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ORIGINAL ARTICLE

Yeasts and moulds contaminants of food ice cubes and their survival in different drinks

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Abstract

Aims: To evaluate the levels of unicellular and filamentous fungi in ice cubes produced at different levels and to determine their survival in alcoholic beverages and soft drinks.
Methods and Results: Sixty samples of ice cubes collected from home level (HL) productions, bars and pubs (BP) and industrial manufacturing plants (MP) were investigated for the presence and cell density of yeasts and moulds. Moulds were detected in almost all samples, while yeasts developed from the majority of HL and MP samples. Representative colonies of microfungi were subjected to phenotypic and genotypic characterization. The identification was carried out by restriction fragment length polymorphism (RFLP) analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5·8S rRNA gene. The process of yeast identification was concluded by sequencing the D1/D2 region of the 26S rRNA gene. The fungal biodiversity associated with food ice was represented by nine yeast and nine mould species. Strains belonging to *Candida parapsilosis* and *Cryptococcus curvatus*, both opportunistic human pathogens, and *Penicillium glabrum*, an ubiquitous mould in the ice samples analysed, were selected to evaluate the effectiveness of the ice cubes to transfer pathogenic microfungi to consumers, after addition to alcoholic beverages and soft drinks. All strains retained their viability.
Conclusions: The survival test indicated that the most common mode of consumption of ice cubes, through its direct addition to drinks and beverages, did not reduce the viability of microfungi.
Significance and Impact of the Study: This study evidenced the presence of microfungi in food ice and ascertained their survival in soft drinks and alcoholic beverages.

Introduction

Fungi are the colonizers of several different environments. This phenomenon is mainly due to their high general competitive saprophytic ability (Griffin 1960). Moisture plays a defining role in the determination of the fungal community distribution and structure (Jacobson 1997). For this reason, yeasts and moulds dominate the microflora on raw fruits and vegetables (Beuchat 2002). Furthermore, some eukaryotic micro-organisms are able to grow at refrigeration temperatures (Li *et al.* 2001; Viljoen 2001). In general, several microfungi such a penicillia and most soil fungi can be considered ubiquitous (Fischer and Dott 2003) and can be easily dispersed in air (Adams *et al.* 2013). Fungi are increasingly gaining importance in view of health hazards and are relevant for indoor hygiene (Fischer and Dott 2003). The primary health impacts from fungi are faced by immunocompromised people, and are caused by inhalation and/or wound infection. Inhalation can lead to worsening of asthma symptoms, pneumonitis or fungal infection. Additionally, exposure of mucosal membranes to fungi may lead to airway infection (Novak Babič *et al.* 2017).

Several studies focused on the emerging pathogenic microfungi, their routes of contamination and the environmental factors that favour the process of colonization

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(Dynowska and Kisicka 2005; Gogu-Bogdan *et al.* 2014). The occurrence of fungi in drinking water has received increased attention in the last decades, and fungi are now generally accepted as drinking water contaminants (Hageskal *et al.* 2009). Furthermore, the presence of yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances has been recently evaluated (Novak Babič *et al.* 2016). Although ingestion is not generally considered a risk factor for fungal infections, fungi in drinks (and ice) may indicate potential risks from other water uses.

Food grade ice is the product obtained through the freezing of potable water. In general, as for water, the hygienic status of ice is determined by the presence of intestinal bacteria (Lateef *et al.* 2006; Ukwo *et al.* 2011) that are faecal indicators. Due to the increased worldwide request of ice cubes for human consumption, commonly added to cool drinks, the number of food ice industries increased proportionally, although a consistent production of ice cubes occurs by low volume ice cube maker machines (self-production), especially in bars, pubs and restaurants (Gaglio *et al.* 2017). The self-production of ice cubes is strictly intended for direct consumption, while industrial productions are packed in bags and commercialized. The Packaged Ice Quality Control Standards manual published by the International Packaged Ice Association provides the quality and processing standards for packaged ice produced by its members, even though the quality and safety of packaged ice products is not consistent (Lee *et al.* 2017).

In general, the microbiological investigations of ice focus on plate counts and the application of biochemical tests for the identification of the major pathogenic bacteria, mainly *Escherichia coli*, *Salmonella* spp., *Yersinia* spp., *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Shigella* spp., *Vibrio cholerae* and *Aeromonas* spp. (Nichols *et al.* 2000; Falcao *et al.* 2002; Gerokomou *et al.* 2011; Noor Izani *et al.* 2012; Awuor *et al.* 2016; Hampikyan *et al.* 2017). However, the presence of micro-organisms in ice cubes is not only due to the contamination of the water used (Lateef *et al.* 2006; Northcutt and Smith 2010), to the scarce hygienic conditions during production and the improper handling (Noor Izani *et al.* 2012), to the packaging containers or bags (Chavasit *et al.* 2011), but it also depends on the contamination by aerosol. Thus, the presence of microfungi deserves attention. In fact, these agents can be pathogenic and possess all characteristics to be spread through ice consumption. To support this thesis, psychrophilic yeasts have been reported in extremely cold environment such as Arctic, Antarctic and alpine snow (Anesio *et al.* 2017). Studies of fungi in drinking water have demonstrated that they are relatively common in water distribution systems. Species of pathogenic, allergenic and toxigenic concern are isolated from water, sometimes in high concentrations (Hageskal *et al.* 2009). The species more frequently found include some opportunistic human pathogens such as *Candida* and *Fusarium* species (Novak Babič *et al.* 2015, 2016).

Based on the above considerations, the aim of the present work was to: (i) evaluate the presence and levels of unicellular and filamentous fungi of food ice produced a three different levels, including small volume (homemade), medium volume (restaurant level) and high volume (industrial level) facilities; (ii) perform phenotypic and genotypic characterization of the isolates; (iii) monitor the survival of representative strains in different alcoholic beverages and soft drinks.

Materials and methods

Sample collection

The samples of ice cubes were collected from domestic freezers, produced at home level (HL) and representing the “from freezer to glass” route without any additional manipulation (Gaglio *et al.* 2017), from ice holding/storage equipment of bars and pubs (BP), for the self-productions performed with low volume ice machines representing the production stored without bags, and from sales packages of ice produced by industrial manufacturing plants (MP) and sold in plastic bags. Five producers per each production level (HL1-HL5, BP1-BP5, MP1-MP5) were selected within the Palermo province (Sicily, Italy), in order to border the source of water supply. Domestic and bar/pub samples were collected aseptically and transferred into sterile stomacher bags, while the industrial samples were left in the manufacturers’ plastic bags during transport. All productions were performed with tap water supplied by the municipal source. Five houses/bars/industrial plants were investigated for each production level and four samples were collected in duplicate (the same day) at a 2-month interval from each plant. Thus, a total of 60 samples were collected to perform this study. The samples were transported into thermal insulated boxes.

Microbiological analyses of ice samples

Depending on the weight of single ice cubes (ranging between 10 and 20 g approximately for the different shapes), about 25–50 cubes corresponding to 500 g were left thawing in a 1 l sterile Dhuram bottle at ambient temperature. Two 100 ml aliquots from each sample collected were separately analysed by membrane filtration in order to investigate unicellular and filamentous fungi. Both eukaryotic microbial groups were inoculated on

malt agar (Oxoid, Milan, Italy) supplemented with 0·1 g l^{-1} chloramphenicol (Sigma-Aldrich, Milan, Italy) to avoid bacterial growth. For the determination of yeasts the plates were incubated at 28°C for 48 h, while for moulds at 25°C for 7 d. When the number of colonies from a given sample exceeded the number of squares (n = 186) present on the membrane grid, or several colonies showed a clear confluent growth, aliquots of 1 ml from this sample were directly inoculated onto malt agar (Oxoid). Two 1-ml aliquots were collected after vigorous manual agitation of thawed ice.

Isolation and grouping of yeasts and moulds

After growth, colonies of yeasts were picked up from the plates. For each morphology (colour, margin, surface and elevation), five identical colonies (or fewer if five were not available or showed confluent growth) were collected. Yeast isolates were subjected to purification after several consecutive subcultures onto malt agar incubated at 28°C for 48 h. The purity of the cultures was verified by an optical microscope and the yeasts were grouped on the basis of their cell morphology.

Several mould colonies were picked up from agar plates and purified to homogeneity after several subculturing steps onto malt agar until each colony reached the diameter of about 2 mm. The isolates were differentiated by macroscopic characterization including colour, texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae, growth rate and topography. The taxonomic investigation was also performed through microscopic characterization of the isolates including the analysis of stipes, vesicles, phialides, metulae and conidia (Barnett and Hunter 1998; Domsh *et al.* 2007).

Genetic identification

Fresh pure cultures of yeasts and moulds were subjected to DNA extraction by lysis applying the Instagene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction and the cell extracts were used as templates for PCRs.

All yeast isolates were preliminary grouped by restriction fragment length polymorphism (RFLP) analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5·8S rRNA gene as reported by Esteve-Zarzoso *et al.* (1999). Each reaction mixture contained 0·5 μmol l^{-1} primer ITS 1 (5′ TCCGTAGGTGAA CCTGCGG 3′), 0·5 μmol l^{-1} primer ITS4 (5′ TCCTCC GCTTATTGATATGC 3′), 10 μmol l^{-1} deoxynucleotides mix (Life Technologies Italia, Monza, Italy), 1·5 mmol l^{-1} $MgCl_2$, and 1 × buffer (Fermentas, MMedical, Milan, Italy). Prior amplification, performed by a T1 Thermocycler (Biometra, Göttingen, Germany), the suspension was heated at 95°C for 15 min and then 1 U of *Taq* DNA polymerase (Fermentas), 2·5 ng of DNA, and Milli-Q® water (Millipore, Billerica, MA, USA) were added to each tube reaching the final reaction volume of 20 μl. The PCR program applied comprised 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 55·5°C, and extension for 2 min at 72°C; the cycles were preceded by denaturation at 95°C for 5 min and followed by extension at 72°C for 10 min. The amplicons (10 μl) were digested overnight at 37°C without further purification with the restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I (Fermentas) The PCR products and their restriction fragments were separated on 1·4% and 3% agarose gels, respectively, with 1 × TAE buffer, stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA), and subsequently visualized by UV transillumination. The GeneRuler 50 bp Plus DNA Ladder (MMedical S.r.l, Milan, Italy) was used as a molecular weight marker.

The isolates representative of each RFLP group were identified at species level by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified and the PCR products visualized as described by Francesca *et al.* (2014). PCR reactions were performed using the primer pair NL1/NL4 (O'Donnell 1993) in a 30 μl reaction volume. PCR mixture contained 0·25 mmol l^{-1} of dNTP mix (Life Technologies Italia), 1·5 U of Taq DNA polymerase (Fermentas), 3 μl of PCR buffer containing 20 mmol l^{-1} $MgCl_2$ (Fermentas), 0·2 μmol l^{-1} of both primers, 25 ng of DNA, and was brought to the final volume with Milli-Q® water (Millipore). PCR program comprised an initial template denaturation step for 5 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 45 s at 52°C and extension for 1 min at 72°C. The final elongation step was for 7 min at 72°C.

The identification of moulds was concluded genetically. Genomic DNA was extracted from single spore cultures following a standard cetyl-trimethyl-ammonium-bromide-based protocol (O'Donnell *et al.* 1998). Sequencing of the 5·8S-ITS rRNA gene were performed as described by Alfonzo *et al.* (2013). PCR reactions were performed using the primer pair ITS1f/ITS4 in a 40 μl reaction volume. PCR mixture contained 0·40 mmol l^{-1} of dNTP mix (Life Technologies Italia), 2·5 U of Taq DNA polymerase (Fermentas), 4 μl of PCR buffer containing 20 mmol l^{-1} $MgCl_2$ (Fermentas), 0·25 μmol l^{-1} of both primers, 25 ng of DNA, and was brought to the final volume with Milli-Q® water (Millipore). PCR program comprised an initial template denaturation step for 2 min at 95°C followed by 34 cycles of denaturation for

30 s at 95°C, annealing for 20 s at 55°C and extension for 1 min at 72°C. The final elongation step was for 5 min at 72°C.

DNA fragments from yeast and mould DNAs were visualized and the amplicons were purified by the QIAquick purification kit (Qiagen S. p.a., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequencing reactions were performed by AGRIVET (Palermo, Italy). The identity of yeast and mould sequences was determined by comparison with the sequences available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov). All sequences were deposited in GenBank under their accession numbers.

Artificial contamination of ice cubes and survival of yeasts and moulds in different systems

The strains of yeasts and moulds found at the highest levels in the ice cubes and belonging to species known as human pathogens were inoculated at the same levels detected in commercial ice cubes and tested for their survival in alcoholic beverages, such as vodka (Keglevich, Plzen, Czech Republic), whisky (Jack Daniel's, Lynchburg, Tennessee) and Martini (Martini and Rossi, SpA, Pessione, Italy) containing 38, 40 and 14·4% vol of alcohol, respectively, and soft drinks, such as peach tea (Conad, Fruttagel SCpA, Alfonsine, Italy), tonic water (Kinley, Sesto S. Giovanni, Italy) and coke (Coca Cola Italia Srl, Sesto S. Giovanni, Italy). These systems were previously chosen by Gaglio *et al.* (2017) as being characterized by different pH, alcohol volume, sugar content, presence or absence of CO_2 and antimicrobial compounds. Ringer's solution (Oxoid) was used to perform the control trial, in order to exclude any inhibitory effect due to the chemical components of the drink systems.

Yeast and mould strains were first cultivated under their optimal growth conditions. Yeast cells were washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy), centrifuged at 5000 × ***g*** for 5 min at 4°C and then suspended singly in sterile (treatment at 121°C for 20 min) still mineral water (Terme di Geraci Siculo S.p.a., Geraci Siculo, Italy). The levels of yeast inocula occurred at the highest cell densities found in the samples analysed which was checked spectrophotometrically (optical density at 600 nm) by a 6400 Spectrophotometer (Jenway Ltd., Felsted, Dunmow, UK) and confirmed by plate count on malt agar. Filamentous fungus propagules were collected after mycelium growth by means of a sterile surgical blade and re-suspended in Ringer's solution. Spore concentration was determined by Burker's chamber (Carl Zeiss, Oberkochen, Germany). In order to ensure the presence of the sole micro-organism to be tested, contaminated ice cubes were produced using autoclaved stainless steel ice cube trays at −32°C. The test was performed as follows: three artificially inoculated ice cubes (corresponding to 60 ml) were added to 100 ml of each drink in 200 ml volume sterile cups (Anicrin, Scorzé, Italy) and left 1 h at room temperature before membrane filtration analysis. Three independent experimentations were carried out.

Statistical analyses

Yeast and mould loads were subjected to one-way analysis of variance (ANOVA). Pair comparison of treatment means was achieved by Tukey's procedure at $P < 0{\cdot}05$. Differences between the production levels (HL, BP and MP) were evaluated with the Generalized Linear Model (GLM) procedure. The statistical analysis was conducted with SAS 9·2 software (Statistical Analysis System Institute Inc., Cary, NC, USA).

Results

Levels of fungi in the ice cubes

The levels of yeasts and moulds in the ice cube samples analysed were expressed as colony forming units (CFU) per 100 ml of thawed ice (t.i.). and are reported in Table 1. Although filtration is not an invasive treatment responsible for the damage of mould colonies, it cannot be excluded that hyphae and conidia from a single colony generated multiple colonies of the same type. Plate counts showed statistical significant differences ($P \leq 0{\cdot}001$) among the samples collected within the three production levels for both groups investigated. Except sample MP1, moulds were always registered with levels ranging between 0·8 and 195·3 CFU per 100 ml t.i., while yeasts developed from the majority of HL and MP samples and only two BP samples. Interestingly, the levels of moulds were below 10 CFU per 100 ml t.i. for 10 ice samples, including all those produced at industrial level. Regarding yeasts, the highest numbers were registered for the domestic samples, in particular, a cell density of 383·8 CFU per 100 ml t.i. was displayed by the samples HL5. The statistical analysis resulting from the interaction of load data for the three ice cube production levels (HL × BP × MP) indicated that yeast and mould levels were significantly different ($P \leq 0{\cdot}001$ and $P \leq 0{\cdot}05$, respectively).

Biodiversity of eukaryotic micro-organisms associated to the ice cubes

After enumeration, colonies of yeasts and moulds were isolated from malt agar. All different morphologies present in plates were collected and purified before

Table 1 Levels of yeasts and moulds of ice cubes produced at different levels†

Ice "samples	Cities‡	Microbial groups§	
		Yeasts	Moulds
HL1	Palermo	0^{A}	$17{\cdot}5 \pm 2{\cdot}4^{D}$
HL2	Villabate	0^{A}	$35{\cdot}5 \pm 4{\cdot}1^{E}$
HL3	Termini imerese	$253{\cdot}5 \pm 24{\cdot}1^{C}$	$1{\cdot}0 \pm 0^{A}$
HL4	Castelbuono	$39{\cdot}5 \pm 2{\cdot}1^{B}$	$12{\cdot}8 \pm 1{\cdot}7^{C}$
HL5	San Giuseppe Jato	$383{\cdot}8 \pm 20{\cdot}5^{D}$	$2{\cdot}3 \pm 1{\cdot}5^{B}$
Statistical significance		***	***
BP1	Palermo	$43{\cdot}5 \pm 14{\cdot}1^{B}$	$7{\cdot}3 \pm 1{\cdot}5^{C}$
BP2	Palermo	0^{A}	$2{\cdot}3 \pm 0{\cdot}5^{B}$
BP3	Palermo	0^{A}	$2{\cdot}0 \pm 1{\cdot}2^{B}$
BP4	Castelbuono	$116{\cdot}5 \pm 6{\cdot}0^{C}$	$0{\cdot}8 \pm 0{\cdot}5^{A}$
BP5	Palermo	0^{A}	$195{\cdot}3 \pm 21{\cdot}4^{D}$
Statistical significance		***	***
MP1	Not available	$0{\cdot}8 \pm 0{\cdot}5^{B}$	0^{A}
MP2	Not available	$16{\cdot}3 \pm 3{\cdot}0^{D}$	$1{\cdot}0 \pm 0{\cdot}8^{B}$
MP3	Not available	$3{\cdot}5 \pm 1{\cdot}7^{C}$	$1{\cdot}3 \pm 1{\cdot}0^{B}$
MP4	Not available	$1{\cdot}3 \pm 1{\cdot}0^{B}$	$6{\cdot}5 \pm 1{\cdot}9^{C}$
MP5	Not available	0^{A}	$0{\cdot}8 \pm 0{\cdot}5^{B}$
Statistical significance		***	***
Interaction among the production levels (HL × BP × MP)		***	*

HL, home level; BP, bars and pubs; MP, manufacturing plants.
Results indicate mean values ± SD of four microbiological counts (carried out in duplicate for two independent sample collections).
Data within a column followed by the same letter are not significantly different according to Tukey's test.
†CFU per 100 ml of thawed ice.
‡All cities are located within Palermo province (Sicily, Italy).
§Yeasts on malt agar incubated for 48 h; moulds on malt agar incubated for 7 days.
*$P \leq 0{\cdot}05$; ***$P \leq 0{\cdot}001$.

microscopic inspection. After the preliminary characterization of the eukaryotic micro-organisms, based exclusively on colony and cell morphology, 153 yeasts and 81 filamentous fungi were selected for the genetic identification.

The combination of band lengths from 5·8S-ITS and the RFLP profiles allowed the identification of nine yeast species (Ac. No. KX609387 – KX609392, KX609394 – KX609396) from the nine ice samples positive for the growth of unicellular fungi (Table 2). A single species was found associated with each HL ice cube production; in particular, *Metschnikowia* sp., *Meyerozyma guilliermondii* and *Cystobasidium slooffiae* were identified from the samples HL3, HL4 and HL5 respectively. A higher biodiversity of yeasts was revealed for the ice cubes collected from bars and pubs, since the only two productions positive for the development of colonies on malt agar after 48 h of incubation contained two species each: *Candida intermedia* and *Cryptococcus curvatus* from sample BP1, while *Pichia guilliermondii* and *Yarrowia lipolytica* from BP4. The most limited yeast diversity was observed for the industrial ice cubes with two species in four samples, *Candida parapsilosis* in MP3 and *Rhodotorula mucilaginosa* in MP1, MP2 and MP4.

The direct comparison of the 5·8S-ITS sequences of filamentous fungi with those available in NCBI indicated that the ice cubes hosted the following species: *Fusarium* sp., *Fusarium solani*, *Hansfordia* sp., *Paecilomyces* sp., *Paecilomyces lilacinus*, *Penicillium glabrum*, *Phoma leveillei*, *Purpureocillium* sp. and *Thanatephorus cucumeris* (Ac. No. KX609397 – KX609405). Regarding their sample of isolation, the home-made ice cubes were contaminated by *Hansfordia* sp. (one sample, HL3) and *P. glabrum* (all samples), those collected from bars and pubs by *Pc. lilacinus*, *Ph. leveillei*, *Purpureocillium* sp. and *T. cucumeris*, almost equally distributed in the different samples, while the four industrial samples hosted *Paecilomyces* sp., and *Fusarium* sp.

Yeast and mould survival in beverages and drinks

The strains used to test the persistence of unicellular and filamentous fungi of ice origin in drink systems characterized by different conditions belonged to the species *C. parapsilosis* (strain ICE214) and *Cr. curvatus* (strain ICE84) among yeasts, since they are reported as human pathogens, while *P. glabrum* (strain ICE139) was chosen among moulds due to its ubiquity among ice samples. Mineral water after autoclave sterilization, as well as all beverages and drinks before ice addition, did not contain any viable micro-organisms as detected by membrane filtration analysis. The three strains were inoculated at the highest levels found for yeasts and moulds during the microbiological ice cube characterization. For this reason, the artificially contaminated ice cubes were produced with yeasts and moulds at levels in the range 200–400 CFU per 100 ml t.i. The survival tests (Fig. 1) showed that all three micro-organisms remained at almost the same levels, since no statistical differences were registered for the cell densities evaluated in the six systems and Ringer's solution (control trial). The negative control represented by noncontaminated ice cubes tested in Ringer's solution did not generate any colony development. Thus, none of the alcoholic beverages and soft drinks affected the viability of *C. parapsilosis* ICE214, *Cr. curvatus* ICE84 and *P. glabrum* ICE139.

Table 2 Molecular identification of fungi

Species	Strain	Ice cube samples	5·8S-ITS PCR	Size of restriction fragments *Cfo*I	*Hae*III	*Hin*fI	% similarity*
Yeasts							
Candida intermedia	ICE86	BP1	390	220 + 170	390	220 + 170	99 (FJ455102·1)
Candida parapsilosis	ICE214	MP3	510	300 + 220	400 + 110	250 + 270	99 (KU316730·1)
Cryptococcus curvatus	ICE84	BP1	510	250 + 275	490	235 + 245	100 (HQ323253·1)
Cystobasidium slooffiae	ICE296	HL5	590	590	590	250 + 340	99 (KC442284·1)
Metschnikowia sp.	ICE192	HL3	390	220 + 90	290 + 100	200 + 190	99 (KT922823·1)
Meyerozyma guilliermondii	ICE210	HL4	600	300 + 250	395 + 125 + 80	300 + 320	99 (LC134306·1)
Pichia guilliermondii	ICE250	BP4	600	300 + 250	400 + 125 + 75	300 + 320	100 (FJ468466·1)
Rhodotorula mucilaginosa	ICE29	MP1, MP2, MP4	600	300 + 225	210 + 400	350 + 220	100 (KR632581·1)
Yarrowia lipolytica	ICE251	BP4	375	205 + 170	375	200 + 175	100 (KF830192·1)
Moulds							
Fusarium sp.,	ICE33	MP2-MP5	n.d.	n.d.	n.d.	n.d.	100 (LC184244·1)
Fusarium solani	ICE31	MP2-MP5	n.d.	n.d.	n.d.	n.d.	99 (AM412594·1)
Hansfordia sp.	ICE303	HL3	n.d.	n.d.	n.d.	n.d.	99 (KF877718·1)
Paecilomyces sp.	ICE30	MP2-MP5	n.d.	n.d.	n.d.	n.d.	96 (GQ229083·1)
Paecilomyces lilacinus	ICE121	BP1-BP5	n.d.	n.d.	n.d.	n.d.	100 (KF367485·1)
Penicillium glabrum	ICE139	HL1-HL5	n.d.	n.d.	n.d.	n.d.	100 (KX664349·1)
Phoma leveillei	ICE122	BP1-BP5	n.d.	n.d.	n.d.	n.d.	99 (KT963795·1)
Purpureocillium sp.	ICE309	BP1-BP5	n.d.	n.d.	n.d.	n.d.	96 (KY318491·1)
Thanatephorus cucumeris	ICE311	BP1-BP5	n.d.	n.d.	n.d.	n.d.	99 (KX928843·1)

n.d., not determined.
All values are given in bp.
*According to BlastN search in NCBI database of D1/D2 26S rRNA gene sequences for yeasts and 5·8S-ITS rRNA gene for moulds.

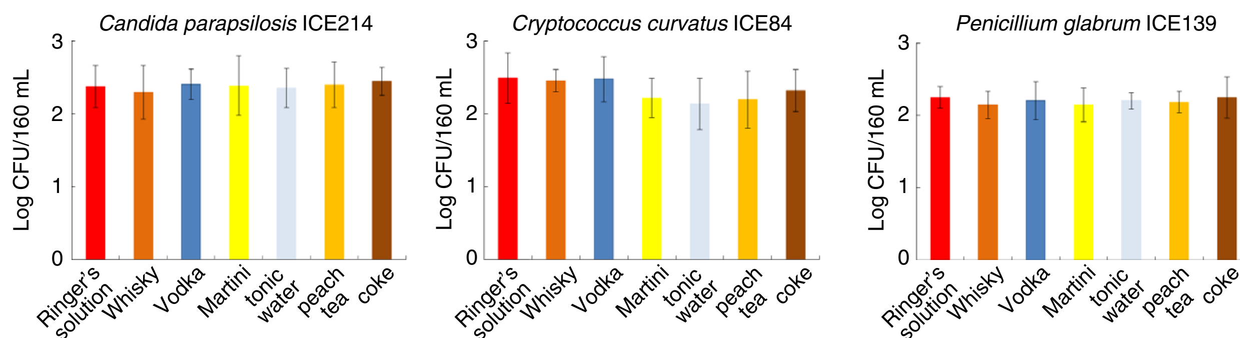

Figure 1 Change in cell density of dominating yeasts (*Candida parapsilosis* ICE214 and *Cryptococcus curvatus* ICE84) and filamentous fungi (*Penicillium glabrum* ICE139) isolated from food ice and added to different alcoholic beverages (whisky, vodka and Martini) and soft drinks (coke, tonic water and peach tea). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

The survival of micro-organisms in ice cubes has been the object of several very recent studies (Awuor *et al.* 2016; Economou *et al.* 2017; Gaglio *et al.* 2017; Hampikyan *et al.* 2017; Lee *et al.* 2017). In general, the resistance of micro-organisms to water freezing temperatures is strictly dependent on their cytoplasm membrane. The alterations in the organization of membrane lipids (Los and Murata 2004) and the function of membrane-associated enzymes and transporters (Avery *et al.* 1995) are the direct consequences of the temperature reduction. The fluidity of membrane lipids is assured by the unsaturation of lipid acyl chains (Sakamoto *et al.* 1998; Sánchez-García *et al.* 2004). Although the principal microbiological risks of food ice are represented by members of *Enterobacteriaceae* family, with *Salmonella*, *Shigella*, *Yersinia* and *Escherichia* being mainly investigated (Falcao *et al.* 2002), some eukaryotic micro-organisms, such as *Cryptosporidium*, are cause of waterborne outbreaks (Betancourt and Rose 2004). However, to our knowledge, no investigation on the presence of

unicellular and filamentous fungi on ice cubes has been conducted so far.

The main aim of this work was to investigate the presence of yeasts and moulds in ice, in order to provide a deepen insight on the complex microbial community associated to food ice cubes. To this purpose, several samples were collected at different levels, including domestic (small volume), restaurant (medium volume) and industrial (high volume) productions. Unicellular and filamentous fungus populations were characterized through a culture dependent polyphasic approach including viable counts, determined applying the membrane filtration method applied for water analysis, phenotypic and genotypic analyses of the isolates.

In this study, home-made ice samples were included in the survey to provide information on the hygiene of the domestic freezers. In these samples, the consistent presence of moulds (all domestic ice samples were positive for colony development) could be attributable to the freezer aerosol continuously contaminated with various foods. *Penicillium* are reported as common inhabitants of the domestic refrigerators (Altunatmaz *et al.* 2012). Thus, the aerosol of refrigerators can easily contaminate the foods stored (Salustiano *et al.* 2003). A similar phenomenon might be supposed for freezers when ice cubes, are not generally covered by a protective envelope.

Only six of the 60 samples of ice cubes did not contain moulds and the range of detection was between 0·8 and 195·3 CFU per 100 ml t.i., with the highest levels found in samples from bars and pubs. Kelley *et al.* (2003) reported an average level of 28 CFU per l in distribution systems. Regarding tap water, Gonçalves *et al.* (2006) reported 4 CFU per l as the highest level of filamentous fungi in samples collected in Portugal, while Hedayati *et al.* (2011) detected a range 1–22 CFU per 100 ml for Iranian samples. A lower number of samples was positive for the presence of yeasts, but their levels, especially in the samples produced at domestic scale, were generally higher than those of moulds. Both eukaryotic groups were investigated at species level in order to better analyse the microbiological characteristics of food ice. Among the nine yeasts identified only *Candida* species, agents of candidal infections (van't Wout 1996), and *Cr. curvatus*, a rare opportunistic pathogen (Dromer *et al.* 1995), represent a risk for humans. Hospital sinks have been reported as a potential nosocomial source of *Candida* infections (Jencson *et al.* 2017). The incidence and epidemiology of *C. parapsilosis* fungemia was evaluated from active population-based surveillance in Barcelona and it was found that this agent accounted for 23% of all fungemias (Almirante *et al.* 2006), highlighting its relevance for the human health. All yeast species detected in this study are of environmental origin. Among filamentous fungi, *Penicillium* was confirmed to dominate the mould community of the home-made samples. Some of the species detected can be agents of human mycosis, in particular, *Fusarium* (Melcher *et al.* 1993), *Pc. lilacinus* (Castro *et al.* 1990) and *Purpureocillium* sp. (Luangsa-ard *et al.* 2011).

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). Hence, in the second part of the work, some strains representative of the yeast and mould community found in ice cubes were evaluated for their survival in several drinks and beverages characterized by different levels of alcohol, CO_2, pH and antibacterial ingredients, in order to monitor the transfer of the microbial load of ice to consumers. Thus, *C. parapsilosis*, *Cr. curvatus* (both reported as human pathogens) and *P. glabrum* (found in the majority of samples) were tested in whisky, vodka, Martini, coke, peach tea and tonic water, after the production of artificially contaminated ice cubes. Unlike bacteria (Gaglio *et al.* 2017), yeasts and moulds did not change their levels, showing that none of the drink/beverage system used the in the survival test exerted a lethal effect on their cell viability.

In conclusion, this study reported the levels and the species composition of unicellular and filamentous fungi hosted in ice cubes produced at three different levels (home-made, restaurant and industrial) finding significant differences among the production systems. The survival test indicated that the most common mode of consumption of ice cubes, through its direct addition to drinks and beverages, does not reduce the viability of these agents. Thus, the presence of potentially pathogenic yeasts and moulds in ice cubes represents a risk for consumers.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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