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# ***Candida* and *Fusarium* species known as opportunistic human pathogens from customer-accessible parts of residential washing machines**

Monika Novak BABIČ<sup>a</sup>, Polona ZALAR<sup>a</sup>, Bernard ŽENKO<sup>b,c</sup>,  
Hans-Josef SCHROERS<sup>d</sup>, Sašo DŽEROSKI<sup>b,c,e</sup>, Nina GUNDE-CIMERMAN<sup>a,c,\*</sup>

<sup>a</sup>Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

<sup>b</sup>Jozef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

<sup>c</sup>Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins (CIPKeBiP), Jamova 39, 1000 Ljubljana, Slovenia

<sup>d</sup>Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia

<sup>e</sup>Jozef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia

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## ABSTRACT

Energy constraints have altered consumer practice regarding the use of household washing machines. Washing machines were developed that use lower washing temperatures, smaller amounts of water and biodegradable detergents. These conditions may favour the enrichment of opportunistic human pathogenic fungi. We focused on the isolation of fungi from two user-accessible parts of washing machines that often contain microbial biofilms: drawers for detergents and rubber door seals. Out of 70 residential washing machines sampled in Slovenia, 79% were positive for fungi. In total, 72 strains belonging to 12 genera and 26 species were isolated. Among these, members of the *Fusarium oxysporum* and *Fusarium solani* species complexes, *Candida parapsilosis* and *Exophiala phaeomuriformis* represented 44% of fungi detected. These species are known as opportunistic human pathogens and can cause skin, nail or eye infections also in healthy humans. A machine learning analysis revealed that presence of detergents and softeners followed by washing temperature, represent most critical factors for fungal colonization. Three washing machines with persisting malodour that resulted in bad smelling laundry were analysed for the presence of fungi and bacteria. In these cases, fungi were isolated in low numbers (7.5%), while bacteria *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Sphingomonas* species prevailed.

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\* Corresponding author. Tel.: +386 1 3203400; fax: +386 1 2565782.

E-mail address: [nina.gunde-cimerman@bf.uni-lj.si](mailto:nina.gunde-cimerman@bf.uni-lj.si) (N. Gunde-Cimerman).

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## Introduction

Infections by opportunistic human pathogenic fungi are becoming an increasing health concern all over the world. The number of patients who are at risk of invasive fungal mycoses (invasive aspergillosis, candidaemia, cryptococcal meningitis) is around 12 million (Parkin et al. 2002; Park et al. 2009; Brown et al. 2012). About 4.8 million patients suffer from allergic bronchopulmonary aspergillosis (Denning et al. 2013), 12 million have allergic fungal sinusitis (To et al. 2012), and 6 million have fungal eye infections (Lam et al. 2002). About 1 billion people around the world suffer from skin, nail, and hair infections (Vos et al. 2012). At the same time, fungal infections are increasing as the number of patients suffering from cancer, AIDS, and autoimmune or chronic diseases increase (Anaissie et al. 2001).

Water environments in nature represent reservoirs for a large spectrum of microorganisms. Some of these invade our homes via the tap-water system (Pereira et al. 2010), and these can represent a potential health risk, particularly to immunocompromised people. Recent studies have shown that they can also invade water-connected household appliances, such as washing machines, and dishwashers. Consumer awareness toward a sustainable use of resources and hazardous chemicals, led also to the development of machines operating at lowered washing temperatures and with reduced amounts of water and biodegradable detergents. The selection of these conditions can promote thermotolerant, oxidative-stress resistant, and generally stress-tolerant microbes and may lead to an accumulation of opportunistic human pathogenic species in equipments and possibly also in other indoor-environments (Gostinčar et al. 2009).

The discovery that dishwashers from residential households can be colonized with the polyextremotolerant and opportunistic human pathogenic black yeast *Exophiala dermatitidis* and other potentially pathogenic fungi (Zalar et al. 2011) received considerable public attention. Although it had been known already earlier that washing machines accommodate bacteria and fungi in visible and non-visible biofilms that can often result in malodour of clothes inside washing machines or laundries in healthcare facilities and residential homes. Different species of the bacterial genera *Acinetobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Pseudomonas*, and *Staphylococcus* have been the most frequently isolated (Robinton et al. 1968; Blaser et al. 1984; Smith et al. 1987; Perry et al. 2001; Panagea et al. 2005). In comparison, fungi have been reported less frequently and belonged to genera such as *Alternaria*, *Aspergillus*, *Candida*, *Capronia*, *Cladosporium*, *Cryptococcus*, *Fusarium*, *Penicillium*, *Rhodotorula*, and *Trichosporum* (Munk et al. 2001; Hamada 2002; Gattlen et al. 2010; Kubota et al. 2012; Stapleton et al. 2013).

Further studies have indicated that malodour usually is associated with the bacterial degradation of various substances present in detergents (Munk et al. 2001). As washing machine colonizing microorganisms can also cross-contaminate clothes during washing cycles, they also present a threat for humans as they may cause cutaneous and other infections and may lead to the development of nosocomial infections in hospital environments (Munk et al. 2001; Gattlen et al.

2010). Such nosocomial infections have been reported for the bacteria *Clostridium difficile*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Rozman et al. 2013), and for dermatophytic fungi and yeasts belonging to the genera *Microsporum*, *Trichophyton*, and *Candida* (Shah et al. 1988; Tanaka et al. 2006).

In the present study, we focused on the presence and diversity of fungi that inhabited 70 residential washing machines in Slovenia. With emphasis on potentially pathogenic species that have not been studied to date, we sampled easily accessible plastic drawers for washing powder and softener, and the rubber door seals. These parts are often covered visibly with persistent, even stained microbial biofilms. As they are also frequently manipulated by consumers, they require special attention. Additionally, three of the sampled washing machines producing strong malodour persistently, were sampled to identify involved fungi and bacteria. Isolated fungal strains were tested for their ability to grow at 37 °C, commercial softener and produce esterases and proteases. Eventually, we used a machine learning approach for the identification of the most important factor that supports fungal growth in washing machines.

## Materials and methods

### Washing machines sampled

Seventy three residential washing machines were sampled for fungi, three of these also for bacteria. The mean time of use of machines was 3.5 y. These washing machines were also used from once per week to once per day, at 30 °C–95 °C, and were from different producers and located in different geographical sites in Slovenia. Samples were taken from the washing powder and fabric softener drawers, and from the rubber door seals around the washing machine doors (Table 1).

### Isolation of fungi and bacteria

Sterile cotton swabs pre-moistened in saline solution (0.9 % w/v NaCl) were used for taking samples by rubbing the surface of the drawers and the rubber door seals. These swabs were kept in sterile tubes and were processed either immediately or stored at 4 °C for up to 7 d. Swabs were rubbed over the surface of malt extract agar (MEA) supplemented with 0.05 g L<sup>-1</sup> chloramphenicol, and incubated at 25 °C and 30 °C for up to 7 d. Visibly developing fungi were then transferred to fresh MEA plates. All pure cultured fungal isolates were tested for their ability to grow at 37 °C on MEA.

Bacteria and fungi were isolated from three washing machines selected due to their intense malodour, and from three washing machines without malodour (Tables 2 and 3), using the same sampling technique as described above. Additional samples were taken from the inner parts of the washing machine drum, the water-supply connector, and the wastewater connector. These swabs were rubbed over the surface of R2A culture medium (Conda, Spain) and nutrient agar (NA; Biolife, Italy), and incubated at both 25 °C and 37 °C for up to 7 d.

**Table 1 – Frequency of occurrence of strains, their sampling sites and GenBank accession numbers of recruited DNA barcodes from the 70 residential washing machines from Slovenia.**

Identification	Frequency of isolation	Representative strain – EXF no.	GenBank Accession no.
<b>Washing powder drawer</b>			
<i>Aureobasidium pullulans</i>	1	EXF-6298	KJ481250 (ITS)
<i>Candida parapsilosis</i>	3	EXF-8293	KJ481229 (LSU)
<i>Cladosporium sphaerospermum</i>	2	EXF-8279	KJ500007 (act)
<i>Exophiala lecanii-corni</i>	1	EXF-6140	KJ481253 (ITS)
<i>Exophiala mesophila</i>	1	EXF-6138	KJ481254 (ITS)
<i>Exophiala phaeomuriformis</i> genotype 1	3	EXF-8235	KJ481255 (ITS)
<i>Fusarium oxysporum</i> species complex (FOSC)	9	EXF-5661	KJ481241 (tef)
<i>Fusarium proliferatum</i>	1	EXF-5664	KJ481246 (tef)
<i>Fusarium solani</i> species complex (FSSC)	2	EXF-5665	KJ481247 (tef)
<i>Fusarium verticillioides</i>	2	EXF-5553	KJ481244 (tef)
<i>Meyerozyma guilliermondii</i>	1	EXF-8240	KJ481231 (LSU)
<i>Mucor circinelloides</i>	1	EXF-6296	KJ481257 (ITS)
<i>Ochroconis</i> sp.	1	EXF-5565	KJ481236 (act)
<i>Penicillium crustosum</i>	2	EXF-8272	KJ481238 (benA)
<i>Phoma radicina</i>	1	EXF-6297	KJ481248 (ITS)
<i>Phoma fimeti</i>	1	EXF-5551	KJ481249 (ITS)
<b>Fabric softener drawer</b>			
<i>Aureobasidium melanogenum</i>	1	EXF-8259	KJ481251 (ITS)
<i>Candida parapsilosis</i>	9	EXF-8289	KJ481227 (LSU)
<i>Cladosporium pseudocladosporioides</i>	1	EXF-5563	KJ500008 (act)
<i>Cladosporium halotolerans</i>	1	EXF-5564	KJ500009 (act)
<i>Exophiala equina</i>	1	EXF-5566	KJ481252 (ITS)
<i>Fusarium oxysporum</i> species complex (FOSC)	7	EXF-8264	KJ481243 (tef)
<i>Mucor racemosus</i>	1	EXF-5556	KJ481258 (ITS)
<i>Penicillium brevicompactum</i>	1	EXF-5558	KJ481240 (benA)
<i>Penicillium crustosum</i>	3	EXF-8276	KJ481239 (benA)
<i>Rhodotorula slooffiae</i>	1	EXF-5557	KJ481234 (LSU)
<b>Rubber door seal</b>			
<i>Candida parapsilosis</i>	2	EXF-8290	KJ481228 (LSU)
<i>Cladosporium bruhnei</i>	1	EXF-5660	KJ500006 (act)
<i>Cryptococcus diffluens</i>	1	EXF-6329	KJ481230 (LSU)
<i>Exophiala phaeomuriformis</i> genotype 1	1	EXF-6326	KJ481256 (ITS)
<i>Fusarium oxysporum</i> species complex (FOSC)	2	EXF-6333	KJ481242 (tef)
<i>Fusarium proliferatum</i>	1	EXF-6330	KJ481245 (tef)
<i>Meyerozyma guilliermondii</i>	1	EXF-6331	KJ481232 (LSU)
<i>Rhodotorula mucilaginosa</i>	3	EXF-6325	KJ481233 (LSU)
<i>Rhodotorula slooffiae</i>	1	EXF-6328	KJ481235 (LSU)
<i>Ochroconis</i> sp.	1	EXF-6327	KJ481237 (act)

EXF, strain accession number in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia); act, partial sequence of the gene encoding for actin; benA, partial sequence of the gene encoding beta tubulin; ITS, internal transcribed spacer region of ribosomal DNA; LSU, large subunit of ribosomal DNA; tef, translation elongation factor 1 alpha partial sequence.

For the six washing machines selected for the malodour comparison, samples of waste water (50 mL) and incoming tap water (500 mL) were also collected. For the isolation of fungi, the water samples were filtered through 0.45- $\mu$ m membrane filters (Merck, Millipore). The filters were then placed on dichloran rose bengal chloramphenicol agar (DRBC; Oxoid, England), and MEA (Biolife, Italy) with addition of chloramphenicol. The culture media were incubated at both 25 °C and 30 °C for up to 7 d. For determination of the bacteria in the incoming and waste water, 10 mL of each were filtered through 0.22- $\mu$ m membrane filters (Merck, Millipore). These filters were placed on NA and R2A culture media and incubated at both 25 °C and 37 °C for up to 7 d. All of the pure microbial cultures obtained are deposited in the Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

#### DNA extraction

DNA was extracted from freshly growing yeast and yeast-like colonies on MEA, and from bacterial colonies on NA, using PrepMan Ultra reagent (Applied Biosystems) according to the manufacturer instructions. DNA from filamentous fungi was extracted after mechanical lysis of 1 cm<sup>2</sup> of mycelium using the protocol of [Gerrits van den Ende & de Hoog \(1999\)](#).

#### Molecular characterization and identification of strains

Filamentous fungi were first identified on the basis of morphological characters that allowed the recognition of genera. Molecular barcodes were then selected for further characterizations and for species typing. For all filamentous fungi and for genotyping *Exophiala* strains, the internal

**Table 2 – Bacteria and fungi isolated from three washing machines characterized with intense malodour.**

Bacteria	Frequency of isolation	Representative strain– EXB no.	Fungi	Frequency of isolation	Representative strain – EXF no.
<b>Washing powder drawer</b>					
<i>Agrobacterium tumefaciens</i>	1	EXB L-612	<i>Sporobolomyces ruberrimus</i>	1	EXF-8989
<i>Pseudomonas putida</i>	1	EXB L-602			
<i>Roseomonas genomospecies</i>	1	EXB L-618			
<i>Sphingobium yanoikuyae</i>	1	EXB L-1236			
<i>Stenotrophomonas maltophilia</i>	1	EXB L-601			
<b>Fabric softener drawer</b>					
<i>Bacillus horneckiae</i>	1	EXB L-598	<i>Sistotrema brinkmannii</i>	1	EXF-8992
<i>Microbacterium</i> sp.	1	EXB L-599			
<i>Micrococcus luteus</i>	3	EXB L-610			
<i>Micrococcus yunnanensis</i>	2	EXB L-617			
<i>Ochrobactrum anthropi</i>	1	EXB L-600			
<b>Drum interior</b>					
<i>Brevibacterium casei</i>	1	EXB L-604	<i>Penicillium chrysogenum</i>	1	EXF-8995
<i>Micrococcus luteus</i>	3	EXB L-619			
<i>Micrococcus yunnanensis</i>	1	EXB L-603			
<i>Paracoccus marcusii</i>	1	EXB L-1232			
<b>RUBBER DOOR SEAL</b>					
<i>Achromobacter xylosoxidans</i>	1	EXB L-613	<i>Penicillium sanquifluum</i>	1	EXF-8994
<i>Brevundimonas diminuta</i>	1	EXB L-605			
<i>Kocuria rhizophila</i>	1	EXB L-622			
<i>Micrococcus luteus</i>	2	EXB L-623			
<i>Pseudomonas aeruginosa</i>	2	EXB L-1228			
<b>Interior of water supply connector tube</b>					
<i>Blastomonas natoria</i>	1	EXB L-1238	<i>Phialophora europaea</i>	1	EXF-8990
<i>Chryseobacterium daecheongense</i>	1	EXB L-625			
<i>Methyloversatilis</i> sp.	1	EXB L-1234			
<i>Sphingomonas koreensis</i>	1	EXB L-606			
<i>Sphingomonas</i> sp.	1	EXB L-1233			
<i>Sphingopyxis chilensis</i>	1	EXB L-1237			
<b>Interior of waste water connector tube</b>					
<i>Massilia timonae</i>	1	EXB L-629	<i>Penicillium chrysogenum</i>	1	EXF-8996
<i>Micrococcus luteus</i>	3	EXB L-626			
<i>Pseudomonas aeruginosa</i>	1	EXB L-1230			
<i>Pseudomonas nitroreducens</i>	1	EXB L-607			
<b>Water from water supply system</b>					
<i>Acinetobacter</i> sp.	1	EXB L-614	<i>Alternaria alternata</i>	1	EXF-8991
<i>Micrococcus</i> sp.	1	EXB L-608	<i>Cladosporium cladosporioides</i>	2	EXF-8997
<i>Sphingomonas yabuuchiae</i>	1	EXB L-1239	<i>Cladosporium pseudocladosporioides</i>	1	EXF-8998
<i>Sphingomonas</i> sp.	1	EXB L-1235	<i>Neosartorya fischeri</i>	1	EXF-8993
			<i>Penicillium chrysogenum</i>	1	EXF-9011
			<i>Sporobolomyces ruberrimus</i>	1	EXF-9005
<b>Waste water from washing machines</b>					
<i>Klebsiella variicola</i>	1	EXB L-1241	<i>Penicillium chrysogenum</i>	1	EXF-9012
<i>Pseudomonas aeruginosa</i>	1	EXB L-1231	<i>Simplicillium chinense</i>	1	EXF-9013
<i>Pseudomonas putida</i>	1	EXB L-609			
<i>Shewanella putrefaciens</i>	1	EXB L-615			

EXB, accession number for Bacteria and EXF- for Fungi in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

transcribed spacer regions 1 and 2 and the 5.8S rDNA was amplified and sequenced using primers ITS5 and ITS4 (White et al. 1990). For *Penicillium* strains, partial beta tubulin gene exons and introns (*benA*) were amplified and sequenced with primers Bt2a and Bt2b (Glass & Donaldson 1995); for *Cladosporium* strains the partial actin gene (*act*) with primers ACT-512F and ACT-783R (Carbone & Cohn 1999); for *Fusarium* strains the nuclear translation elongation factor 1-alpha (*tef*) with primers EF1 and EF2 (O'Donnell et al. 1998). Yeast were

identified based on their large subunit ribosomal DNA (LSU) sequence (partial 28S rDNA, D1/D2 domains), which were amplified and sequenced with primers NL1 and NL4 (Boekhout & Kurtzman 1996). BigDye terminator cycle sequencing kits were used in the sequence reactions (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were obtained with an ABI Prism 3700 Big Dye Sequencer (Applied Biosystems) at Microsynth AG, Switzerland. The sequences were assembled with the software FinchTV 1.4 (Geospiza, PerkinElmer, Inc.) and

**Table 3 – Bacteria and fungi isolated from three washing machines not associated with malodour.**

Bacteria	Frequency of isolation	Representative strain– EXB no.	Fungi	Frequency of isolation	Representative strain – EXF no.
<b>Washing powder drawer</b>					
<i>Halomonas hamiltonii</i>	2	EXB L-1347	<i>Fusarium oxysporum</i>	1	EXF-9794
<i>Micrococcus luteus</i>	1	EXB L-1340			
<b>Fabric softener drawer</b>					
<i>Brevibacterium casei</i>	1	EXB L-1355	<i>Candida parapsilosis</i>	2	EXF-9781
<i>Micrococcus luteus</i>	1	EXB L-1339	<i>Meyerozyma guilliermondii</i>	1	EXF-9786
<b>Drum interior</b>					
<i>Bacillus amyloliquefaciens</i>	1	EXB L-1349	<i>Cladosporium cladosporioides</i>	1	EXF-9795
<i>Bacillus pumilus</i>	1	EXB L-1350	<i>Cladosporium langeronii</i>	1	EXF-9796
<i>Micrococcus luteus</i>	3	EXB L-1357	<i>Penicillium viridicatum</i>	1	EXF-9797
<b>Rubber door seal</b>					
<i>Acinetobacter</i> sp.	1	EXB L-1358	<i>Aspergillus fumigatus</i>	1	EXF-9799
<i>Bacillus</i> sp.	1	EXB L-1343	<i>Penicillium bialowiezense</i>	1	EXF-9800
<i>Bacillus subtilis</i>	1	EXB L-1354	<i>Penicillium expansum</i>	1	EXF-9798
<i>Micrococcus luteus</i>	1	EXB L-1342	<i>Penicillium glabrum</i>	1	EXF-9792
<i>Pseudomonas pseudoalcaligenes</i>	1	EXB L-1353			
<b>Interior of water supply connector tube</b>					
<i>Blastomonas natatoria</i>	1	EXB L-1344	<i>Aureobasidium pullulans</i>	1	EXF-9785
<i>Brevundimonas aurantiaca</i>	1	EXB L-1359			
<i>Sphingobacterium spiritivorum</i>	1	EXB L-1360			
<b>Interior of waste water connector tube</b>					
<i>Acinetobacter</i> sp.	1	EXB L-1361	<i>Debaryomyces hansenii</i>	1	EXF-9780
<i>Pseudoxanthomonas</i> sp.	1	EXB L-1345	<i>Exophiala phaeomuriformis</i>	1	EXF-9788
			<i>Ochroconis constricta</i>	1	EXF-9793
<b>Water from water supply system</b>					
<i>Micrococcus luteus</i>	1	EXB L-1351	<i>Aspergillus versicolor</i>	1	EXF-8692
<i>Pseudomonas pseudoalcaligenes</i>	1	EXB L-1352	<i>Aureobasidium melanogenum</i>	1	EXF-8428
			<i>Trichoderma citrinoviride</i>	1	EXF-6299
<b>Waste water from washing machines</b>					
<i>Pseudomonas aeruginosa</i>	1	EXB L-1229	<i>Penicillium oxalicum</i>	1	EXF-8693

EXB, accession number for Bacteria and EXF- for Fungi in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

automatically aligned. The alignments were manually adjusted using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al. 2011). Taxa were identified through BLAST searches (Altschul et al. 1990) by recruiting the sequence database at <http://www.ncbi.nlm.nih.gov/> in June 2014.

### Machine-learning analysis

For the investigation of differences between samples in terms of absence or presence of different fungal genera, machine-learning methods were used. The J48 algorithm for the induction of decision trees in the WEKA data-mining package (Witten et al. 2011) was used, which is a reimplementation of the well-known C4.5 algorithm (Quinlan 1993). Default parameter settings for J48 were applied, but reduced error pruning of the tree was used instead of the standard C4.5 pruning. The dependent (class) variable in our analysis was the absence or presence of fungi: 'no' if no fungi were present, and 'yes' if any fungus was present in the sample. The independent variables (attributes) were age of washing machine (years), frequency of washing machine use (times per week), use of detergents (yes/no), use of fabric softeners (yes/no), temperature programmes used for washing (one binary attribute for 30 °C, 40 °C, 60 °C and 90 °C as yes/no and two numeric

attributes for minimum and maximum temperatures) and temperature of isolation of fungi (one binary attribute for 25 °C, one for 30 °C as yes/no and one for the actual temperature value).

### Growth of fungal strains from washing machines on selected softener

Solid culture media containing a selected commercial fabric softener were prepared with distilled water, with the fabric softener as the only source of nutrients. The fabric softener was diluted to concentrations of 50 %, 25 %, 10 %, 5 %, and 1 % (v/v). Medium prepared only with distilled water and agar was used as a negative control. The diameters of fungi on media with different concentrations of softener were measured after 3 weeks of incubation at 25 °C and 30 °C and compared to negative controls. When the diameter on media with softener was larger than in the negative controls, the growth was assigned as positive. As a replacement for softener, consumers often use acetic acid, and thus, fungal growth was also tested on liquid yeast nitrogen base medium supplemented with 1 % (v/v) acetic acid. Fungal growth observed on MEA was used for comparisons. The media were inoculated with 10 µL fungal suspensions, prepared with saline and incubated at 25 °C and 37 °C for 2 weeks.

## Production of different extracellular enzymes

For testing whether the fungal strains can break down the substrates containing fatty acids and proteins, one representative isolate of each species from the washing machines was tested for the production of extracellular proteases and esterases. Proteolytic activity was tested using agar supplemented with milk, while for testing of the esterase activity, agar with the addition of Tween 80 was used (Paterson & Bridge 1994).

## Results

### Different communities of fungi known also as opportunistic human pathogens inhabiting drawers for washing powder, fabric softener, and rubber door seals

No fungi were isolated in 21 % ( $n = 15$ ) of the 70 sampled washing machines. The mean age of these washing machines was 6 y and the temperature most often used for washing within this group was 60 °C. In the 79 % of washing machines that were positive for fungi, the most often used temperature for washing was 40 °C. Drawers for fabric softeners were fungus positive in 80.6 % cases; drawers for powder in 74.4 % and rubber door seals in 52.6 %. This sampling resulted in a total of 72 fungal isolates (Table 1), of which 43 (60 %) had earlier been reported as an opportunistic human pathogen (Table 4). The most frequently isolated fungi were members of the *Fusarium oxysporum* species complex (FOSC; 19 strains) and *Candida parapsilosis* (14 strains), followed by different species of black yeasts from genus *Exophiala* (7 strains).

Most often, there were three different types of fungal communities for each single sampling site. Filamentous fungi prevailed over yeasts in drawers for washing powder. This filamentous fungal community was composed of members of the FOSC and *Fusarium solani* species complex (FSSC) that occurred together with *Cladosporium sphaerospermum*, *Exophiala phaeomuriformis* genotype 1, *Exophiala mesophila*, *Exophiala lecanii-corni*, *Meyerozyma guilliermondii*, *Candida parapsilosis*, and *Penicillium crustosum*. The most frequently isolated species were from the FOSC, followed by *Exophiala* species and *C. parapsilosis* (Figs 1 and 2). The second most common community was observed most frequently in the drawers for fabric softener, and it was represented by a predominance of *C. parapsilosis*, followed by members of the FOSC and *P. crustosum*. The third most common community was dominated by yeasts, and was mainly observed on the washing machine rubber door seals. This was represented by the red yeast *Rhodotorula mucilaginosa*, the white yeast *C. parapsilosis*, the black yeast-like *E. phaeomuriformis*, and *Ochroconis* species. In this community *R. mucilaginosa* and *C. parapsilosis* prevailed.

### Increased occurrence of fungi in washing machines primarily correlates with use of fabric softener

The occurrence of fungi in drawers for washing powder and softener and on rubber door seals were statistically analysed with the machine-learning model (Fig 3). Correlations of

fungal diversity and key variables such as the age and frequency of use of washing machines, the temperatures used in washing cycles, and the use of detergents were tested. The accuracy of the model when used for predicting the presence or absence of fungi was 82.5 % when evaluated on the training data and 73.2 % when evaluated with the 10-fold stratified cross-validation procedure. The 10-fold stratified cross-validation gives a more realistic estimate if the model is used for predictions of unknown samples. The decision tree suggests that the type of washing machine, its time of use, and its frequency of use are not important parameters for the presence of fungi (i.e., they do not appear in the tree). The key variables that most influenced the presence of fungi at these sampled sites were the use of fabric softener, the washing temperature, and the temperature used for the isolation of the fungi. When both detergents were used (washing powder and fabric softener; first leaf in the decision tree in Fig 3), the diversity of fungi was higher than in cases where one or both of these detergent types were not used. In four cases when neither of these detergent types was used, no fungi were isolated from the chosen sample sites. The temperature of cultivation also appeared to be important - incubation at 25 °C resulted mainly in the isolation of filamentous fungi, while at 30 °C, filamentous fungi and white and black yeasts were isolated.

### Characterization of the fungal isolates for selected virulence factors

The isolated fungal strains were characterized in terms of their growth at 25 °C and 37 °C, their proteolytic and esterase activities, and their use of softener as sole source of carbon (Table 5). When examined for their thermotolerance, all fungi grew well at 25 °C on MEA because they formed solid colonies within 2 weeks incubation. As the ability to grow at 37 °C is an important factor for fungal pathogenesis in human (Anaissie et al. 2001), the isolated fungi were also tested for their ability to grow at this temperature. *Aureobasidium pullulans*, *Mucor racemosus*, *Ochroconis* spp. and the species of *Cladosporium*, *Penicillium*, and *Phoma* did not grow at 37 °C, while all other isolated taxa grew well at 37 °C (Table 5). Another important fungal virulent factor is the production of esterases and proteases (Ishida et al. 2012). All of the tested fungi showed esterase activities at 25 °C, while *A. melanogenum*, *E. phaeomuriformis*, FOSC and FSSC members, *Fusarium proliferatum*, *Fusarium verticillioides*, *M. guilliermondii* produced esterases at 37 °C. The tested strains of *A. melanogenum*, *Cladosporium bruhnei*, *C. sphaerospermum*, *F. solani*, *F. verticillioides*, *Mucor circinelloides*, *P. crustosum*, and *Penicillium brevicompactum* produced proteases at 25 °C. At 37 °C, protease activity was measured only for *A. melanogenum*, *F. solani*, *F. verticillioides*, and *M. circinelloides*.

All tested strains, listed in Table 5, except *Phoma fimeti* grew well on the medium with 1 % fabric softener (Fig 4), while *F. verticillioides* and *P. crustosum* even grew on the medium containing 5 % fabric softener. No growth was observed at higher concentrations of fabric softener. The growth of 26 of the isolated strains was tested on medium with 1 % acetic acid but none of the tested fungi developed colonies.

**Table 4 – Fungal species isolated in this and other studies from different parts of washing machines, along with their natural habitats and opportunistic pathogenic potential.**

Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
<i>Alternaria</i> sp.	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, toxin production	Air, water	<a href="#">Gattlen et al. 2010.</a>
<i>Aspergillus ochraceus</i>	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, pulmonary infections, antrromycosis, toxin production	Air, water	<a href="#">Gattlen et al. 2010.</a>
<i>Aspergillus versicolor</i>	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, pulmonary infections, toxin production	Air, water	<a href="#">Gattlen et al. 2010.</a>
<i>Aureobasidium pullulans</i> var. <i>pullulans</i>	Drawer for washing powder	Air, water, soil, limestone, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	This study
<i>Aureobasidium pullulans</i> var. <i>melanogenum</i>	Drawer for fabric softener	Air, watery habitats, soil, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	This study
<i>Aureobasidium</i> sp.	Different parts	Air, water, soil, limestone, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	Hamada 2005
<i>Candida albicans</i>	Laundered towels	Soil, water, human skin	2	Invasive candidiasis, catheter infections, urinary tract infections, vulvovaginitis, endocarditis, peritonitis, joint infections, meningitis	Water, hands	<a href="#">Blaser et al. 1984.</a>
<i>Candida parapsilosis</i>	Drawer for washing powder and softener, rubber	Soil, water, marine environment, plants, insects, human skin	1	Invasive candidiasis, catheter infections, urinary tract infections, vulvovaginitis, endocarditis, peritonitis, joint infections, meningitis	Water, skin, insects	This study
<i>Candida</i> sp.	Different parts, laundered towels, lab coats, clothes	Soil, water, marine environment, plants, insects, human skin	1	Candidiasis, catheter infections, urinary tract infections, vulvovaginitis, meningitis	Water, skin, insects	<a href="#">Stapleton et al. 2013,</a> <a href="#">Neely &amp; Orloff 2001</a>
<i>Capronia coronata</i>	Metal parts	Water, wood, plants	1	Unknown	Water	<a href="#">Gattlen et al. 2010.</a>
<i>Cladosporium bruhnei</i>	rubber	Air, water, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study
<i>Cladosporium halotolerans</i>	Drawer for fabric softener	Air, water, bathrooms, salterns	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study
<i>Cladosporium cladosporioides</i>	Different parts	Air, water, soil, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	Hamada 2005
<i>Cladosporium pseudocladosporioides</i>	Drawer for fabric softener	Air, water, soil	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study

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Table 4 – (continued)						
Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
<i>Cladosporium sphaerospermum</i>	Drawer for powder, rubber	Air, water, bathrooms, salterns	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study, <a href="#">Gattlen et al. 2010.</a>
<i>Cladosporium</i> sp.	Plastic parts, water from WM	Air, water, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	<a href="#">Gattlen et al. 2010, Hamada 2002.</a>
<i>Cryptococcus diffluens</i>	rubber	Air, flowers, trees, sea water	1	Subcutaneous cryptococcosis, skin, nail infections	Air, water	This study
<i>Cryptococcus</i> sp.	Metal parts	Air, flowers, trees, sea water	1	Subcutaneous cryptococcosis, skin, nail infections	Air, water	<a href="#">Gattlen et al. 2010.</a>
<i>Exophiala alcalophila</i>	Different parts	Soil, water, bathrooms	1	Unknown	Water	Hamada 2005
<i>Exophiala equina</i>	Drawer for fabric softener, soap dispenser	Water, cold blooded animals	1	Subcutaneous phaeohyphomycosis	Water	This study, <a href="#">Isola et al. 2013.</a>
<i>Exophiala lecanii-corni</i>	Drawer for washing powder, soap dispenser	Water, biofilms from water supply	2	Cutaneous, subcutaneous phaeohyphomycosis, systemic infections	Water	This study, <a href="#">Isola et al. 2013.</a>
<i>Exophiala mesophila</i>	Drawer for washing powder, soap dispenser	Water, steam bath, bathrooms	1	Cutaneous, subcutaneous phaeohyphomycosis	Water	This study, <a href="#">Isola et al. 2013.</a>
<i>Exophiala phaemuriformis</i> genotype 1	Drawer for washing powder, rubber	Water, natural hot springs, steam bath, bathrooms, dishwashers	2	Cutaneous, subcutaneous and systemic infections	Water	This study
<i>Fusarium oxysporum</i> species complex	Drawer for washing powder and softener, rubber, other parts, towels	Air, water, soil, plant material, animals	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants	This study, <a href="#">Stapleton et al. 2013.</a>
<i>Fusarium proliferatum</i>	Drawer for washing powder, rubber	Air, water, soil, plant material	1	Sinusitis, endophthalmitis, onychomycosis, pneumonia, subcutaneous infections	Air, water, plants, soil	This study
<i>Fusarium solani</i> species complex	Drawer for washing powder, different parts, towels	Air, water, soil, plant material, animals	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants, soil	This study, <a href="#">Stapleton et al. 2013.</a>
<i>Fusarium</i> sp.	Washed laundry	Air, water, soil, plant material	1	Sinusitis, endophthalmitis, onychomycosis, pneumonia, subcutaneous infections	Air, water, plants, soil	Neely & Orloff 2001



<i>Fusarium verticillioides</i>	Drawer for washing powder	Air, water, soil, plant material	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants, soil	This study
<i>Meyerozyma guilliermondii</i>	Drawer for washing powder, rubber	Soil, sea water, dishwashers, rubber	1	Blood stream, pulmonary, cutaneous infections	Air, water	This study
<i>Microsporium canis</i>	Washed laundry	Soil, animals	2	Hair, skin infections	Water, clothes, animals	<a href="#">Shah et al. 1988</a> , <a href="#">Blaser et al. 1984</a> .
<i>Mucor circinelloides</i>	Drawer for washing powder	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	This study
<i>Mucor racemosus</i>	Drawer for washing softener	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	This study
<i>Mucor</i> sp.	Washed laundry	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	Neely & Orloff 2001
<i>Ochroconis humicola</i>	Soap dispenser	Water, soil, air, animals	1	Pulmonary infections	Air, water	<a href="#">Isola et al. 2013</a>
<i>Ochroconis</i> sp.	Drawer for washing powder, rubber	Water, soil, air, animals	1	Pulmonary infections	Air, water	This study
<i>Penicillium brevicompactum</i>	Drawer for washing powder	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water, soil	This study
<i>Penicillium crustosum</i>	Drawer for washing powder and softener, rubber	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water, soil	This study
<i>Penicillium</i> sp.	Rubber, plastic parts of	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water	<a href="#">Gattlen et al. 2010</a> .
<i>Phialophora olivacea</i>	Soap dispenser	Water, fruits, air	1	Unknown	Water, air	<a href="#">Isola et al. 2013</a> .
<i>Phoma fimeti</i>	Drawer for washing powder	Plant material, marine environment, water, cement	1	Unknown	Air, water	This study
<i>Phoma radicina</i>	Drawer for washing powder	Plant material, marine environment, water, cement	1	Unknown	Air, water	This study
<i>Rhodotorula minuta</i>	rubber	Soil, water, sea water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	<a href="#">Gattlen et al. 2010</a> .
<i>Rhodotorula mucilaginosa</i>	rubber, plastic, metal	Soil, water, air, fruits, bathrooms, dishwashers	1	Fungemia, endocarditis, meningitis	Air, water	This study, <a href="#">Gattlen et al. 2010</a> .
<i>Rhodotorula slooffiae</i>	Drawer for washing softener, rubber, metal parts	Soil, water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	This study, <a href="#">Gattlen et al. 2010</a> .

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Table 4 – (continued)

Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
<i>Rhodotorula</i> sp.	Different parts, towels	Soil, water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	Stapleton et al. 2013.
<i>Scolecobasidium constrictum</i>	Different parts	Water, soil, air, bathrooms	1	Pulmonary infections	Air, water	Hamada 2005
<i>Trichosporon domesticus</i>	Plastic parts	Soil, water, human skin	2	Superficial, subcutaneous, systemic infections, pneumonitis	Water	Gattien et al. 2010.
<i>Trichophyton mentagrophytes</i>	Laundered socks	Soil, human skin, animals	2	Onychomycosis, hair, skin infections, <i>Tinea pedis</i>	Clothes, animals	Tanaka et al. 2006.

BSL, Biosafety level (de Hoog et al. 2009).

### Fungi are not the causative agents of malodour in washing machines

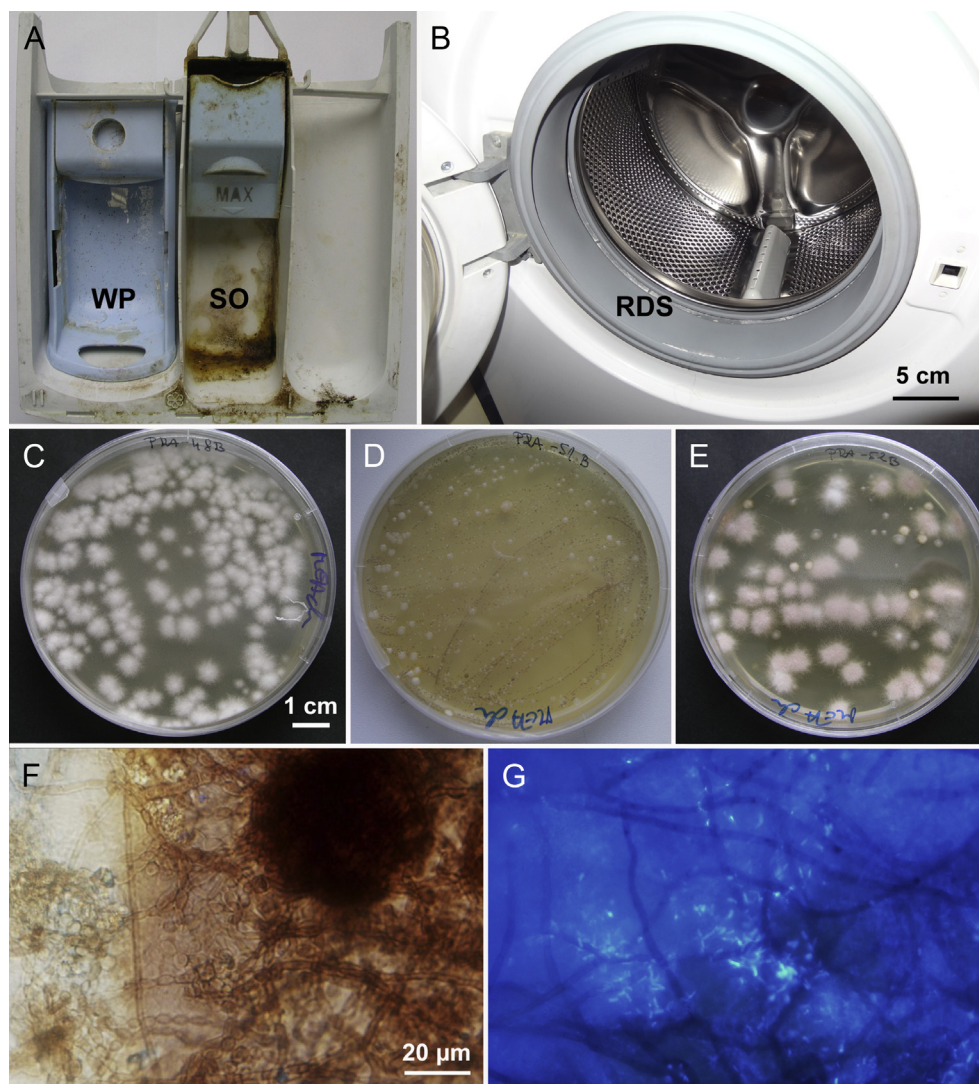
The six residential washing machines tested for bacteria and fungi had been used from 1 month up to 10 y with different frequencies of use. In one of these six cases, fabric softener had never been used. In all six cases, the most frequently used temperature program ran at 40 °C.

While fungi prevailed over bacteria in the sampled tap water, very low numbers of fungal colony forming units (CFU) were recovered from washing machines with malodour, and in waste water samples (Fig 5). Although the microbiota isolated from the different parts inside the washing machines was very diverse, it was dominated by *Micrococcus luteus*, followed by different *Pseudomonas* and *Sphingomonas* species in washing machines with malodour. Only a few species from the genera *Pseudomonas*, *Klebsiella*, and *Shewanella* were isolated from the waste water. Fungi of the genera *Penicillium* and *Cladosporium* occurred rarely, if at all (Table 2). In comparison, the three washing machines that did not have intensive malodour were mainly colonized with *M. luteus*, different *Bacillus* species, and *Pseudomonas pseudoalcaligenes*, with only *P. aeruginosa* isolated from waste water. Fungi were detected more often in these machines, compared with those with malodour. Here, different *Penicillium* species were isolated, followed by the fungal genera *Aspergillus*, *Cladosporium*, and *Aureobasidium*. Also white and black yeasts from the genera *Candida*, *Meyerozyma*, *Debaryomyces*, and *Exophiala* were detected, which were not isolated from the machines with malodour (Table 3).

### Discussion

Infections with opportunistic human fungi can occur in many ways (de Hoog et al. 2009). Presence of fungi that are known also as opportunistic human pathogens in household appliances might represent a so-far largely overlooked risk factor for fungal infections (de Hoog et al. 2009). Dishwashers and washing machines may have become more microbe-friendly environments than they used to be in the past because they use now lower temperatures and less aggressive detergents without bleach (Beadle & Verran 1999) promoting the selection of species that are stress-resistant and have become known as opportunistic human pathogens (Gostinčar et al. 2011). This ecological trend is confirmed also in the present study, as 40 °C was the washing temperature of choice for most users. Gram-negative bacteria in planctonic form can survive washing temperatures up to 50 °C, while Gram-positive bacteria can survive up to 60 °C (Munk et al. 2001). Although there is little data on the highest temperatures that fungi can survive during the washing cycle, it has been reported that *Candida albicans* strains can be successfully recovered at lower temperatures, while 60 °C is needed for the inactivation of *Trichophyton rubrum* (Hammer et al. 2011). It is also known that certain filamentous fungi and yeasts can survive temperatures above 60 °C, or even near to 100 °C (Rogers et al. 1994; Sterflinger 1998).

The main way for fungi to enter washing machines is via the water supply system and/or dirty laundry. This study did



**Fig 1 – Fungi in biofilm formation from selected parts of washing machines. (A)** Drawers for washing powder (WP) and fabric softener (SO) covered with visible dark brown blemishes. **(B)** Rubber door seal (RDS). **(C–E)** Isolation culture media (MEA with chloramphenicol): **(C)** With members of the *Fusarium oxysporum* species complex. **(D)** With dense colonies of *Exophiala phaeomuriformis* genotype 1 among white yeast colonies of *Candida parapsilosis*. **(E)** With pink colonies of *Fusarium oxysporum* accompanied with colonies of *Candida parapsilosis*. **(F, G)** Fungal/bacterial biofilms viewed with light microscopy **(F)** and fluorescent microscopy **(G)**: Autofluorescence of fungi and bacteria. Scale bar in **(B)** (5 cm) applies also for **(A)**. Scale bar in **(C)** (1 cm) applies also for **(D, E)**. Scale bar in **(F)** applies also for **(G)**.

not focus on dermatophytes, which are transferred mainly via the laundry, but primarily on water-borne fungi. Fungi entering household appliances via the tap-water system, might present a health risk, since they are enriched within the devices such as washing machines. Thus, many investigations focused on the presence of microbes in groundwater and in domestic water systems and pipes. Spores of filamentous fungi from the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma*, black yeasts from the genera *Aureobasidium*, *Cladophialophora*, *Exophiala*, and *Phialophora*, white yeasts from the genera *Candida*, *Meyerozyma*, *Pichia*, and *Saccharomyces*, and red yeasts from the genera *Rhodotorula* and *Sporobolomyces* have all been retrieved from tap water (Anaisie et al. 2001; Göttlich et al. 2002;

Gonçalves et al. 2006; Hageskal et al. 2007, 2009; Sammon et al. 2010). Previous studies of fungi in different sites of washing machines revealed contamination with filamentous species from the genera *Alternaria*, *Aspergillus*, *Capronia*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichosporon*, and of yeasts from the genera *Candida*, *Cryptococcus*, and *Rhodotorula* (Gattlen et al. 2010; Stapleton et al. 2013). Hamada (2002) analysed rinsing and washing water from washing machines for fungal contamination, and reported the presence of *Exophiala*, *Phoma*, *Cladosporium*, *Scolecobasidium*, *Penicillium*, and *Phialophora*.

During the washing cycle, water-borne fungi entering a washing machine may become completely inactivated, retain their viability without colonizing surfaces, or become

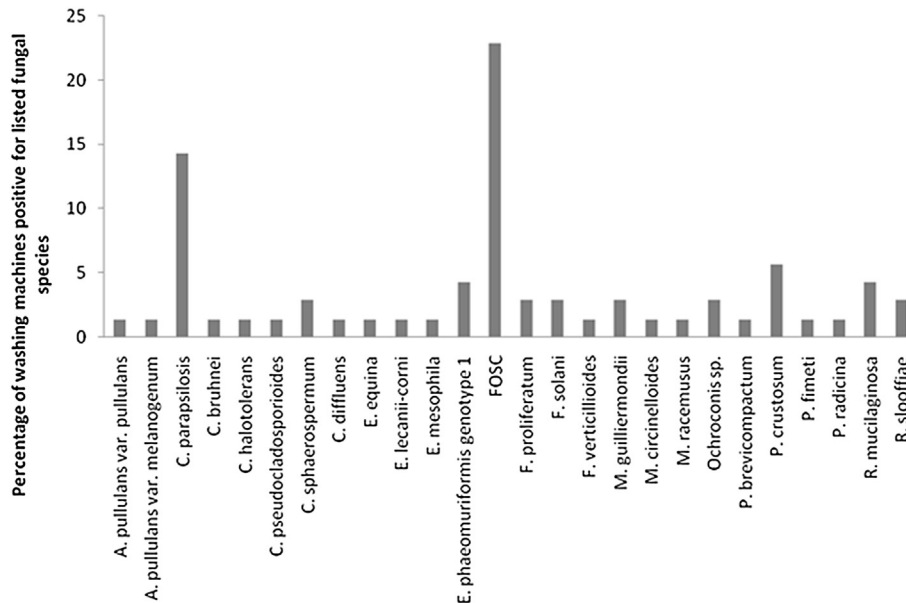


Fig 2 – Occurrence of different fungal species in washing machines. Members of *Fusarium oxysporum* species complex (22.9 %) were detected most often, followed by *Candida parapsilosis* (14.3 %), *Penicillium crustosum* (5.7 %), *Exophiala phacomuriformis* (4.3 %) and *Rhodotorula mucilaginosa* (4.3 %).

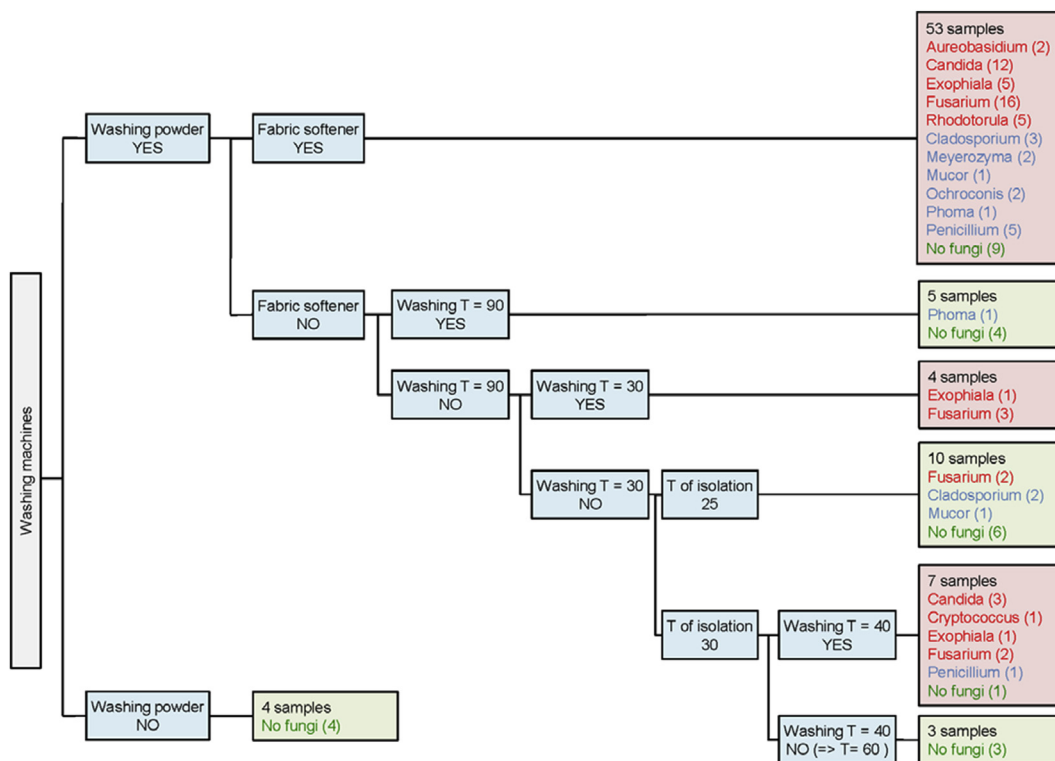
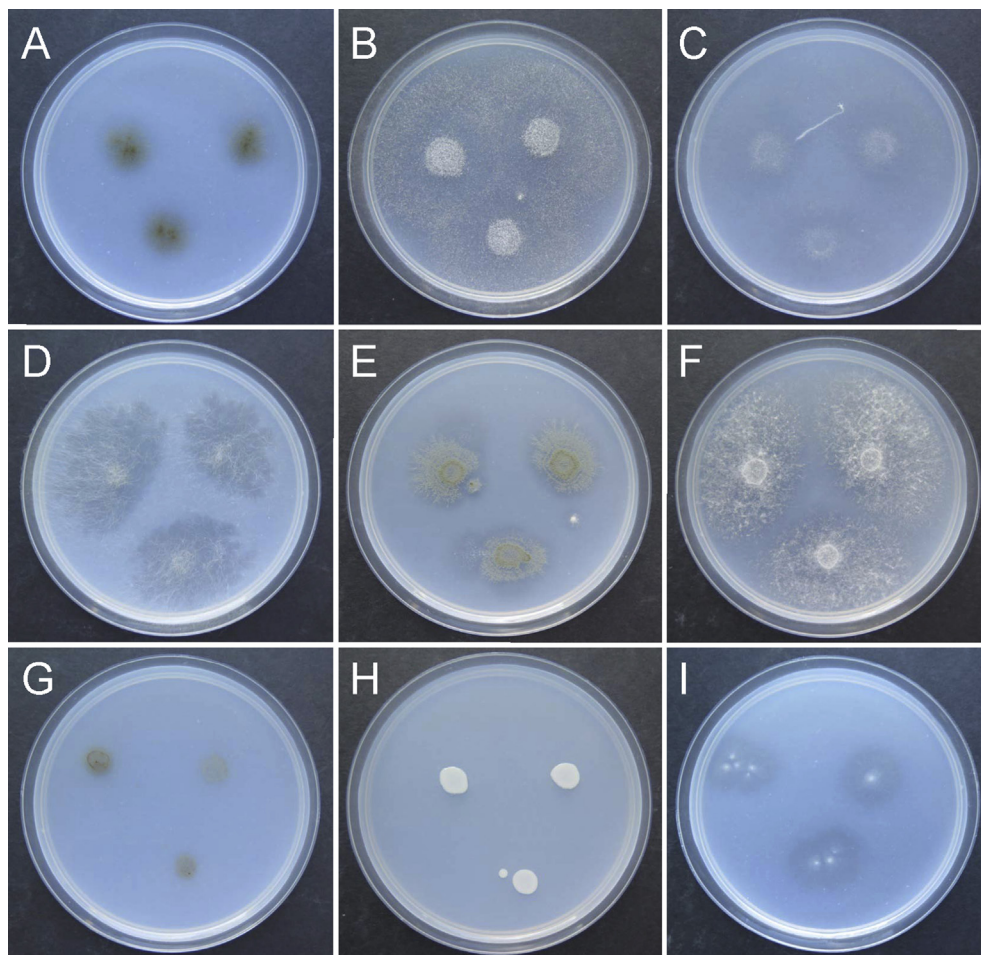


Fig 3 – Decision tree for samples from 70 washing machines not showing malodour, generated with J48 machine-learning method. Internal nodes (blue boxes) represent conditions for values (or presence) of different factors including use of washing powder and fabric softener, washing temperature, and temperature used during taxon isolation. Tree leaves (red and green boxes) contain the samples that satisfy all of the conditions on the path from the tree root to the given leaf. Each leaf provides information of total number of samples in a leaf (in black), fungal genera in these samples with numbers of samples for each genus (in red and blue), and number of samples where no fungi were found (in green). Red colour indicates genera with possible pathogenic potential, while blue colour indicates fungi without pathogenic potential. The red colour of the leaf means that the majority of samples contained at least one fungal genus, while the green colour of the leaf means that in the majority of samples no fungi were found.

**Table 5 – Selected growth characteristics of fungi isolated from washing machines.**

Fungal species	EXF- no.	Growth/activity under different temperature conditions											
		Malt extract medium		Esterase activity		Proteolytic activity		1 % Acetic acid		Water agar with 1 % fabric softener		Water agar with 5 % fabric softener	
		25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C
<i>Aureobasidium melanogenum</i>	8259	+	+	+	+	+	+	-	-	+	-	-	-
<i>Aureobasidium pullulans</i>	6298	+	-	+	-	+	-	-	-	+	-	-	-
<i>Candida parapsilosis</i>	8293	+	+	+	-	-	-	-	-	+	-	-	-
<i>Cladosporium bruhnei</i>	5660	+	-	+	-	+	-	-	-	+	-	-	-
<i>Cladosporium halotolerans</i>	5564	+	-	+	-	-	-	-	-	+	-	-	-
<i>Cladosporium pseudocladosporioides</i>	5563	+	-	+	-	-	-	-	-	+	-	-	-
<i>Cladosporium sphaerospermum</i>	8279	+	-	+	-	+	-	-	-	+	-	-	-
<i>Cryptococcus diffluens</i>	6329	+	+	+	-	-	-	-	-	+	+	-	-
<i>Exophiala equina</i>	5566	+	+	+	-	-	-	-	-	+	-	-	-
<i>Exophiala lecanii-corni</i>	6140	+	+	+	-	-	-	-	-	+	-	-	-
<i>Exophiala mesophila</i>	6138	+	+	+	-	-	-	-	-	+	-	-	-
<i>Exophiala phaeomuriformis</i> genotype 1	8235	+	+	+	+	-	-	-	-	+	+	-	-
<i>Fusarium oxysporum</i>	5661	+	+	+	+	-	-	-	-	+	+	-	-
<i>Fusarium proliferatum</i>	5664	+	+	+	+	-	-	-	-	+	+	-	-
<i>Fusarium solani</i>	5665	+	+	+	+	+	+	-	-	+	+	-	-
<i>Fusarium verticillioides</i>	5553	+	+	+	+	+	+	-	-	+	+	+	+
<i>Meyerozyma guilliermondii</i>	8240	+	+	+	+	-	-	-	-	+	+	-	-
<i>Mucor circinelloides</i>	6296	+	+	+	-	+	+	-	-	+	-	-	-
<i>Mucor racemosus</i>	5556	+	-	+	-	-	-	-	-	+	-	-	-
<i>Ochroconis</i> sp.	5565	+	-	+	-	-	-	-	-	+	-	-	-
<i>Penicillium brevicompactum</i>	5558	+	-	+	-	+	-	-	-	+	-	-	-
<i>Penicillium crustosum</i>	8272	+	-	+	-	+	-	-	-	+	-	+	-
<i>Phoma fimeti</i>	5551	+	-	+	-	-	-	-	-	-	-	-	-
<i>Phoma radicina</i>	6297	+	-	+	-	-	-	-	-	+	-	-	-
<i>Rhodotorula mucilaginosa</i>	6325	+	+	+	-	-	-	-	-	+	-	-	-
<i>Rhodotorula slooffiae</i>	5557	+	+	+	-	-	-	-	-	+	+	-	-

Isolates indicated in bold grew at 37 °C and had both proteolytic and esterase activities.



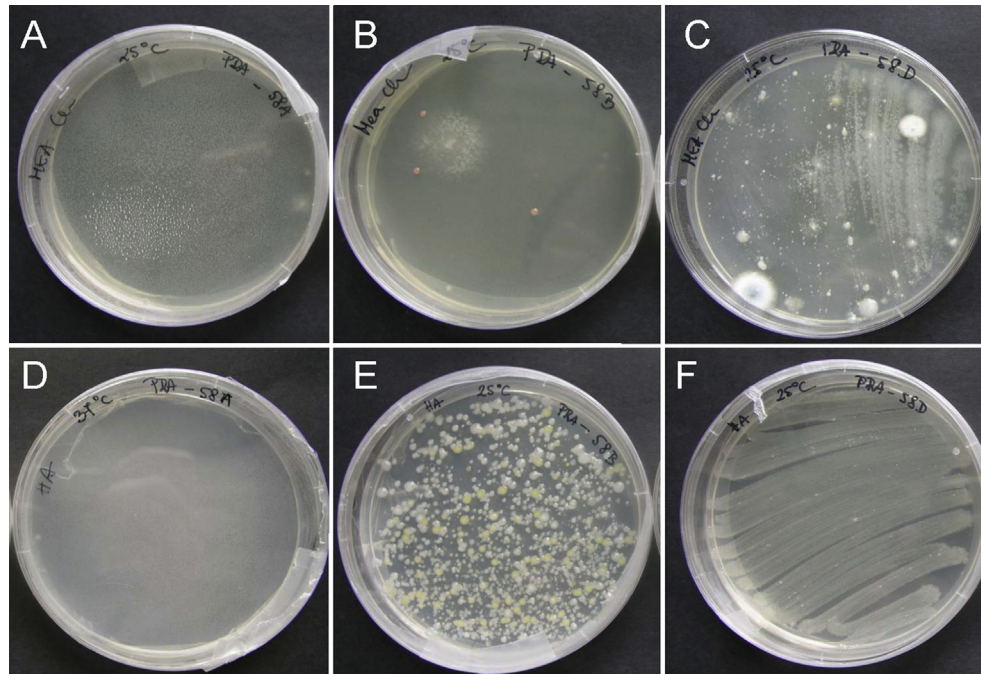
**Fig 4 – Growth of pure cultured fungi after 2 weeks at 25 °C on water agar containing 1 % commercial fabric softener. (A) *Cladosporium bruhnei*. (B) *Fusarium verticillioides*. (C) *Fusarium oxysporum*. (D) *Mucor circinelloides*. (E) *Penicillium crustosum*. (F) *Fusarium solani*. (G) *Exophiala phaeomuriformis* genotype 1. (H) *Meyerozyma guilliermondii*. (I) *Aureobasidium pullulans*.**

selectively enriched. Compared to their planktonic form, microorganisms in biofilms can survive higher temperatures and are more resistant to the ‘cleaning’ effects of detergents (Gattlen et al. 2010), and thus the development of biofilms is favoured inside washing machines. Biofilm formation is further influenced by the presence of the appropriate nutrients and conditions, such as moisture and the type of material (Lund & Ormerod 1995; Hageskal et al. 2007), production of extracellular polysaccharides, and diversity of the microorganisms present (Doggett 2000; Steenbergen et al. 2001; Pereira et al. 2002; Kinsey et al. 2003).

The potential build-up of biofilm formation in washing machines and on laundry can result in persistent malodour (Munk et al. 2001). Past studies have identified the main cause of malodour as a result of bacterial degradation of different substances including detergents and dirt on clothes (Munk et al. 2001), which results in the production of volatile organic compounds, and in particular dimethyl disulphide (Stapleton et al. 2013). The fungal species *R. mucilaginosa*, *F. oxysporum*, and *F. solani* have also been investigated for the production of volatile organic compounds; however, they were not classified as producers of such (Gattlen et al. 2010; Stapleton et al.

2013). In different studies, bacteria from the genera *Brevundimonas*, *Micrococcus*, *Moraxella*, *Ochrobactrum*, *Pseudomonas*, *Roseomonas*, *Shewanella*, *Sphingobacterium*, *Sphingomonas*, and *Stenotrophomonas* have been connected with washing machine malodour (Legnani & Leoni 1997; Labows et al. 1999; Gattlen et al. 2010; Kubota et al. 2012; Stapleton et al. 2013). Munk et al. (2001) observed adhesion and survival of *Staphylococcus epidermis*, *Escherichia coli*, and *P. aeruginosa* to textiles at low temperatures of washing (less than 60 °C) and with use of detergents without bleach. Almost all of the isolated bacterial species were previously reported from freshwater or from household surfaces such as shower curtains, kitchen sponges, and dish racks (Munk et al. 2001).

The 73 washing machines included three with persistent malodour. When the fungi were isolated from the inner parts of these machines, only six fungal isolates (*Penicillium chrysogenum*, *Penicillium sanquifluum*, *Phialophora europaea*, *Sistotrema birkmannii*, and *Sporobolomyces ruberrimus*) were retrieved and bacterial communities consisting of *Micrococcus*, *Pseudomonas*, and *Sphingomonas* clearly dominated. By means of contrast, the three washing machines without malodour accommodated 15 fungal strains from ten different genera, mainly



**Fig 5 – Growth of fungi and bacteria from different sites of washing machines with malodour. The different samples were smeared either on MEA supplemented with chloramphenicol for the isolation of fungi (A–C), or on nutrient agar for bacteria (D–F). (A, D) Sample from washing powder drawer, showing no fungi (A) and no bacteria (D). (B, E) Sample from fabric softener drawer showing several yeast colonies (B) and numerous bacteria (E). (C, F) Sample from rubber door seal, showing several yeast (C) and numerous bacterial colonies (F).**

different *Penicillium* species, followed by *Cladosporium* species and *Candida parapsilosis*. *Micrococcus* and *Bacillus* species were the most frequently isolated bacteria, while other bacterial genera were only sporadically identified. These findings indicate that the malodour is associated with the presence of bacteria, especially from the genera *Pseudomonas*, *Shewanella*, and *Sphingomonas*. The presence of fungi does not have any effects on malodour formation. *Pseudomonas* species are typical waterborne opportunistic bacteria that have been previously reported from washing machines (Legnani & Laoni 1997), and in particular as biofilms on elastomeric and polyethylene surfaces, and less so on metal surfaces (Moritz et al. 2010). These are known producers of dimethyl polysulphides and ammonium, which can cause a ‘swampy’ odour. In our study, *P. aeruginosa* and *P. putida* prevailed, and both of these bacteria originate from water and soil. *Pseudomonas aeruginosa* is known as a human pathogen that can cause wound infections (Kelly et al. 2004; Feazel et al. 2009; Gattlen et al. 2010), while *P. putida* can break down aliphatic and aromatic hydrocarbons and organic toxins of the herbicide atrazine, and it is not classified as a human pathogen (Palleroni 1992). Bacteria from genus *Sphingomonas*, which mainly originate from water, can cause wound and respiratory infections in immunocompromised people, and the corrosion of metals (White et al. 1996). *Micrococcus luteus*, which was the most frequently detected bacteria in the present study, are commensals and are only rarely pathogenic. They can be isolated from water, dust, and human skin. On the surface of skin, they break down fatty acids to produce volatile organic compounds that result in malodour (James et al. 2004).

Microbial degradation of detergents might be a reason for malodour and biofilm persistence. Detergents are mixtures of different chemical components that include aromatic hydrocarbons (polyvinylpyrrolidone), alcohols (terpineol, sorbitol), surfactants (anionic, non-ionic, cationic, zwitterionic), fragrances (citral, lymonene), enzymes (amylase, protease, lipase), and bleaches (sodium percarbonate) (ZPS 2009; Isola et al. 2013). Not only bacteria, but also fungi have been described as having the ability to degrade washing detergents. Hamada & Abe (2009) tested the growth of different bathroom-colonizing fungi on media containing different components of detergents like fatty acids and anionic and non-ionic surfactants. Most of these fungi grew on fatty acids and anionic surfactants, while the growth on non-ionic surfactants varied from species to species. Detergents containing bleach successfully prevent both bacterial and fungal growth (Beadle & Verran 1999; Hamada & Abe 2009).

The machine-learning analysis used in our study indicates that in washing machines where both washing powder and fabric softener are used, the diversity of fungi is significantly higher than in washing machines where only one or none of these are used. The use of fabric softener presents a key parameter that influences fungal colonization of washing machines. To the best of our knowledge, there have been no reports on the growth of fungi on commercial fabric softeners. In contrast to washing powder, softeners do not include bleach. When we tested the growth of 26 of the most representative fungal isolates from the washing machines on media that contained a commercial fabric softener, all of the tested fungi, except *P. fimeti*, assimilate the softener at least to

a concentration of 1 %. Acetic acid at 1 %, which is in some cases used as an alternative for commercial fabric softeners, completely inhibited the growth of these same fungal strains.

Amongst these fungi isolated from washing machines, filamentous fungi prevailed over yeast, in contrast to the mycoflora detected in dishwashers (Zalar et al. 2011). Surprisingly, around 30 % of the washing machines in the present study were colonized with species from the genus *Fusarium*: FOSC, FSSC, *F. proliferatum*, and *F. verticillioides*. The FOSC and FSSC fungi are causative agents of approximately 80 % of human fungal infections (O'Donnell et al. 2010; Sutton & Brandt 2011; Garnica & Nucci 2013). Members of FOSC and FSSC are also known for their ability to form biofilms on surfaces of contact lenses and polyvinyl chloride pipes (Short et al. 2011), and thus these are often involved in eye (mycotic keratitis) or catheter-related (Wey & Colombo 1997; Mukherjee et al. 2012) infections. In nature, representatives of FOSC and FSSC have been isolated from plants, plant materials, soil, air, and water, and have primarily been seen as plant pathogens and soil inhabitants (O'Donnell et al. 2004; Zhang et al. 2006; Smith 2007).

*Cladosporium pseudocladosporioides* and *C. sphaerospermum* at least occasionally colonize washing machines. Both are examples of stress-resistant cosmopolitan fungi disseminated through air and colonizing water and bathrooms and habitats with lowered water activities such as salterns (de Hoog et al. 2000; Zalar et al. 2007; Pereira et al. 2010). They were also isolated from the water distribution system of hospitals (Hayette et al. 2010). Representatives of the cosmopolitan genus *Penicillium* were also isolated. *Penicillium crustosum* and *P. chrysogenum*, which dominated among the fungi in the washing machines with malodour, are not recognized as opportunistic human pathogens (de Hoog et al. 2009); instead, they are primarily known as food spoilage organisms.

Species of the genus *Exophiala* were isolated in 8.5 % of cases. Different species of the genus *Exophiala* are oligotrophic and can be commonly found on rocks and in water (Sterflinger 1998), and also in water-related human-made environments, such as bathrooms, water pipes for taps, and saunas (Matos et al. 2002; Biedunkiewicz & Schulz 2012). The majority of *Exophiala* species are classified as opportunistic pathogens that can cause cutaneous and subcutaneous infections, and lung infections (de Hoog et al. 2009) and known from biofilms (Hamada & Abe 2009; Isola et al. 2013; Heinrichs et al. 2013). The present study resulted in the isolation of *E. phaeomuriformis*, *E. mesophila*, *E. equine*, and *E. lecanii-corni*, which have all been reported as human pathogens. All of these species are able to cause infections in humans (Woo et al. 2013; Najafzadeh et al. 2013). They decompose aromatic hydrocarbons (Isola et al. 2013), assimilate different detergents (Hamada & Abe 2009) and survive high temperature and high pH (Zalar et al. 2011).

*Candida parapsilosis* has been reported as an emerging pathogen (van Asbeck et al. 2009; Miceli et al. 2011). It was the second-most frequently detected species (14.3 %) in washing machines investigated here and in dishwashers (Zalar et al. 2011). *Candida parapsilosis* is a ubiquitous microorganism that can be isolated from soil, water, and plants (Deresinski et al. 1995) and occurs on catheters and other prosthetic materials (Levin et al. 1998). It is a causative agent of opportunistic

fungemia in immunocompromised patients (Barone & Branchini 1998; de Hoog et al. 2009). *Rhodotorula mucilaginosa* prevailed amongst the red-pigmented yeasts in dishwashers (Zalar et al. 2011) and in the present study of washing machines. Members of the genus *Rhodotorula* are known to form biofilms (Gattlen et al. 2010) and have been involved in catheter-related infections (Neofytos et al. 2007) and fungemia in cancer and AIDS patients (Pfaller et al. 2007). It is capable of behaving in a vigorous and highly competitive manner and therefore dominates various habitats (Cray et al. 2013). These red yeasts are very common in the environment and have been isolated from air, soil, food, and saline water (Wirth & Goldani 2012).

We were able to show that the majority of the analysed washing machines were colonized with various fungal species of which several are known also as opportunistic human pathogens. Fungi and bacteria commonly occur in water and water supply systems as single propagules, however, typically in low numbers. Within washing machines, they can become established as colonies and in biofilms that may release cells or conidia during washing cycles. Accordingly, washing machines may present a reservoir for these fungi from where they are further disseminated to clothes and wastewater. It appears that cloth washing at temperatures below 60 °C, mild detergents and commonly used fabric softeners can lead to an increased presence of microbial diversity in washing machines. The processes during washing may allow the selective enrichment of thermotolerant species and are not capable of eliminating non-thermotolerant species. Washing regimes recruiting reduced amounts of water, lowered water temperatures and biodegradable detergents may increase the diversity and quantity of microbes in households and could present a health risk specifically for immunocompromised people. The regular cleaning of washing powder drawers with bleach or bleach containing cleaners helps to restrict or remove microbial biofilms. Performances of such cleaning procedures are recommended by washing machine manufacturers.

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