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Cultivable microorganisms associated with honeys of different geographical and botanical origin

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ABSTRACT

In this study, the composition of the cultivable microbial populations of 38 nectar honey and honeydew honey samples of different botanical and geographical origin were assessed. After growth in specific media, various colonies with different appearance were isolated and purified before phenotypic (morphological, physiological and biochemical traits) and genotypic [randomly amplified polymorphic DNA (RAPD), repetitive DNA elements-PCR (rep-PCR) and restriction fragment length polymorphism (RFLP)] differentiation. The identification was carried out by 16S rRNA gene sequencing for bacteria and, in addition to RFLP, by sequencing the D1/D2 region of the 26S rRNA gene for yeasts and the 5.8S-ITS rRNA region for filamentous fungi. The results showed the presence of 13 species of bacteria, 5 of yeasts and 17 of filamentous fungi; the species most frequently isolated were *Bacillus amyloliquefaciens*, *Zygosaccharomyces mellis* and *Aspergillus niger* for the three microbial groups, respectively. The highest microbial diversity was found in multifloral honeys. No correlation among the microbial species and the botanical/geographical origin was found, but some strains were highly adapted to these matrices since they were found in several samples of different origin.

1. Introduction

According to the European Union Legislation (DLgs 179/2004) and the *Codex Alimentarius* (CODEX STAN 12-1981) honey is the natural sweet substance produced by *Apis mellifera* L. bees from the nectar of plants, secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature.

Honey is the most ancient sweetener used by mankind, appreciated throughout the word, embraced by religious and cultural beliefs and today considered not only a food sources, but also a homeopathic treatment alternative for wounds, burns, oral healthcare and even a potential help in cancer treatment (Lay-Flurrie, 2008; Bardy et al., 2008). It is a super saturated sugar solution characterized by a low water activity to support microbial growth (Malika et al., 2004). The natural acidity of this product, the low protein content and the high viscosity, that limit the atmospheric oxygen penetration, are particularly stressing for several microorganisms.

Honeys also possess antimicrobial properties due to several components such as glucose oxidase (Bogdanov et al., 2008), flavonoids, phenolic derivatives (Ceauşi et al., 2009) and 3-phenyllactic acid (2-hydroxy-3-phenylpropanoic acid or β-phenyllactic acid) (PLA) active against bacteria (Ohhira et al., 2004), yeasts (Schwenninger et al., 2008) and a wide range of mould species, including some mycotoxigenic species (Lavermicocca et al., 2003). However, these beneficial effects may vary depending on the product origin (Voidarou et al., 2011).

Despite the numerous inhibiting factors, some microorganisms can survive in honey, at least as latent forms and may represent a mean for their transfer to consumers. Snowdon and Cliver (1996) showed that different microbial species in honey may reach a concentration of some thousands forming unit (CFU) per gram. Studies on French (Tysset and Rousseau, 1981) and Argentinian honeys (Iurlina and Fritz, 2005) showed an average value about 200–250 CFU/g for bacteria and 100–150 CFU/g for fungi. Regarding Italian honeys, lower values of both microbial groups were reported (Piana et al., 1991), even though Farris et al. (1986), which specifically analyzed the bacterial component of Sardinian honeys, detected *Bacillus* spp. at concentrations of approximately 1000 spores/g. So far, the microorganisms detected in honey belong

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to several bacterial (Rozanska, 2011) as well as filamentous fungal (Kačániová et al., 2009) and yeast (Carvalho et al., 2006) species. Other studies carried out on this topic have been mainly forwarded to the hygienic implications and many authors focused on the presence of *Clostridium botulinum* (Saraiva et al., 2012) due to the risk of infant botulism for children below one year old.

Honey is often used as a food ingredient and its microbial load may be transferred to complex matrices where some microorganisms may found the optimal conditions to develop. The knowledge of the microbial composition and the level of the species (and strains), relevant during transformation and/or conservation of the food matrices, may assume a paramount importance for the correct management of the process. Furthermore, the progressive market penetration of foreign honeys, often with a lower quality, increased the interest towards the complete characterization in order to check quality, sanitation and authenticity of the local product.

In light of the above reasons, the quality of honey depends not only on the physical and chemical properties well defined by EC Directive 2001/110, but also on the microbiological aspects largely ignored by the EU legislation. The aim of this study was to deepen the knowledge on the microbial community of untreated nectar honeys and honeydew honeys of different botanical and geographical origin collected in southern Italy.

2. Materials and methods

2.1. Sample collection

A total of 38 artisanal honey (31 nectar honeys and 7 honeydew honeys) samples were purchased from producers, transported to our laboratories and stored in dark conditions at refrigeration temperature until analysis. All honey samples were not apparently characterized by any sign of alteration.

2.2. Microbial counts

Osmophilic microorganisms were counted after homogenization of samples (25 g) in a 30% (w/v) glucose solution (sample/diluent 1:9) following the indications ISO 21527-2 to avoid shock of cells and to recover sub-lethally injured cells. The first dilution of nectar honeys and honeydew honeys was obtained with a stomacher (BagMixer 400, Interscience, Saint Nom, France) for 2 min at the highest speed. Cell suspensions were spread plated and incubated as follows: total (osmophilic and osmotolerant) yeasts (TY) on tryptone glucose yeast extract agar (TGY), incubated aerobically at 25 °C for 7 d (Beuchat et al., 2001); osmophilic bacteria (OB) on De Whalley Agar (DWA), incubated aerobically at 25 °C for 120 h (Justè et al., 2008); osmophilic yeasts (OY) on DWA, incubated aerobically at 25 °C for 7 d.

All other microorganisms were recovered by homogenization of samples (25 g) in peptone water. Cell suspensions were plated and incubated as follows: total mesophilic count (TMC) spread on plate count agar (PCA), incubated aerobically at 30 °C for 72 h; filamentous fungi (FF) spread on potato dextrose agar (PDA), incubated aerobically at 25 °C for 21 d; lactic acid bacteria (LAB) poured on glucose M17 (GM17) agar, incubated anaerobically with the AnaeroGen AN25 system at 30 °C for 72 h; *Enterobacteriaceae* poured on violet red bile glucose agar (VRBGA), incubated anaerobically by overlay agar at 37 °C for 24 h; clostridia on reinforced clostridial medium (RCM) by 3 × 3 Most Probable Number (MPN) procedure (FDA BAM, 2006).

Except VRBGA, all media used for bacterial growth were supplemented with cycloeximide (170 ppm) and biphenyl (1 g/L) to inhibit the growth of yeasts and moulds, while all media used for fungal growth were supplemented with chloramphenicol (0.1 g/L) to inhibit bacteria growth. Media were purchased from Oxoid (Basingstoke, UK) and chemicals by Sigma–Aldrich (Milan, Italy). Analyses were performed in duplicate.

2.3. Isolation, grouping and identification of bacteria

After growth, approximately five colonies with the same appearance (colour, morphology, edge, surface and elevation) were collected from count plates of each sample. Presumptive *Enterobacteriaceae* and clostridia were not isolated. The isolates were purified by successive sub-culturing and the purity was checked microscopically. After growth, the cultures were phenotypically characterized by cell morphology, Gram reaction (KOH method) and catalase (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5%, v/v). Rod, Gram positive, catalase positive bacteria were further characterised for spore formation: cell suspensions were treated at 85 °C for 15 min, (1:10) diluted in Ringer's solution and aliquots of 0.1 ml were spread plated onto Nutrient Agar (NA) (Oxoid) incubated at 32 °C for 48 h.

Otherwise all isolates, excepted Gram-ones, were subjected to biochemical assays and about 40% of the isolates of each phenotypic group was subjected to molecular analyses.

Genotypic differentiation of selected isolates was first performed by means of randomly amplified polymorphic DNA (RAPD). Genomic DNA for PCR assays was prepared after overnight growth in broth media at 30 °C. Cells were harvested and DNA was extracted by the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as templates for PCR reactions. RAPD analysis was carried out in a 25-μL reaction mix using primer M13 (Stenlid et al., 1994). Amplifications were performed by means of T1 Thermocycler (Biometra, Göttingen, Germany). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualized by UV transillumination after staining with SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA). GeneRuler 100bp Plus DNA ladder (M·Medical Srl, Milan, Italy) was used as a molecular size marker.

All isolates that showed growth onto NA after treatment at 85 °C for 15 min, recognised as spore forming bacteria (SFB), were also analysed by repetitive DNA elements-PCR (rep-PCR) analysis using $(GTG)_5$ and BOXA1R primer set (Versalovic et al., 1994; Gevers et al., 2001) corresponding to $(GTG)_5$- and BOX-like elements in bacterial DNA, respectively. All patterns were analyzed using the Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium). BOXA1R and $(GTG)_5$ profiles were combined and compared by UPGMA clustering method.

The isolates representative of each cluster were subjected to 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991). DNA fragments were visualized and the amplicons of about 1600 bp were purified by the QIA-quick purification kit (Quiagen S.p.a., Milan, Italy) and both strands were sequenced using the same primers employed for PCR amplification. DNA sequencing reactions were performed by PRIMM (Milan, Italy). The sequences obtained with forward and reverse primers from each strain were edited and merged into a single sequence covering the entire 16S rRNA gene. In addition, SFB isolates were analysed by restriction fragment length polymorphism (RFLP) of 16S rRNA gene fragment using *Rsa*I, *Cfo*I and *Hinf*I endonucleases (MBI Fermentas, St. Leon-Rot, Germany) (Jeyaram et al., 2011). The sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database.

2.4. Isolation and identification of yeasts

After growth, five colonies per morphology from each sample, or 1–2 isolates for the less numerous groups, were purified onto