Diversity of fungi in bottled water in Jeddah, Saudi Arabia

Fuad Ameen, Alhanouf Albejad, Rukaia Gashgari, S. Murialdo and A. Al-Sabri

ABSTRACT

The occurrence of fungi in drinking water systems has received increased attention over recent decades and fungi are now generally accepted as drinking water system contaminants. However, fungal contamination of bottled water has received little attention. Forty unopened bottled water samples, of different trademarks, were collected from various localities in Jeddah city, Saudi Arabia and analyzed for fungal contamination: (1) immediately after opening the bottles; and (2) after closing and storing them for 180 and 365 days. The fungal species were identified under a compound microscope followed by molecular sequencing. At least one fungal species were found in 58% of the bottles. In total, 18 fungal species belonging to 11 fungal genera were identified. *Rhizopus nigricans* and seven different species of *Aspergillus* were found to frequently contaminate the bottled water samples. *Penicillium* sp. were found in one sample. The 180 days storage of opened and reclosed bottles did not substantially affect the abundance of fungi or the species found. Some of the fungi identified may be pathogenic and the contamination of fungi in bottled water should be considered during the processing of water.

Key words | bottled water, contamination, drinking water, fungi, water

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INTRODUCTION

Fungi are ubiquitous in nature and are able to survive and grow in water sources, including drinking water. Fungi were observed to survive through the drinking water disinfection process in the 1980s (Niemi *et al.* 1982). Recently, potentially pathogenic species have frequently been isolated from drinking water systems (Paterson & Lima 2005; Paterson *et al.* 2009; Hageskal *et al.* 2011; Oliveira *et al.* 2013; Babič *et al.* 2016; Hurtado-McCormick *et al.* 2016). More than half (66%) of the fungal species identified in different drinking water sources in Brazil were considered potential pathogens (Oliveira *et al.* 2013). An emerging pathogen, *Aspergillus calidoustus*, has been frequently isolated from Norwegian water systems (Hageskal *et al.* 2011). Several fungal species found in drinking waters are known to cause infectious diseases (Paterson & Lima 2005; Paterson *et al.* 2009) but no report about any acute disease caused by fungal contamination in purified drinking water was found in a recent review (Hageskal *et al.* 2009). However, health effects are not fully understood and several articles have regarded fungal contamination as a possibly underestimated problem in drinking water distribution systems (Hageskal *et al.* 2006; 2009; 2012; Kanzler *et al.* 2008; Pereira *et al.* 2009; 2010; Siqueira *et al.* 2011; Al-gabr *et al.* 2014; Skaar & Hageskal 2015). Also a recent review of Babič *et al.* (2017) concludes that harmful health effects of pathogenic fungi are possible especially for immunocompromised people. In addition to health effects, fungal contamination may also be responsible for the mycotoxins that possibly cause organoleptic defects and allergenic reactions (Mata *et al.* 2015; Skaar & Hageskal 2015; Bai *et al.* 2017).

The use of bottled water, as a safe substitute for tap water, has increased in the past few decades, but the possible microbial contamination of bottled water has been studied very little. It has been reported that bottled water

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may be contaminated with bacteria and fungi (Cabral & Pinto 2002; Criado *et al.* 2005; Yamaguchi *et al.* 2007). However, only a few studies have reported the contamination of bottled water at the species level, although diseases, mycotoxins, pigment and odor formation have been associated more with the individual species than with the genus (Siqueira *et al.* 2011; Oliveira *et al.* 2013). *Cladosporium cladosporioides, Penicillium* sp. and *Alternaria alternata* were found in bottled water in Buenos Aires, Argentina (Cabral & Pinto 2002; Criado *et al.* 2005). In Brazil, 20% of the bottled water samples were contaminated by fungi; three species of the genus *Candida* were found (Yamaguchi *et al.* 2007). The review of Babič *et al.* (2017) reports ten fungal species identified from bottled water during 30 years. These species were *Aspergillus fumigatus, A. versicolor, Aureobasidium pullulans, Debaryomyces hansenii, Exophiala spinifera, Penicillium chrysogenum, P. glabrum, Talaromyces rugulosus, Trichoderma longibrachiatum,* and *Filobasidium magnum*. In addition they report three genera, namely *Cladosporium*, *Fusarium* and *Paecilomyces*.

We aim to fill the knowledge gap in fungal contamination of bottled waters and analyze the occurrence and diversity of fungi as contaminants in waters. Purity of water is especially important in places where people drink mainly bottled water, for instance, in Saudi Arabia where the land is poor in natural water resources. We collected forty bottled waters, of international trade marks, and analyzed them using both classical and molecular techniques. The results give information on the occurrence of potentially harmful pathogenic fungi in bottled drinking water.

MATERIALS AND METHODS

Forty unopened water bottles were randomly collected from different markets in Jeddah, Saudi Arabia in 2012–2013. The origin and the water processing information are provided in Table 1. Nine of the bottles were described as being ozone treated (ozone) while 31 bottles had no mention of the treatment (no-ozone). The details of the ozone treatments are not known. The *t*-test was used to study the difference between the ozone and no-ozone treated bottles.

Table 1 | Information about the bottled water samples and the production companies

Sample ID	Trade mark	Production company	Origin	Package size ml/l	Information on the package	Origin of water
W1	AL-Higra	Hijaz water company	Saudi Arabia, Makkah	250	–	
W2	ALien	Delta water factory	Saudi Arabia	330	Ozone treatment Fluoride Added	
W3	Alwadi	Al-Amoodi Industry Co. refreshments	Saudi Arabia, Makkah, Fatima Valley	650	–	Valley
W4	Nova	Manufactory Health Water Bottling Company	Saudi Arabia	330	–	Ground water
W5	Hana	National factory of health water	Saudi Arabia, Boriedah	330	Fluoride Added	Ground water
W6	Acquafina	Saudi Industrial beverage company	Saudi Arabia, Jeddah	330	Fluoride Added	
W7	Panda	National Factory for healthy water company (HANA)	Saudi Arabia, Boriedah (Green oasis)	330	–	
W8	Dalla	Arab company Modern Industries	Saudi Arabia	600	–	
W9	Bin Dawood	Delta factory of water	Saudi Arabia	330	–	
W10	Fihaa	Wells Ozone treatment	Saudi Arabia	330	–	Ground water
W11	Mozen	South water factory	Saudi Arabia, Wadi Nakhlan, Jazan	300	–	Valley

(*continued*)

Table 1 | continued

Sample ID	Trade mark	Production company	Origin	Package size ml/l	Information on the package	Origin of water
W12	Safa	Makkah company of water	Saudi Arabia, Makkah	330	Floride Added	
W13	Arwa	Saudi Arabia Coca-Cola Bottling	Saudi Arabia	500	–	
W14	Massafi	Massafi company	United Arab Emiratis, Ras-Alkhima	500	–	Ground water
W15	Al-Qassim	Qassim health Factory Co.	Saudi Arabia, Al-Qassim	200	–	
W16	Qobaa	AL-Madina Water Company Limited and juices	Saudi Arabia, Almadina	330	Ozone treatment	
W17	Taiba	Madina Factory of water (Taiba)	Saudi Arabia, Al-Madina	300	Ozone treatment	
W18	Fifaa	Factory of Jazan company for development	Saudi Arabia, Jazan	330	–	
W19	Nestle	Limited Nestle company of water	Saudi Arabia, Riyadh	330	–	Ground water
W20	Al-khirat	AL-Khyrat factory of water	Saudi Arabia, Jeddah	250	–	
W21	Tanweerin	–	Lebanon	500	Sodium added	
W22	Sahaab	–	Saudi Arabia	400		
W23	Bambieni	Delta factory of water	Saudi Arabia	330	Ozone treatment without floride	
W24	Water1	National factory of health water in Qassim	Saudi Arabia, Boriedah	330	Ozone treatment	
W25	Alhadaa	Alhadaa limited company of water	Saudi Arabia, Makkah	330	Ozone treatment	
W26	Artweena	Artweena factory of water	Saudi Arabia, Onizah	620	Ozone treatment	Ground water
W27	Mater	Al-Qassim production	Saudi Arabia, Al-Qassim	1,900	–	
W28	Aleion	Aleion company of water	Saudi Arabia, Jeddah	330	–	
W29	ALtharwat	Sama Food Industries Co.	Jordan	600	–	Ground water
W30	Manahel	Manahel Al-Qassim factory of healthy water	Saudi Arabia, Al-Qassim	330	–	Ground water
W31	Yaqeen	–	Syria	500	–	Water fountains
W32	Eliet	Lofinac factory of water	Croatia	500		Water fountains
W33	Alrie	Alrie factory of water	Saudi Arabia, Jeddah	4 Gallons	Ozone and ultraviolet treatment	
W34	Alnojoom Dawrq	Alnojoom Dawrq factory	Saudi Arabia, Jeddah	4 Gallons	–	
W35	Alnaqaa	–	Saudi Arabia, Jeddah	4 Gallons	–	
W36	Aljoob	Aljoop factory of healthy water	Saudi Arabia, Jeddah	4 Gallons	Ozone treatment	
W37	Aquatic	–	Saudi Arabia	4 Gallons	–	
W38	Nord	Nord Water Ltd	Finland	500	–	Water fountains
W39	Highland	–	Scotland	500	Free gas and calories	
W40	Evian	Evian Company of mineral water	France	330	–	Water fountains

The bottles were stored unopened at room temperature (28 ± 2°C) until studied (0 d). All work after opening the bottles was performed with aseptic techniques under sterile conditions and all possible contamination outside the bottles was avoided. The bottles were sterilized outside with ethanol before entering the sterile environment and the lips were sterilized with ethanol after opening the bottle. No growth was observed in the blanks inoculated with sterile water. Five of the bottles were randomly chosen for further analysis after they had been stored for 180 d and 365 d at room temperature in order to find out the potential of fungi to reproduce in bottled water. Three replicate analyses were performed, the mean colony forming units (CFU) was calculated and the species results were combined to represent the trademark.

The membrane filtering technique was used (Pereira *et al.* 2010). An aliquot of 100 ml of water was filtered through a 0.45 μm membrane. The membrane was placed on the surface of sterilized petri dishes containing autoclaved potato dextrose agar medium (PDA). The plates were incubated at 28 ± 2°C for one week. The number of colonies was counted and the grown fungal mycelia were collected for identification. The isolated fungi were maintained on PDA slants at 4°C. All the media used in this study were obtained from HiMedia, Mumbai, India.

For the classic morphological identification, the fungi were sub-cultured in suitable agar media (Dichloran Rose Bengal Chloramphenicol agar (DRBC), Czapek Yeast extract agar (CYA), Czapek Dox agar (CZ), Synthetic Nutrient Medium (SNM)) according to Samson & Frisvad (2004). Slide preparations were stained with lactofuchsin, with or without alcohol, lactic acid or in double distilled water. The fungi were phenotypically identified to the genus level, or to species level when possible, under a light microscope (Barnett & Hunter 1972; De Hoog *et al.* 2000; Klich 2002). The identifications were checked for consistency with the latest diagnoses. In total, 35 fungal isolates were identified either morphologically or using molecular techniques (13 isolates).

For the molecular identification, an aliquot of 2 ml of potato dextrose broth (PDB) was poured into the PDA slants containing well grown fungi and shaken thoroughly. The PDB containing spores of each fungi were poured individually into flasks containing 100 ml of sterilized PDB. The flasks were incubated at room temperature without shaking for a week. The grown mycelium in the broth culture was collected aseptically by filtration and ground in liquid nitrogen in a sterile mortar to obtain a mycelium powder. Further, the DNA was extracted from 20 mg of mycelium powder using a cycle-sequencing kit (Applied Biosystems, Darmstadt, Germany).

The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR) with the primers ITS1-F(CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990; Gardes & Bruns 1993). PCR amplifications were performed in a final volume of 50 μl by mixing 2 μl of DNA with 0.5 μM of each primer, 150 μM of dNTP, 6 U of Taq DNA polymerase and PCR reaction buffer. Amplification was conducted in a thermal cycler with an initial denaturation of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension of 10 min at 72°C. Aliquots of PCR products were checked by electrophoresis on agarose gel (1%) revealed with ethidium bromide and visualized by UV trans-illumination. The PCR products were purified by ExoSAP-IT (USB Corporation, under license from GE Healthcare) based on the manufacturer's instructions. The purified products were sequenced using an automated DNA sequencer (ABI PRISM 3700) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) following the manufacturer's instructions. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). They will be deposited in the World Data Centre for Microorganisms (http://new.wfcc.info/ccinfo/index.php/collection/by_id/907).

Sequences obtained in this study were compared with the GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). DNA sequences were first aligned with Clustal X_2 for Windows (version 1.3b), which was used to construct a neighbor-joining tree using the Jukes-Cantor model.

RESULTS AND DISCUSSION

Fungal contamination was found in 58% of the bottled water samples; 23 out of 40 bottles were contaminated with fungi. The contamination frequency was high compared to

previous studies where 20–33% of the bottles had been contaminated in Brazil (Yamaguchi *et al.* 2007) and Argentina (Cabral & Pinto 2002). The reason for the higher contamination frequency in Saudi Arabia than in Brazil or Argentina is not evident. In the first place, all samples represented different international trademarks. Thus, many more trademarks were studied in Saudi Arabia than in Brazil or Argentina. Although the membrane filtration technique was used in all studies, the different media used for fungal enumeration may explain the results.

The bottles described as being treated with ozone (ozone, $n = 9$) did not differ significantly from the non-ozonated bottles (no-ozone, $n = 31$). Up to three species were identified in both treatment groups (Table 2). The mean CFU was lower, although not significantly (*t*-test), in the ozone bottles (0.5 ± 5.7 CFU in 100 ml, mean ± SD) than in the no-ozone bottles (2.6 ± 5.7). The variation was high and the counts started from 0 CFU in both groups. The maximum CFU counted was lower (3.5) in the ozone group than in the non-ozone group (20). The ozone bottles were less frequently (44%) contaminated than the no-ozone bottles (61%). It seems possible that the ozone treatments used had reduced fungal contamination. An ozone treatment has been observed to be the most effective treatment against fungi in general (Hageskal *et al.* 2012). However, the efficiency of the treatment has depended on the dose of ozone used (Hageskal *et al.* 2012). We had no information on the ozone dose used in our bottles and thus, we cannot verify its effect reliably. The susceptibility of species to ozone treatment varied a lot in the study of Hageskal *et al.* (2012), who also found different species to those we did. In summary, there was an indication that the ozone treatment may reduce fungal contamination in bottled waters. However, this conclusion is highly speculative because more detailed information on the treatments used as well as a more balanced study design would have been needed in order to confirm this.

Table 2 | Number of species identified and total CFU in bottled water, described as being treated with ozone or not treated with ozone, collected from different markets in Jeddah, Saudi Arabia

Treatment	Ozone	No-ozone
Number of bottles	9	31
Number of species	1–3	1–3
CFU mean in 100 ml (mean, SD)	0.5 (5.7)	2.6 (5.7)
CFU min – CFU max	0–3.5	0–20
Contaminated bottles (number, %)	4 (44%)	19 (61%)

The total CFUs observed were relatively low. Up to 20 CFU were detected in 100 ml water (Table 3). The level of CFU was about the same as in bottled waters in the study of Cabral & Pinto (2002). Much higher levels, up to 3,000 CFU, have been observed in drinking water systems (Hurtado-McCormick *et al.* 2016; Oliveira *et al.* 2016). However, comparison between the studies is difficult because of the slight differences in the methods and the sensitivity of microbial growth to growing conditions. A more reliable comparison is presented in Yamaguchi *et al.* (2007), where the same conditions were used for tap water and bottled water. The authors conclude that the tap water samples had a clearly lower fungal count and contamination frequency because bottled waters are mostly unique natural products that cannot be treated, nor can any exogenous elements be added to them. As a summary, the variation in fungal contamination can be assessed to be high throughout the world.

Our focus was on the identification and diversity of species found in bottled drinking waters. Different drinking water resources and drinking water systems have been reported to be contaminated with a high variety of fungal genera and species, reviewed by Hageskal *et al.* (2009). The species composition seems to be determined by the concentrations of inorganic ions, such as calcium, magnesium and nitrate in water (Babič *et al.* 2016).

The genera isolated most frequently are *Penicillium* and *Aspergillus* both in drinking water systems and in bottled waters (Hageskal *et al.* 2009; Oliveira *et al.* 2013; 2016; Babič *et al.* 2016; Fish *et al.* 2016). In our study, the 35 fungal isolates found belonged to 11 fungal genera (Table 3). The 14 identified species were *Aspergillus niger, A. flavus, A. terreus, A. fumigatus, A. caespitosus, A. tubingensis, A. chevalieri, Cladophialophora bantiana, C. sphaerospermum, Exophiala cancerae, Gliomastix murorum, Penicillium crustosum, Rhizopus nigricans* and *Sarocladiumim plicatum*. In addition, *Mycelium sterilium*, a fungal strain that cannot be identified, and three unidentified species from the genera *Geotrichum*, *Periconia* and *Phialocephala* were observed. A recent review reports ten fungal species and three genera identified in bottled waters during 30 years (Babič *et al.*

Table 3 | Fungal identification and their counts (CFU) isolated in the bottled water samples collected from Jeddah, Saudi Arabia

Sample	CFU in 100 ml	Isolated genera and species	Sample	CFU in 100 ml	Isolated genera and species
W1	18.6	*C. bantiana*	W21	12.99	*E. cancerae*
W2	0.50	*Periconia* sp. *R. nigricans*	W22	0.00	–
W3	0.16	*Geotrichum* sp.	W23	0.00	–
W4	0.33	*S. implicatum R. nigricans*	W24	0.00	–
W5	0.16	*R. nigricans*	W25	0.00	–
W6	0.16	*R. nigricans*	W26	0.00	–
W7	8.66	*A. niger*	W27	0.00	–
W8	0.83	*R. nigricans*	W28	0.16	*A. caespitosus*
W9	0.00	–	W29	0.33	*M sterilium*
W10	1.00	*A. flavus A. terreus R. nigricans*	W30	0.00	–
W11	0.33	*A. niger R. nigricans*	W31	19.99	*G. murorum*
W12	0.00	–	W32	0.00	–
W13	0.00	–	W33	3.50	*A. tubingensis A. chevalieri M. sterilium*
W14	0.00	–	W34	0.00	–
W15	0.50	*R. nigricans A. fumigatus*	W35	0.00	–
W16	0.16	*R. nigricans*	W36	0.00	–
W17	0.16	*R. nigricans*	W37	0.33	*C. sphaerospermum R. nigricans*
W18	0.00	–	W38	14.33	*Phialocephala* sp. *M. sterilium P. crustosum*
W19	0.50	*R. nigricans*	W39	0.33	*R. nigricans*
W20	1.16	*C. bantiana R. nigricans*	W40	0.00	–

2017). Compared to that result, we identified a large variety of species in our sampling. This may be first of all explained with the techniques developed to identify the species.

Rhizopus nigricans was the most frequently found species occurring in 14 samples, which is 61% of the contaminated samples. The total counts of *R. nigricans*, however, were

Table 4 | Internal transcribed spacer rDNA sequence similarity between the fungal isolates and the closest type strain of valid described species

S. no.	Accession number in GenBank	Closely related fungal sequence	Similarity %	Genus/Species
1	KSU-1(LN812958)	LN482450.1	99%	*Aspergillus caespitosus*
2	KSU-2(LN813023)	LN482490.1	99%	*A. flavus*
3	KSU-3(LN812957)	KF986804.1	99%	*Sarocladium implicatum*
4	KSU-4(LN813024)	JQ697532.1	99%	*A. terreus*
5	KSU-5(LN813026)	AM745112.1	95%	*A. tubingensis*
6	KSU-6(LN813025)	KY310641.1	100%	*Cladophialophora bantiana*
7	KSU-7((LN813027)	LT670923.1	99%	*A. chevalieri*
8	KSU-8(LN813029)	NR_137766.1	99%	*Exophiala cancerae*
9	KSU-9(LN813028)	AB540540.1	99%	*Gliomastix murorum*
10	KSU-10(LN813030)	GU827487.1	100%	*Geotrichum* sp.
11	KSU-11(LN813031)	KU847869.1	95%	*Penicillium crustosum*
12	KSU-12(LN813032)	KJ933421.1	99%	*Periconia* sp.
13	KSU-13(LN812959)	AB752276.1	99%	*Phialocephala* sp.

relatively low, a maximum of 0.5 CFU in 100 ml. The highest total counts, over 10 CFU in 100 ml, were observed for *G. murorum, C. bantiana, E. cancerae and Phialocephala* sp. They, however, occurred only in one or two samples each. The genus *Aspergillus* occurred in six samples. *Aspergillus niger* occurred in two samples and six different *Aspergillus* species occurred each in one sample.

Several species found in drinking waters have been reported as emerging pathogens. Hageskal *et al.* (2012), reported *Aspergillus calidoustus*, *Penicillium spinulosum*, *Trichoderma viride* and *Fusarium solani* as potential pathogens and common drinking water system contaminants in Norway. According to their experiment on possible pathogenicity, Oliveira *et al.* (2013) classified several *Penicillium* and *Trichoderma* species as potential pathogens, but we did not identify exactly the same species in our samples. *Aspergillus niger*, which we identified, was classified as non-pathogenic by Oliveira *et al.* (2013). We identified an *Aspergillus* species in 26% of the contaminated samples. The genus is a common drinking water contaminant; it has been reported in several studies (Anaissie *et al.* 2003; Hageskal *et al.* 2006; 2007; 2009; Kennedy & Williams 2007; Kanzler *et al.* 2008; Pires-Gonçalves *et al.* 2008; Gashgari *et al.* 2013; Oliveira *et al.* 2016; Ma 2017).

In addition, many of these studies have reported the genus *Penicillium* to occur in drinking water samples. We observed it only in one sample. As a summary, we observed several species that, however, seemed to occur at low frequencies and are probably mostly non-pathogenic. Moreover, most of these potentially pathogenic fungi are only pathogenic after inhalation as opposed to ingestion (De Hoog *et al.* 2000; Zhou *et al.* 2007). Therefore, it seems that the fungal contamination in bottled water is not a great health risk to humans. However, a recent finding that the resistance to disinfection of *Penicillium* and *Aspergillus* species could facilitate their survival in drinking water systems (Ma 2017), raises a need for further studies about fungal contamination also in bottled waters.

The ITS rDNA sequences of the isolated fungal strains (submitted to the European Molecular Biology Laboratory (EMBL) were compared with published sequences at GenBank (Table 4). The GenBank accession numbers and the closest relatives of the isolates are listed in Table 4. The phylogenetic tree was established (Figure 1). The detected fungal strains

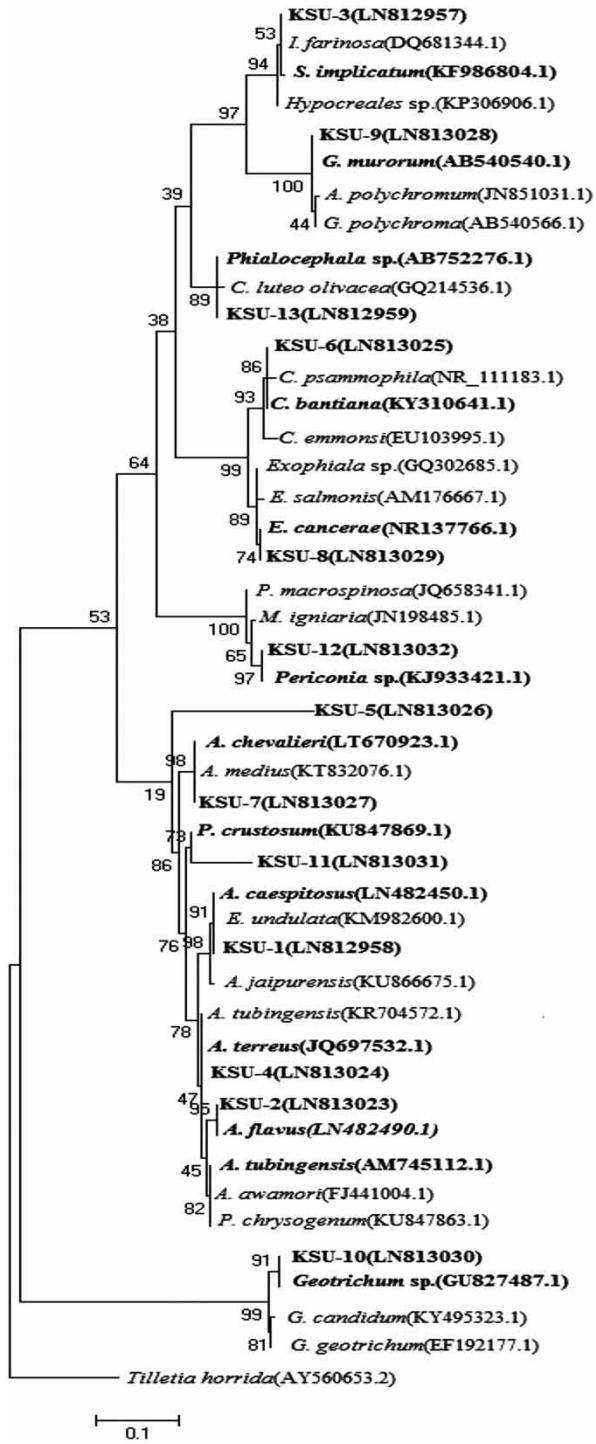

Figure 1 | Phylogenetic tree of ITS rDNA sequences of the fungi isolated from the bottled water and the selected reference sequences from public databases. Sequences obtained in the present study and their GenBank accession numbers are shown in bold. The tree was constructed by the neighbor-joining algorithm using the maximum composite likelihood model. Bootstrap percentages from 1,000 replicates are shown. The tree is rooted with *Tilletia horrida* [AY560653.2] as the out-group.

Table 5 | Fungal species and counts after storing the bottled waters for 0, 180 and 365 days

	0 Day		180 days		365 days	
S. no.	CFU in 100 ml	Fungal species	CFU in 100 ml	Fungal species	CFU in 100 ml	Fungal species
W33	3.50	*A. tubingensis* *A. chevalieri* *M. sterilium*	0.66	*A. tubingensis* *A. chevalieri* *M. sterilium*	0.66	*M. sterilium*
W34	0.00	–	0.00	–	0.00	–
W35	0.00	–	0.16	*P. crustosum*	1.99	*M. sterilium*
W36	0.00	–	0.00	–	0.00	–
W37	0.33	*C. sphaerospermum* *R. nigricans*	0.16	*M. sterilium*	0.33	*M. sterilium*

were classified as members of the subphylum Pezizomycotina and Saccharomycotina (phylum Ascomycota). All detected fungal strains were placed in six orders, Eurotiales (*A. tubingensis, A. chevalieri, A. caespitous, A. terreus, A. flavus* and *P. crustosum*), Chaetothyriales (*Exophiala cancerae* and *Cladophialophora bantiana*), Helotiales (*Phialocephala* sp.), Hypocreales (*Sarocladium implicatum* and *Gliomastix murorum*), Pleosporales (*Periconia* sp.), and Saccharomycetales (*Geotrichum* sp.) (Figure 1). Most fungal contaminants belonged to the Ascomycetes. This is in accordance with the previous finding of Cabral & Pinto (2002), who associated the contamination of eight different commercial brands of bottled water in Argentina mainly with Ascomycetes. More recently, Gashgari *et al.* (2013) reported that most mycobiota in four different drinking water distribution points in Jeddah City (Saudi Arabia) belonged to the Ascomycetes.

Five samples were selected to study the effect of storage on the growth of fungi. Two of the samples remained negative for fungal growth during the storage. In one sample, all three species identified at the beginning (0 days) survived for 180 d (Table 5). However, the fungi were not able to reproduce effectively, most likely due to the lack of nutrients. The CFU of the two species, *A. tubingensis and A. chevalieri*, and the unidentifiable species *M. sterilium*, decreased from 3.5 at the beginning (day 0) to 0.66 in 100 ml water after 180 d storage. After 365 d storage, the total count of *M. sterilium* was 0.66 in 100 ml water. Unidentified species (*M. sterilium*) were found in three out of the five 365 d stored bottled waters. Our results are only tentative because of the low number of replicates, but they support the interpretation of Morais & Da Costa (1990) and Ferreira *et al.* (1994) that microbial growth and species might change during storage due to the presence of oxygen and increasing surface area (mass of microbes) inside the packaging. However, fungi seem not to be able to grow in bottled water to any great extent. Thus, we suggest that even long storage bottled water does not increase the health risks for humans.

CONCLUSION

This is one of few studies about fungal contamination in bottled waters. The samples were of international trademarks and bought in Saudi Arabian markets. The diversity of fungi (18 species belonging to 11 fungal genera) occurring in bottled water seems to be relatively high. The species that frequently contaminated bottles were *Rhizopus nigricans* and seven different species of *Aspergillus*. *Penicillium* sp. were found in one sample. Although some species are known as pathogens, most of the species seemed to be non-pathogenic and thus, we conclude that the fungal contamination in bottled water seems to be a low risk to human health. Harmful health effects seem to be possible, mostly for immunocompromised people. However, the link to health effects is still not fully understood. Because we observed fungal contamination in more than half of the bottled waters studied, the fungi should be taken into account in the bottled water purification processes and in the quality control assessment. The ozone treatment may reduce fungal contamination in bottled waters. Future studies should focus on the mycotoxins the fungi are producing in water.

AUTHOR CONTRIBUTIONS

FA, AA and RG designed the experiment. FA performed the laboratory analyses and conducted data analyses. FA drafted the manuscript. All authors revised and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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