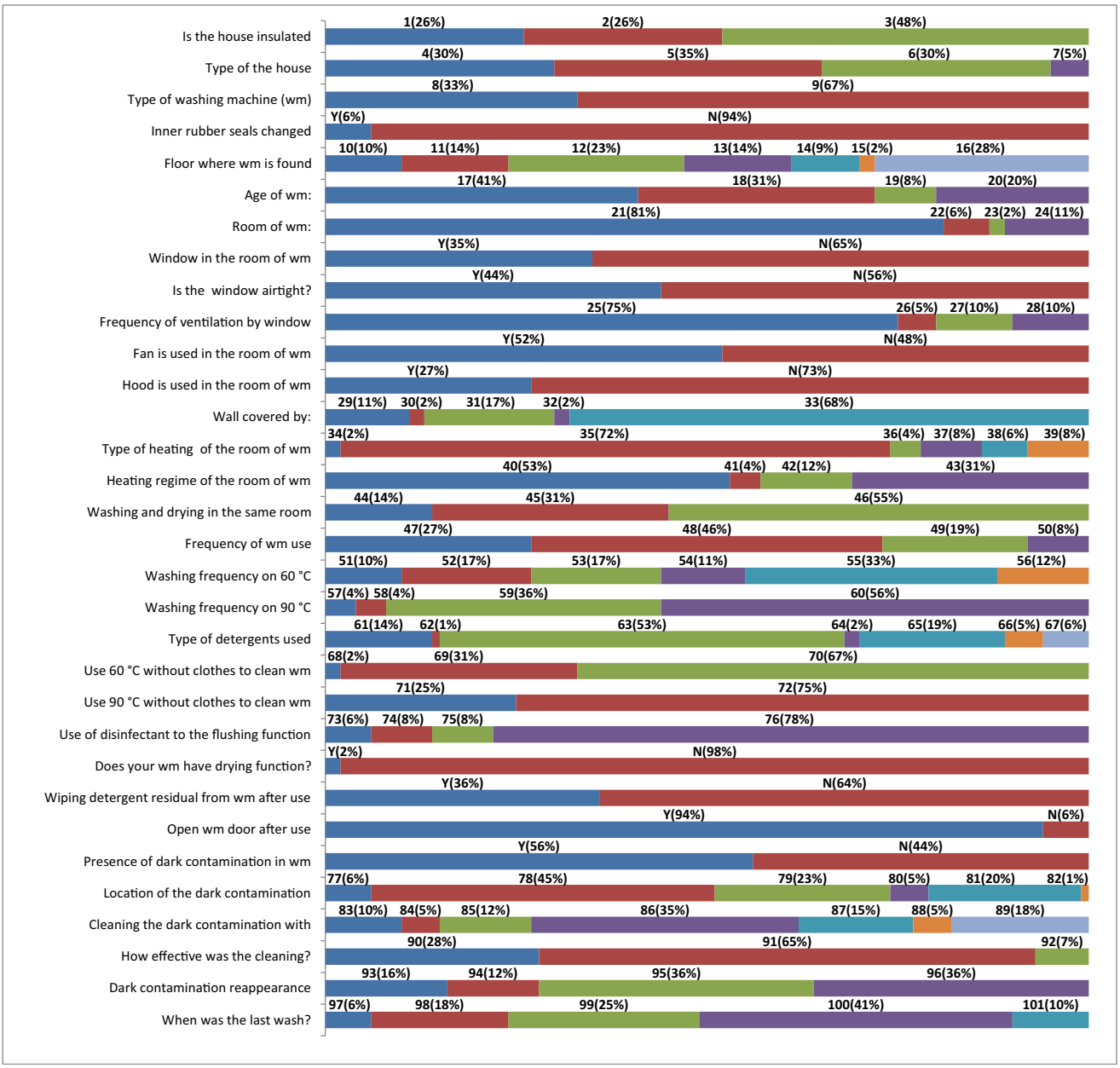

Fig. 2. Results of the questionnaire filled by washing machine users (1: yes; 2: partially; 3: no; 4: family house; 5: block of flats; 6: house panel; 7: no data (ND); 8: front-loader; 9: top-loader; 10: ground floor; 11: first floor; 12: second floor; 13: third floor; 14: fourth floor; 15: fifth floor; 16: ND; 17: 0–5 y; 18: 6–10 y; 19: 11–15 y; 20: 16 + years; 21: bathroom; 22: kitchen; 23: cellar/storeroom; 24: other; 25: many times a day; 26: once a day; 27: many times a week; 28: less often; 29: concrete; 30: whitewash; 31: dispersion/plastic dye; 32: anti-mould dye; 33: tile/ceramics; 34: unheated with cooling outer wall; 35: radiator heating; 36: floor heating; 37: heater; 38: unheated without cooling outer wall; 39: gas convector; 40: continuously; 41: daytime heating, but not in the evening; 42: daytime heating only for 0.5–1 h; 43: no heating; 44: yes; 45: partially; 46: no; 47: daily; 48: 2 or 3 times a week; 49: once a week; 50: biweekly; 51: daily; 52: 2 or 3 times a week; 53: once a week; 54: biweekly; 55: less often; 56: never; 57: once a week; 58: biweekly; 59: less often; 60: never; 61: stain remover; 62: other; 63: liquid detergent; 64: lanoline; 65: washing powder; 66: washing soda; 67: washing nuts; 68: yes, monthly; 69: yes, less often than monthly; 70: no; 71: yes, less often than monthly; 72: no; 73: yes, weekly; 74: yes, monthly; 75: yes, less often than monthly; 76: no; 77: on the cover; 78: in the detergent/fabric softener dispenser; 79: rubber door seals; 80: cauldron; 81: inner rubber seals; 82: other; 83: chlorine containing chemicals; 84: organic cleaning product; 85: other chemicals; 86: scrubbing; 87: wash out on high temperature; 88: other ways; 89: did not try; 90: completely; 91: partially; 92: ineffective; 93: within a few days; 94: 1–2 weeks; 95: 1 m; 96: more than a month; 97: less than 2 h ago; 98: today; 99: a day ago; 100: 2–3 d ago; 101: a week ago).

hypothesis needs to be examined in further studies. Forty six percent of the observed equipments were colonised also by opportunistic human pathogenic species; 68 % of the total isolates can be human pathogen.

Most of the responders participating in our survey preferred low temperature washing programs, similarly to the results deriving from the examination of Slovenian washing machines (Babič et al., 2015). Based on our results, high temperature (60–90 °C) washing programs without clothes can reduce fungal colonization abilities. Generally, washing temperatures of 40 °C and 60 °C inhibit fungal growth.

The probability of fungal colonisation was significantly higher in washing machines kept in the kitchen than in those placed in other rooms. In the kitchen the equipment can be more easily contaminated by airborne spores originating from vegetables, waste, etc. Moreover, in the kitchens more nutrients are available for microorganisms, like starch (Magyar et al., 2017), which is a common component of house dust (Lioy et al., 2002). A study preformed in

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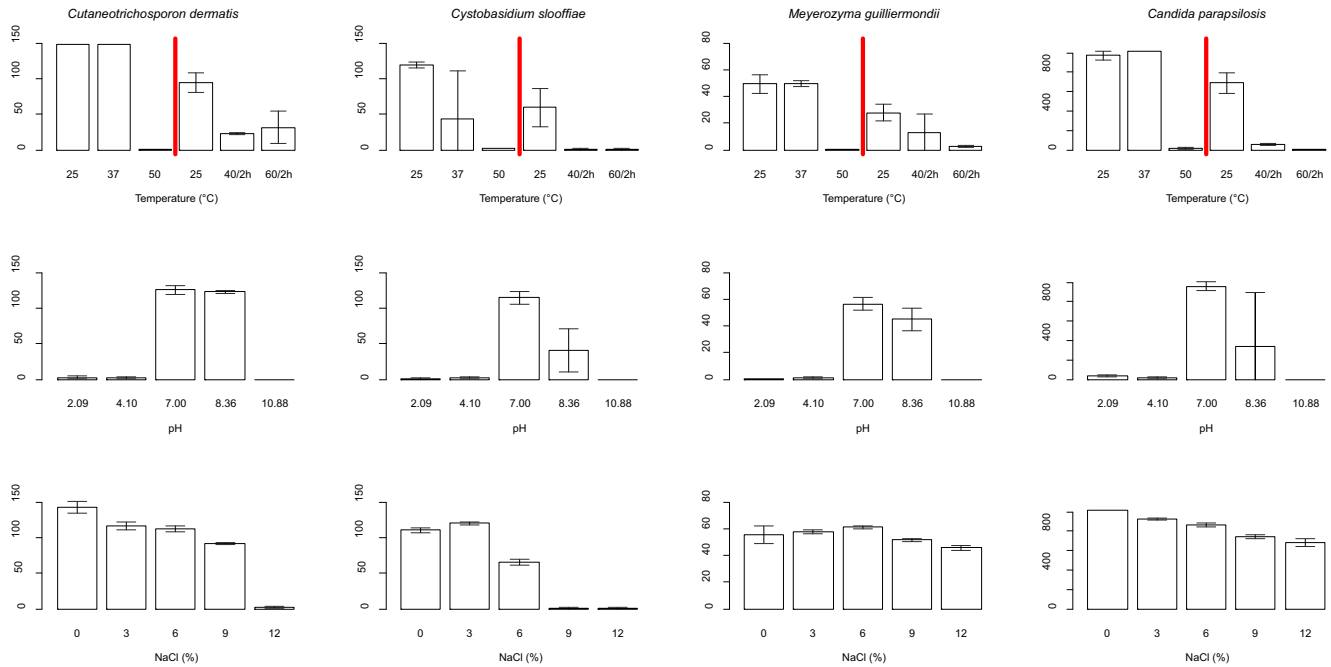

Fig. 3. Results of the tolerance tests (thermo-, pH and halotolerance) with four yeasts.

Hungarian households showed that 90.5 % and 100 % of the air samples collected in bathrooms contained *Cladosporium* and *Penicillium* spp., respectively. On the other hand, 100 % and 80 % of the air samples collected in kitchens contained *Cladosporium* and *Penicillium* spp. (Magyar et al., 2017).

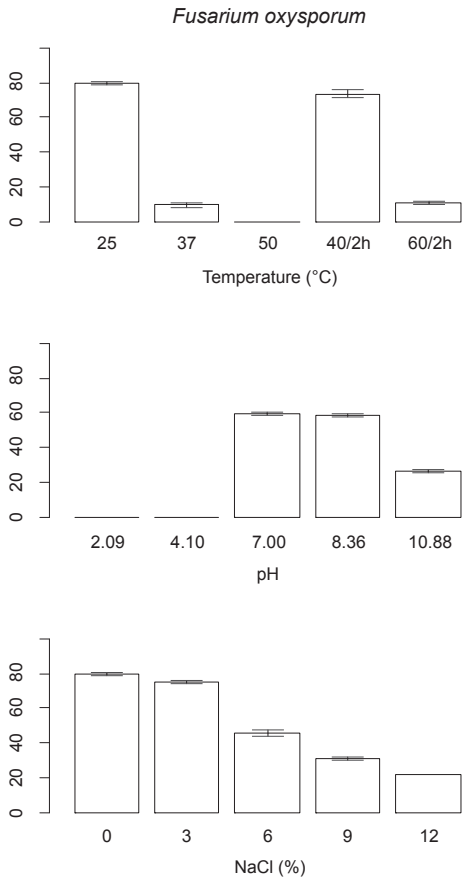

Fig. 4. Results of the tolerance tests (thermo-, pH and halotolerance) with one filamentous fungal strain.

More than one third of our questionnaire's responders wiped the detergent and moisture rests from the surfaces of their washing machines. Reduction of humidity suppresses fungal growth. Wet surfaces can be dried, if the users leave the door of the machine open after washing, which is the custom of 94 % of the users. Two third of the owners daily ventilates the room where the washing machine is located, while less than one third operates a dehumidifier. Apparently, the presence of fungal species inside the washing machines depends on their desiccation tolerance. Certain types of washing machines have surfaces which are difficult to dry and stagnant water remains frequently in their inner part, e.g. in the tubes and under the washing drum, promoting the colonisation of fungal species. In front-loader washing machines the rubber door seals dry slower than in top-loaders due to the stagnant water in the seals. The allergenic *Cladosporium* genus proved to be common in the stagnant water at the bottom of the front-loader washing machines' door. This fungus also appears frequently in the condensation water on windows and heat bridges of the buildings, as it prefers moisture but also tolerates drought. *Cladosporium sphaerospermum* is a xerotolerant fungus with a wide water activity regime ($\geq$0.82), furthermore it is known as a stress tolerant and cosmopolitan species (Segers et al., 2015). Thirty percent of the responders keep their washing machines in unheated rooms, where the species number was higher inside the devices, possibly since their inner surfaces dry slower at lower temperatures.

Different fungi found in household environments can cause several types of human infections such as allergic bronchopulmonary aspergillosis (Agarwal et al., 2013), allergic sinusitis (To et al., 2012), keratitis (Kredics et al., 2015), and different kinds of skin and nail infections (Vos et al., 2012). Building-related illnesses are often caused by indoor moulds and yeasts (Khan and Karuppayil, 2012). These species occupy buildings as alternative habitats and they are well adapted to indoor environments. Moulds and yeasts colonising washing machines may originate from water-conduit, air or clothes. Fungi colonising household equipment mean an increased risk for

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atopic patients, immunocompromised people and patients with cystic fibrosis (De Hoog and Guarro, 1995). Our research revealed that 62 % of the isolated strains are potentially pathogenic to humans. These include the taxa *C. dermatis*, *Exophiala* sp., *Fusarium* spp., *A. niger*, *C. parapsilosis*, *M. guilliermondii* and *R. mucilaginosa*. Twenty-two percent of the samples from the examined washing machines contained one or more of these pathogenic fungi. Babič et al. (2015) isolated 72 fungal strains from residential washing machines in Slovenia and 60 % of them were opportunistic human pathogens. The studied taxa can tolerate alkaline pH values but are sensitive to acidic environment, and their growth is reduced at higher salinity.

The genus *Candida* includes the most common pathogenic yeast species. *M. guilliermondii* is rarely recognized as an invasive pathogen, it is part of the normal microbiota of the skin and mucosa. Infections caused by this species are particularly common in immunocompromised people (Clancy and Calderone, 2012; Tseng et al., 2017). *C. parapsilosis* was one of the most common white yeasts in our samples. It is a really frequent opportunistic human pathogen, which is responsible for e.g. device-associated bloodstream infections (Garzillo et al., 2017) and frequently occurs in hospitals. Levin et al. (1998) isolated this species from catheters and other kinds of laboratory equipment. It often forms biofilms in tubes. *R. mucilaginosa* may also infect via catheters (Neofytos et al., 2007). Black yeasts such as *Exophiala* spp. and *Cadophora* spp. were also isolated from other water-connected places (e.g. plumbing system, air-conditioning system) and tap water (Göttlich et al., 2002; Hageskal et al., 2007, Kärkäinnen et al., 2009). *Cadophora* species are not yet known to cause human or animal infections, but black yeasts from the genus *Exophiala* are known as opportunistic human pathogens able to colonize the lungs of humans suffering from cystic fibrosis (Kennes and Veiga, 2004). This genus prefers high temperatures and humidity, therefore it may colonize saunas, jacuzzis, Turkish steam baths, etc. (Matos et al., 2002). Black yeasts have the ability to recolonize their habitat from resting cells by meristematic growth. The new cells have multi-layered cell walls which help them to cope with abiotic stress (Bell and Wheeler, 1986; Rehnstrom and Free, 1996; Kogej et al., 2007). Fungi having pigmented cell wall are more resistant to lytic enzymes and phagocytosis (Kuo and Alexander, 1967). Yeasts of the genus *Cutaneotrichosporon* are generally occurring in soil, but certain species are also known as members of the normal human skin microbiota. Out of the 38 *Cutaneotrichosporon* species known, 13 are potential pathogens of humans (Chagas-Neto et al., 2008), capable of infecting the gastrointestinal tract and the skin. Trichosporonosis has spread during the past few decades due to the increase in the number of patients with immune deficiency disorders (Ruan et al., 2009). Rodriguez-Tudela et al. (2005) isolated eight different *C. dermatis* strains from skin, nail and blood of 49 Spanish and Argentine patients suffering from trichosporonosis.

Fusarium is a common filamentous fungal genus which includes mainly soil-inhabiting fungi, but also species with phyto-, zoo- and/or human pathogenic potential. Some of them are able to produce mycotoxins such as fumonisins and trichothecenes (Samson, 2010). The most common disease caused by them is keratitis (Thomas, 2003), an eye infection, the development of which depends on climatic conditions, being the most frequent in tropical and subtropical areas. In Hungary only one case has been reported in details (Dóczi et al., 2004). In immunocompromised people, members of the FOSC, the *F. solani* species complex (FSSC), *F. proliferatum* and *Fusarium verticillioides* may cause allergic pulmonary mycosis as well as systemic diseases (Nolting and Fegeler, 1987; De Hoog and Guarro, 1995). *Fusarium* species frequently produce mycotoxins such as fumonisins and trichotecenes. Similarly to fusaria, certain members of the genus *Apergillus* are also able to produce mycotoxins (Samson, 2010). In Hungarian households, black aspergilli are sometimes present (Varga et al., 2014). They relatively rarely colonize walls, but spores were frequently detected in house dust (Magyar et al., 2017). Black aspergilli are also known to cause otomycosis (Szigeti et al., 2012).

Babič et al. (2015) also isolated *C. parapsilosis, Exophiala* sp., FOSC, FSSC, *M. guilliermondii, Ochroconis* sp., *P. crustosum, C. halotolerans* and *R. mucilaginosa* from Slovenian washing machines, with *C. parapsilosis* and FOSC being the most frequent species in their samples. Apart from these taxa, they also isolated other pathogenic fungi such as *Alternaria*, *Exophiala*, *Cryptococcus* and *Aureobasidium* species. The isolated *Fusarium* sp., *Exophiala phaeomuriformis* and *Rhodotorula slooffiae* showed significant growth in culture media containing 1 % of fabric softener. Gattlen et al. (2010) isolated *R. mucilaginosa*, *R. slooffiae* and *Rhodotorula minuta* from biofilms formed in washing machines in the USA, Switzerland, South Korea and Germany.

Fungi which can survive the extreme conditions like those found in washing machines can be considered as extremotolerant species. The five microscopic fungi that were included in the tolerance tests are potentially human pathogenic fungi with the exception of *C. slooffiae*. These species were remarkably frequent in our samples. The species were unable to grow at 50 °C. Fungi studied in the frame of the tolerance tests are non-thermophilic species (Cooney and Emerson, 1964; Crisan, 1964). In order to simulate the real temperatures in washing machines, we applied two-hours-long treatments at 40 and 60 °C per day. These treatments decreased the growth of fungal species except FOSC. This species complex includes tropical strains which are able to grow at 40 °C (Booth, 1971). The 60 °C washing cycle did not have a significant effect on the presence of fungi. This could be due to the scarcity of the usage of such programs; most of the questionnaire respondents use 40 °C washing cycle to save clothes. Thus the sample size for comparing the contamination specific to the cold programs with the warm ones is not sufficient. Stritzke (1970) showed that water temperature of 60 °C (140 °F) is effective to remove *Trichophyton mentagrophytes* from sock fabric in washing machines. Babič et al. (2015) raised that washing temperature below 60 °C, mild detergents and fabric softeners could lead to an increased microbial diversity in washing machines. Household vinegar (pH = 2–3; Bruckner, 1961) is often used to clean the washing machines. Many people dilute vinegar with water, thus its acidity decreases, therefore we included two acidic pH values (2.09, 4.10) in the pH tolerance test performed. The pH values of the detergents range from 7 to 11. Liquid detergents are less basic (~pH 7–8.5), while the washing powder has higher pH values (~10–11). In our experiments there were two alkaline pH treatment groups (8.36, 10.88). The five taxa involved in the tolerance tests were able to tolerate slightly alkaline pH values, but unable to tolerate acidic pH. Acids can therefore be efficient chemicals to suppress microscopic fungi in washing machines.

No research has been conducted to find out if fungi in washing machines could threat human health. Babič et al. (2015) hypothesized that washing machines might act as reservoirs for fungal contaminations. However, their study did not focus at this question, rather it was an assumption made by them. It is not clear whether these machines could act as a source of the indoor contamination. On the other hand it is highly possible that the source of fungal contamination in the washing machines are soiled clothes, tap water (Babič et al., 2017) and indoor air (Magyar et al., 2017).

5. Conclusions

The present study revealed that a high percentage of washing machines are polluted by fungi, drawing attention to hygienic and

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possible health concerns. The isolated species are well adapted to washing conditions. Based on our results and the literature, the fungal contamination of washing machines can be suppressed by appropriate user habits. Since these species tolerate mild alkaline environments more than acidic ones, they could be suppressed by acidic chemicals such as acetic acid. They were unable to grow above 50 °C and at the 60 °C treatment, therefore the owners are strongly advised to regularly perform washing cycles above 60 °C without clothes and use acidic chemicals for cleaning the machine. Dryness is also unfavourable to fungi, so it is worth to keep the door of the washing machine open, as well as to heat and ventilate the room where the washing machine is placed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.05.010.

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