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Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks

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ABSTRACT

This study aimed to evaluate the levels of enteric bacteria in ice cubes produced in different environments (home-made, prepared in bars and pubs with ice machines and produced in industrial plants) and to determine their survival in different alcoholic beverages and soft drinks. Members of the *Enterobacteriaceae* family were found in almost all samples analysed. All industrial and the majority of home-made samples did not contain coliforms. Enterococci were not identified in domestic samples while they were detected in two industrial and three bar/pub samples. The samples collected from bars and pubs were characterized by the highest levels of enteric bacteria. Fourteen strains representing 11 species of eight bacterial genera were identified, some of which are known agents of human infections. The most numerous groups included *Enterococcus* and *Stenotrophomonas.* The survival of *Enterococcus faecium* ICE41, *Pantoea conspicua* ICE80 and *Stenotrophomonas maltophilia* ICE272, that were detected at the highest levels (100–400 CFU/100 mL thawed ice) in the ice cubes, was tested in six drinks and beverages characterized by different levels of alcohol, CO_2, pH and the presence of antibacterial ingredients. The results showed a species-specific behaviour and, in general, a reduction of the microbiological risks associated with ice after its transfer to alcoholic or carbonated beverages.

1. Introduction

The production of ice, especially ice cubes, is increasing notably due to a continuous and high demand for ice cubes in bars, pubs and restaurants. High volumes of this product are produced by ice cube maker machines (self-production) or by specialized industries. The self-production of ice cubes is strictly intended for direct consumption, while industrial productions are packed in bags and commercialized.

"Ice which comes into contact with foods or which may contaminate foods has to be made from potable water or, when used to chill whole fishery products, clean water. It has to be made, handled and stored under conditions that protect it from contamination" (Commission Regulation, 2004). Hence, food grade ice, or simply "food ice", is a product that after melting becomes potable water and must have the same chemical and microbiological characteristics of the water before freezing (Falcao et al., 2002). Thus, in order to produce food ice, the industrial process must follow specific and stringent guidelines (INGA, 2015).

The resistance of microorganisms to low temperatures is strictly dependent on their cytoplasmic membrane. Temperature reductions cause alterations in the organization of membrane lipids (Los and Murata, 2004) and alter the functionality of membrane-associated enzymes and transporters (Avery et al., 1995). To maintain the appropriate fluidity of membrane lipids the unsaturation of lipid acyl chains occurs in several microorganisms at low temperatures (Sakamoto et al., 1998; Sánchez-García et al., 2004). Bacteria synthesize long chain fatty acids (Russell, 1984) and higher proportions of unsaturated or branched fatty acids facilitate phase transition at a lower temperature (Freedman, 1981). For this reason, the water used to produce food ice must not contain pathogenic microorganisms that survive during the freezing process (Dickens et al., 1985). Generally, the microbiological risk factors of ice are represented by members of the *Enterobacteriaceae* family, mainly belonging to the genera *Salmonella*, *Shigella*, *Yersinia* and

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Escherichia (Falcao et al., 2002).

The microbiological investigation of ice used for foods and drinks in several countries revealed that it could be the cause for human health concerns due to the occurrence of gastroenteritis (Falcao et al., 2004; Gerokomou et al., 2011; Graman et al., 1997; Lateef et al., 2006; Nichols et al., 2000; Noor Izani et al., 2012; Wilson et al., 1997). The presence of pathogenic microorganisms in ice cubes is attributable to the contamination of the water used (Lateef et al., 2006; Northcutt and Smith, 2010), poor sanitary conditions during production, improper handling (Noor Izani et al., 2012) and/or the final packaging containers or bags (Chavasit et al., 2011).

Due to the hygiene issues related to ice production, the demand for industrially manufactured ice cubes applying high process standards is growing and, consequently, the number of food ice industries is increasing proportionally (Stucchi, oral communication). According to the International and European Packaged Ice Association (I.P.I.A and E.P.I.A, respectively) current production volumes in Europe is estimated at 500,000 tonnes per year. Considering the increasing trend for production in this sector, there is an urgent need to evaluate the hygienic quality of the products available on the market.

Based on these considerations, the present study aimed to (1) evaluate the presence and levels of enteric bacteria of different samples of ice cubes produced at different levels; (2) perform phenotypic and genotypic characterization of the isolates and; (3) monitor the survival of representative strains in different drink systems.

2. Materials and methods

2.1. Collection of ice cubes

The ice cubes analysed in this study were collected from three different production levels i.e. domestic freezers (DF), ice machines (IM) in bars and pubs and industrial ice production facilities (IN). The water supply for all ice productions was the municipal source which is subjected to the Italian guidelines "D. Lgs. n° 31, 02/02/2001" for the implementation of directive 98/83/EC on the quality of water for human consumption. The samples ranged from 1 kg (DF) to 5 kg (IM and IN) while the individual ice cube weight ranged from 10 to 20 g, depending on the shape. DF ice was produced from tap water, while IM and IN productions were obtained from water stored in tanks. DF and IM samples were collected from stock boxes and transferred into sterile stomacher bags. IN samples were provided in the manufacturers' plastic bags. Samples were transported into thermal insulated boxes. Five samples of each production level (DF1-DF5, IM1-IM5, IN1-IN5) were collected in duplicate (the same day) with a 2-month interval (April and June 2016), forming a total of 60 samples. All ice producers were located within the Palermo province (Sicily, Italy).

2.2. Determination of the level of contamination of ice samples by enteric bacteria

Each ice sample was thawed in a 1 L sterile Dhuram bottle at room temperature and subjected to membrane filtration analyses (250 mL). The following enteric bacterial groups were investigated: members of the *Enterobacteriaceae* family on violet red bile glucose agar (VRBGA), incubated at 37 °C for 24 h; coliforms on violet red bile agar (VRBA), incubated at 37 °C for 24 h; enterococci on kanamycin aesculin azide (KAA) agar, incubated at 37 °C for 24 h. All media and supplements were purchased from Oxoid (Milan, Italy). When the colonies were uncountable (n > 186, corresponding to the number of squares on the membrane grid) and showed a confluent growth, aliquots of 1 mL from samples were directly inoculated into agar media.

2.3. Isolation and grouping of bacteria

After growth, five identical colonies (or fewer if five were not available or showed confluent growth) of each observed morphology (color, margin, surface and elevation) were selected for further analysis. All isolates were purified after several consecutive subcultures on the same media and under the same growth conditions as those used for plate counts. The purity of the cultures was verified by an optical microscope.

In order to perform a phenotypic grouping of bacteria, all isolates were preliminarily characterized using the KOH test (Gregersen, 1978) to determine the type of cell wall and the catalase test by addition of H_2O_2 (5%, w/v) to the colonies. Furthermore, the cell morphology and motility were evaluated by microscopic inspection.

2.4. Genetic characterization and identification

Genomic DNA for PCR assays was prepared from bacterial cultures after their overnight growth in broth media at 37 °C. The isolates from VRBGA and VRBA were developed in Brain Heart Infusion (Oxoid), while enterococci in M17 (Oxoid). Cells were harvested by centrifugation at 5000 × g for 5 min and subjected to DNA extraction using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The concentration of each DNA sample was adjusted to 25 ng/μL.

The differentiation of the bacterial cultures at strain level was performed by random amplification of polymorphic DNA (RAPD)-PCR using single primers M13 (Stenlid et al., 1994), AB111, and AB106 (van den Braak et al., 2000) in a 25-μL reaction mix. Each reaction mixture contained 0.2 mM of dNTP mix (Life Technologies Italia, Monza, Italy), 0.625 U of *Taq* DNA polymerase (Fermentas, M-Medical, Milan, Italy), 2.5 μL of PCR buffer (Fermentas), 2.5 mM of $MgCl_2$, 2 μM of each primer, 2.5 ng of DNA, and Milli-Q® water (Millipore, Billerica, MA, USA) to reach the final reaction volume. The PCR program applied for all primers comprised 40 cycles of denaturation for 2 min at 94 °C, annealing for 20 s at 40 °C, and extension for 2 min at 72 °C; the cycles were preceded by denaturation at 94 °C for 2 min and followed by extension at 72 °C for 5 min. The amplifications were performed using a T1 Thermocycler (Biometra, Göttingen, Germany) and the amplicons were separated by electrophoresis on a 2% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France), stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA), and subsequently visualized by UV transillumination. The GeneRuler 100 bp Plus DNA Ladder (M-Medical S. r.l, Milan, Italy) was used as a molecular weight marker. RAPD patterns were analysed using Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium). A dendrogram showing the relationships among ice enteric bacteria was constructed with the combination of the RAPD profiles obtained with the three primers. The combined patterns were analysed using the Pearson's product moment correlation coefficient and the unweighted pair-group method using arithmetic average (UPGMA). The reproducibility of the RAPD-PCR fingerprints was assessed by comparing the PCR products obtained with the primers M13, AB106 and AB111 using DNA extracted from three separate cultures of two strains (ICE41 and ICE80).

The identification of the strains at species level was obtained by 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991) using the primer pair fD1/rD1 in a 30-μL reaction volume. PCR mixture contained 0.25 mM of dNTP mix (Life Technologies Italia), 1.5 U of *Taq* DNA polymerase