Food Microbiology 38 (2014) 284–294

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Cultivable microorganisms associated with honeys of different geographical and botanical origin

Milko Sinacori, Nicola Francesca, Antonio Alfonzo, Margherita Cruciata, Ciro Sannino, Luca Settanni, Giancarlo Moschetti*

Department of Agricultural and Forestry Science, University of Palermo, Viale delle Scienze 4, 90128 Palermo, Italy

ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form
9 July 2013
Accepted 30 July 2013
Available online 11 August 2013

Keywords:
Bacteria
Culture-dependent approach
Filamentous fungi
Honey
Molecular characterisation
Yeasts

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

* Corresponding author. Tel.: +39 (0)91 23896050; fax: +39 (0)91 6515531.
E-mail address: giancarlo.moschetti@unipa.it (G. Moschetti).

0740-0020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2013.07.013

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

the same isolation medium, grouped on the basis of appearance and subjected to genetic characterization.

DNA extraction of all selected isolates was performed as above reported and a first differentiation of yeasts were obtained by RFLP of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso et al., 1999) and subsequently digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas) at 37 °C for 8 h. ITS amplicons as well as their restriction fragments were analysed twice on agarose gel using at first 1.5% (w/v) agarose and then 3% (w/v) agarose in 1× TBE buffer and visualized as above reported. Standard DNA ladders were 1 kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas). One isolate per group was further processed 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 with primers NL1 and NL4 (Kurtzman and Robnett, 1998). PCR products visualization and identification was realized as above reported.

2.5. Isolation and identification of filamentous fungi

Fungal colonies were collected and streaked onto PDA, observed at 24-h intervals and once they reached a diameter of approximately 2 mm, transferred to a new Petri dish containing the same substrate. Subsequently, the colonies with different appearance (colour, texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae, growth rate and topography) were purified

Table 1
Microbial loads (CFU/g) of honey and honeydew honey samples of different botanical and geographical origin.

Sample code	Source	Sites of sampling	Media							
			PCA	GM17	DWA-B	VRBGA	RCM[a]	TGY	DWA-y	PDA
Miel 01	*Er. japonica*	Ciaculli (Pa)	7.0 ± 2.8	3.0 ± 0.0	–	–	0.36	5420.0 ± 882.0	18600.0 ± 1790.0	–
Miel 02	Multifloral	Belmonte Mezzagno (Pa)	3.5 ± 2.1	–	–	–	–	1.0 ± 1.4	–	3.5 ± 2.1
Miel 03	Multifloral	Naro (Ag)	–	–	–	–	0.36	255.0 ± 17.7	290.0 ± 45.2	2.5 ± 2.1
Miel 04	*Citrus* spp.	Sciacca (Ag)	–	–	–	–	–	1.0 ± 0.0	2.0 ± 0.0	4.0 ± 1.4
Miel 05	Multifloral	Trabia (Pa)	2.0 ± 0.0	1.0 ± 0.0	–	–	0.36	–	–	5.0 ± 1.4
Miel 06	Multifloral	Trabia (Pa)	4.0 ± 0.0	–	–	–	–	–	–	27.0 ± 4.2
Miel 07	*Citrus* spp.	Bolognetta (Pa)	47.0 ± 18.0	–	–	–	0.36	476.0 ± 65.1	121.0 ± 29.7	5.5 ± 2.1
Miel 08	Multifloral	Belmonte Mezzagno (Pa)	6.5 ± 2.1	–	–	–	–	2.0 ± 1.4	23.0 ± 2.8	3.0 ± 0.0
Miel 09	*Hed. coronarium*	Santa Cristina (Pa)	3.0 ± 1.4	–	–	–	0.36	5.5 ± 2.1	216.0 ± 55.2	2.0 ± 1.4
Miel 10	*Citrus* spp.	Partinico (Pa)	1.0 ± 1.4	–	–	–	0.36	8.5 ± 2.1	361.0 ± 14.8	5.5 ± 3.5
Miel 11	*R. pseudoacaciae*	Caronia (Me)	2.0 ± 1.4	–	–	–	0.36	–	–	10.5 ± 2.1
Miel 12	*H. honey*	Somma Vesuviana (Na)	39.0 ± 1.4	–	–	–	–	–	–	1.0 ± 0.0
Miel 13	*H. honey*	Somma Vesuviana (Na)	54.0 ± 12.7	–	–	–	0.92	6.5 ± 2.1	6.5 ± 2.1	–
Miel 14	*H. honey*	Castel di Tusa (Me)	5.5 ± 2.1	–	–	–	0.36	–	–	1.0 ± 0.0
Miel 15	Multifloral	Benevento (Bn)	20.5 ± 3.5	–	–	–	–	6.5 ± 3.5	3.5 ± 2.1	5.0 ± 0.0
Miel 16	Multifloral	Somma Vesuviana (Na)	9.5 ± 2.1	1.0 ± 1.4	–	–	0.36	17.5 ± 3.5	292.0 ± 61.5	–
Miel 17	Multifloral	Portici (Na)	7.0 ± 0.0	2.0 ± 0.0	–	–	–	156.0 ± 22.6	306.0 ± 36.8	–
Miel 18	*Cas. sativa*	Monteforte (Sa)	7.5 ± 2.1	2.0 ± 0.0	–	–	0.36	–	–	–
Miel 19	*H. honey*	Somma Vesuviana (Na)	64.0 ± 2.8	13.5 ± 2.1	–	–	0.36	19.0 ± 7.1	550.0 ± 8.5	–
Miel 20	*H. honey*	Somma Vesuviana (Na)	30.5 ± 2.1	3.0 ± 0.0	–	–	0.36	63.5 ± 23.3	288.0 ± 45.3	–
Miel 21	*Euc. camaldulensis*	Naro (Ag)	1.0 ± 0.0	–	–	–	–	3.0 ± 0.0	43.0 ± 1.4	–
Miel 22	*Citrus* spp.	Sciacca (Ag)	1.0 ± 1.4	–	–	–	–	1.0 ± 0.0	–	4.5 ± 2.1
Miel 23	*Car. defloratus*	Naro (Ag)	1.0 ± 1.4	–	–	–	0.92	–	–	1.0 ± 1.4
Miel 24	Multifloral	Naro (Ag)	1.0 ± 0.0	–	–	–	–	–	2.0 ± 0.0	2.0 ± 1.4
Miel 25	*Euc. camaldulensis*	Lercara Friddi (Pa)	3.0 ± 0.0	–	–	–	–	782.0 ± 31.1	874.0 ± 65.1	1.0 ± 1.4
Miel 26	*Car. defloratus*	Roccapalumba (Pa)	318.0 ± 1.4	88.0 ± 8.5	–	–	–	19.0 ± 5.7	–	–
Miel 27	*H. honey*	Finale di Pollina (Pa)	14.0 ± 4.2	1.0 ± 0.0	–	–	–	7.5 ± 2.1	540.0 ± 84.9	5.5 ± 2.1
Miel 28	*Citrus* spp.	Santa Flavia (Pa)	108.0 ± 14.1	10.0 ± 2.8	–	2.0 ± 1.4	–	16.5 ± 4.95	56.0 ± 5.7	22.0 ± 8.49
Miel 29	*Euc. camaldulensis*	Piana degli Albanesi (Pa)	9.5 ± 3.5	–	–	–	–	–	–	3.0 ± 1.4
Miel 30	Multifloral	Castronovo di Sicilia (Pa)	–	–	–	–	–	–	–	1.0 ± 1.4
Miel 31	Multifloral	Montemaggiore (Pa)	1.0 ± 0.0	–	–	–	–	–	–	–
Miel 32	Multifloral	Portella di mare (Pa)	1.0 ± 0.0	–	–	–	–	–	–	3.0 ± 1.4
Miel 33	*Hed. coronarium*	Balata di Baida (Tp)	3.0 ± 0.0	–	–	–	–	–	–	3.0 ± 1.4
Miel 34	*Hed. coronarium*	Balata di Baida (Tp)	3.0 ± 0.0	–	–	2.5 ± 2.1	0.36	2.0 ± 0.0	1.0 ± 1.4	4.0 ± 1.4
Miel 35	*H. honey*	Boscotrecase (Na)	35.5 ± 2.1	6.5 ± 2.1	–	–	–	741.0 ± 12.7	1600.0 ± 148.0	–
Miel 36	Multifloral	Boscotrecase (Na)	8.5 ± 3.5	21.5 ± 2.1	–	–	–	–	–	2.0 ± 0.0
Miel 37	Multifloral	Balata di Baida (Tp)	–	–	–	–	–	–	–	–
Miel 38	*Euc. camaldulensis*	Balata di Baida (Tp)	–	–	–	–	–	–	–	2.0 ± 0.0

Results indicate mean values ±S.D.

[a] As estimated by MPN. Abbreviation: PCA, plate count agar added with cycloeximide and biphenyl for total mesophilic counts; DWA-b, De Whalley Agar added with cycloeximide and biphenyl for osmophilic bacteria counts; GM17, glucose M17 added with cycloeximide and biphenyl for lactic acid bacteria counts; TGY, Tryptone Glucose Yeast extract agar added with chloramphenicol for total (osmophilic and osmotolerant) yeasts; DWA-y, De Whalley Agar added with chloramphenicol for osmophilic yeast counts; PDA, Potato Dextrose Agar added with chloramphenicol for filamentous fungi counts; Me, Messina; Pa, Palermo; Ag, Agrigento; Na, Napoli; Tp, Trapani; Bn, Benevento; *R.*, *Robinia*; *Car.*, *Carduncullus*; *Cas.*, *Castanea*; *Euc.*, *Eucalyptus*; *H.*, *Honeydew honey*; *M.*, Multifloral; *Er.*, *Eriobotrya*; *Hed.*, *Hedysarum*. Symbols: –, under the detection limit.

Table 2
Morphological and biochemical characterization of presumptive LAB found in honeys and honeydew honeys.

Character	Clusters		
	A	B	C
	$n = 28$	$n = 1$	$n = 10$
Morphology	Coccus tetrads	Coccus short chain	Coccus short chain
Mobility	−	−	−
Catalase	−	−	−
Gram reaction	+	+	+
Growth			
15 °C	+	+	+
45 °C	−	−	+
pH 9.2	+	+	+
6.5% NaCl	+	−	+
CO_2 from glucose	−	−	−
Growth in presence of pentose sugars	−	−	−

Symbols: +, positive; −, negative to the test.

to homogeneity after several sub-culturing steps onto PDA. All colonies were subjected to microscopic analysis (Barnett and Hunter, 1998) through a light microscope (Carl Zeiss Ltd). Five isolates, or less for the less numerous morphological groups, were subjected to genetic analysis. Genomic DNA was extracted from single-spore cultures following a standard cetyl-trimethyl-ammonium-bromide (CTAB) based protocol (O'Donnell et al., 1998). Filamentous fungi were analyzed by RFLP of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). The amplicons were then digested with the endonucleases *Cfo*I and *Hae*III (MBI Fermentas) at 37 °C for 8 h. ITS products, as well as restriction fragments were analyzed on agarose gels using 2% (w/v) agarose in 1× TBE buffer. Gels were treated and visualised as above reported. One isolate per group was processed by sequencing of the 5.8S-ITS rRNA region to confirm the morphological identification.

2.6. Biodiversity indexes

The biodiversity of the overall microbial load was evaluate with Margalef's index of genotypes richness (R), Shannon-Weaver's index of diversity (H′) and Simpson's index of dominance (D), calculated as proposed by Ventorino et al. (2007).

3. Results

3.1. Microbial counts

The viable counts of the different microbial groups investigated in this study are reported in Table 1. Although growth of osmophilic bacteria on DWA resulted negative, a positive bacterial development was registered on PCA for the majority of samples. In several samples, only one colony contributed to TMC. However, except sample Miel 36, the highest levels of microorganisms were detected on PCA. *Enterobacteriaceae* were found only in two samples. The presence of clostridia was found in 15 samples, with the highest values estimated (0.92 MPN/g) for sample Miel 13 and Miel 23. The sample Miel 26 showed the highest bacterial richness. In general, yeasts counted onto DWA were at higher numbers than those registered on TGY, but an opposite observation was made for samples Miel 07, Miel 15, Miel 26 and Miel 34. Several samples resulted positive for the presence of moulds, but only samples Miel 6, Miel 11 and Miel 28, showed concentrations higher than 10 CFU/g. Twelve samples showed the presence of presumptive LAB and their concentrations were in the range 1.0 ± 0.0–88.0 ± 8.5 CFU/g. Members of the *Enterobacteriaceae* family were found only in samples Miel 28 and Miel 34.

3.2. Isolation and identification of bacteria

A total of 464 pure cultures were isolated and purified to homogeneity on the same media used for the plate counts. All cultures were subjected to a preliminary microscopic inspection and separated in three main groups: 423 rod shaped, Gram-positive, catalase positive, spore forming bacteria considered as presumptive *Bacillus* spp.; two rod shaped, Gram-negative, catalase-positive bacteria; 39 coccus shaped, Gram-positive, catalase negative bacteria considered as presumptive LAB. Due to the limited number of isolates and their different isolation sample, the Gram-negative isolates were subjected to the 16S rRNA gene sequencing without any differentiation at strain level: both isolates were identified as *Klebsiella pneumonia* (Acc. No. KC692177, KC692181). All other strains were further subjected to biochemical analysis that allowed the subdivision of the presumptive LAB and presumptive *Bacillus* spp. into three (Table 2) and seven (Table 3) groups, respectively. Furthermore, about 40% of the isolates of each phenotypic group was subjected to RAPD analysis. The resulting dendrograms (Figs. 1 and 2), at a similarity level of 80%, showed that presumptive LAB were divided into three clusters, while presumptive *Bacillus* spp.

Table 3
Morphological and biochemical characterization of aerobic spore forming bacilli found in honey and honeydew honeys.

Characters	Clusters						
	D ($n = 198$)	E ($n = 50$)	F ($n = 10$)	G ($n = 68$)	H ($n = 95$)	I ($n = 1$)	L ($n = 1$)
Cell and spore morphology:							
Vegetative cells	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Spores	+	+	+	+	+	+	+
Biochemical tests:							
Catalase	+	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	+
Anaerobic growth	−	+	+	−	−	+	+
Nitrate reduction	+	+	+	V	−	+	+
Voges-Proskauer	+	+	+	−	+	−	+
Starch hydrolysis	+	+	+	+	−	+	+
Growth at 50 °C	+	−	+	−	+	−	−
Growth at 65 °C	−	−	−	−	−	−	−
Growth in 7% NaCl	+	+	+	+	+	+	−
Acid and gas in glucose	−	−	−	−	−	−	+

Symbols: V, variable; +, positive to the test; −, negative to the test.

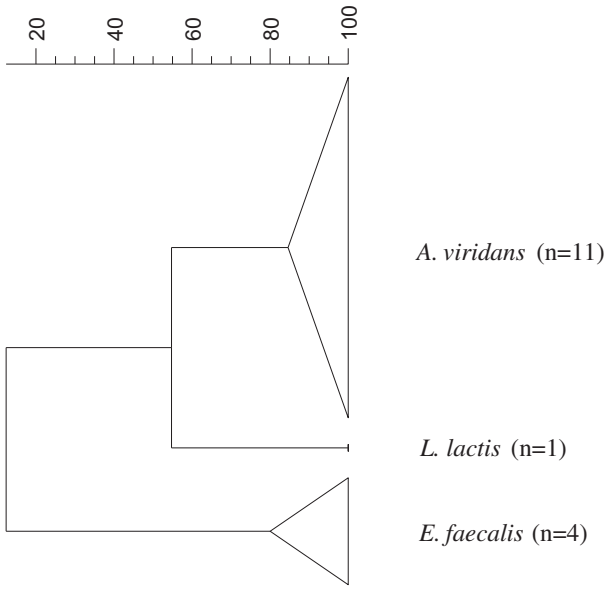

Fig. 1. Dendrogram obtained from RAPD-PCR patterns of presumptive LAB. The values between brackets refer to the number of isolates in each cluster. Upper line indicates the percentage of similarity.

into 33 clusters. The 16S rRNA gene of each presumptive LAB strain was sequenced and the following species were identified: *Aerococcus viridans* (phenotypic group A, Acc. No. KC692208); *Lactococcus lactis* (phenotypic group B, Acc. No. KC692209); and *Enterococcus faecalis* (phenotypic group C, Acc. No. KC692178, KC692183). The presumptive *Bacillus* spp. strains were further investigated by REP-PCR; $(GTG)_5$ and BOXA1R profiles were combined and the comparison among all isolates recognized 42 different clusters (Table 4). The representative strains of each cluster were analyzed by 16S rRNA gene sequencing (Table 4). The direct comparison performed by BLAST search determined the identification at species level of only five strains: *Paenibacillus polymyxa* (ML227), *Bacillus simplex* (ML384), *Bacillus pumilus* (ML374) and *Bacillus licheniformis* (ML103A and ML104B). For the other strains indeed also the RFLP analysis was performed and the results were compared with restriction fragments size proposed by Jeyaram et al. (2011). The species *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus licheniformis*, *Bacillus megaterium* and *B. pumilus* were clearly identified, but the strain ML582, characterized by a 16S rRNA gene sequence homology close to *B. amyloliquefaciens*/*B. subtilis* and a RFLP profile similar to *B. megaterium*, remained unspeciated.

3.3. Isolation and identification of yeasts

Based on morphology, 1027 yeast colonies were collected from TGY and DWA (409 and 618, respectively). Three hundred and twenty-three isolates were then subjected to the molecular identification. After restriction analysis of 5.8S-ITS region, the isolates were clustered in nine groups (Table 5). The isolates of groups III and IX were identified as *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* by comparison of restriction bands with those available in literature (Esteve-Zarzoso et al., 1999; Carvalho et al., 2006). The other groups could not be identified by RFLP analysis and the identification at species level was concluded by sequencing of D1/D2 domain of the 26S rRNA gene which recognised three species: *Zygosaccharomyces mellis* for the isolates included into the groups IV, V, VI, VII and VIII; *Aureobasidium pullulans* for group I; *Cryptococcus uzbekistanensis* for group II. *Z. mellis* (group IV) was the most isolated species and it represented the majority of yeast population in the great part of samples. On the contrary, the other species were less numerous and they were detected only from six different honeys. A total of 23 samples were positive for yeast presence and the samples Miel 19 and Miel 35, both honeydew honeys, showed the highest yeast diversity at strain level.

3.4. Isolation and identification of filamentous fungi

A total of 117 filamentous fungi were collected and divided into 17 groups after microscopic inspection (Table 6). The results of the restriction analysis of 5.8S-ITS region using the endonucleases *Cfo*I and *Hae*III confirmed the subdivision of the filamentous fungi into 17 groups. One isolate per group was subjected to the sequencing of the 5.8S-ITS rRNA gene that clearly identified the species *Alternaria alternata* (group I), *Aspergillus niger* (group III), *Aspergillus proliferans* (group IV), *Aspergillus spelunceus* (group V), *Chaetomium globosum* (group VI), *Cladosporium cladosporioides* (group VII), *Daldinia concentrica* (group VIII), *Emericella discophora* (group IX), *Emericella qinqixianii* (group X), *Penicillium corylophilum* (group XII), *Penicillium decumbens* (group XIII), *Penicillium italicum* (group XIV), *Penicillium polonicum* (group XVI) and *Penicillium echinulatum* (group XVII). The isolates of groups II, XI and XV were identified at genus level as *Arthrinium*, *Emericella* and *Penicillium*, respectively. The species most frequently isolated were *Pen. corylophilum* and *Asp. niger*. The samples Miel 7 (*Citrus* spp.) and Miel 3, Miel 5 and Miel 15 (multifloral) were the richest sources of fungal diversity among the 26 samples positive to fungal isolation.

3.5. Distribution of strains and biodiversity indexes

The distribution of the microorganisms isolated from honeys is reported in Table 7. *B. amyloliquefaciens*, *B. megaterium*, *B. pumilus*, *Aspergillus niger*, *Penicillium corylophilum* and *Z. mellis* were commonly found in several samples of different botanical origin. Margalef's, Shannon-Weaver's and Simpson's indexes (Table 8) showed the highest microbial diversity for multifloral, *Citrus* and *He. coronarium* honeys. In particular, the highest R and H′ and the lowest D values were registered for the multifloral honeys.

4. Discussion

Honey retains a natural image and the increasing trend in consumption could be also attributed to a new life style with people demanding more natural foods for the beneficial effects of these products (Arvanitoyannis and Krystallis, 2006). However, the quality of honey could be compromised by the hygienic practices during harvest and extraction as well as time and conditions of storage (Snowdon and Cliver, 1996). The intrinsic properties of honey affect the growth and survival of several microorganisms; thus, they influence the species proportions, but some microbial species, especially bacteria with pathogenic characteristics, have been isolated from honey samples. Hence, the interest towards the microbial composition of commercial honeys is of paramount importance, even though it is considered a food ingredient and it is consumed in low amounts. In order to better examine the living microbial communities associated with honey, 38 samples were collected in different cities of southern Italy and analysed by culture-dependent methods. This because the presence of undesired microorganisms that are able to develop colonies undoubtedly provide a clear indication of the hygienic safety of honey.

Despite the stressing conditions of honey, the microbiological analyses revealed the presence of several microbial groups. The results of our study, conducted on honey and honeydew honey samples, showed that 33 of them hosted low loads of bacteria, with

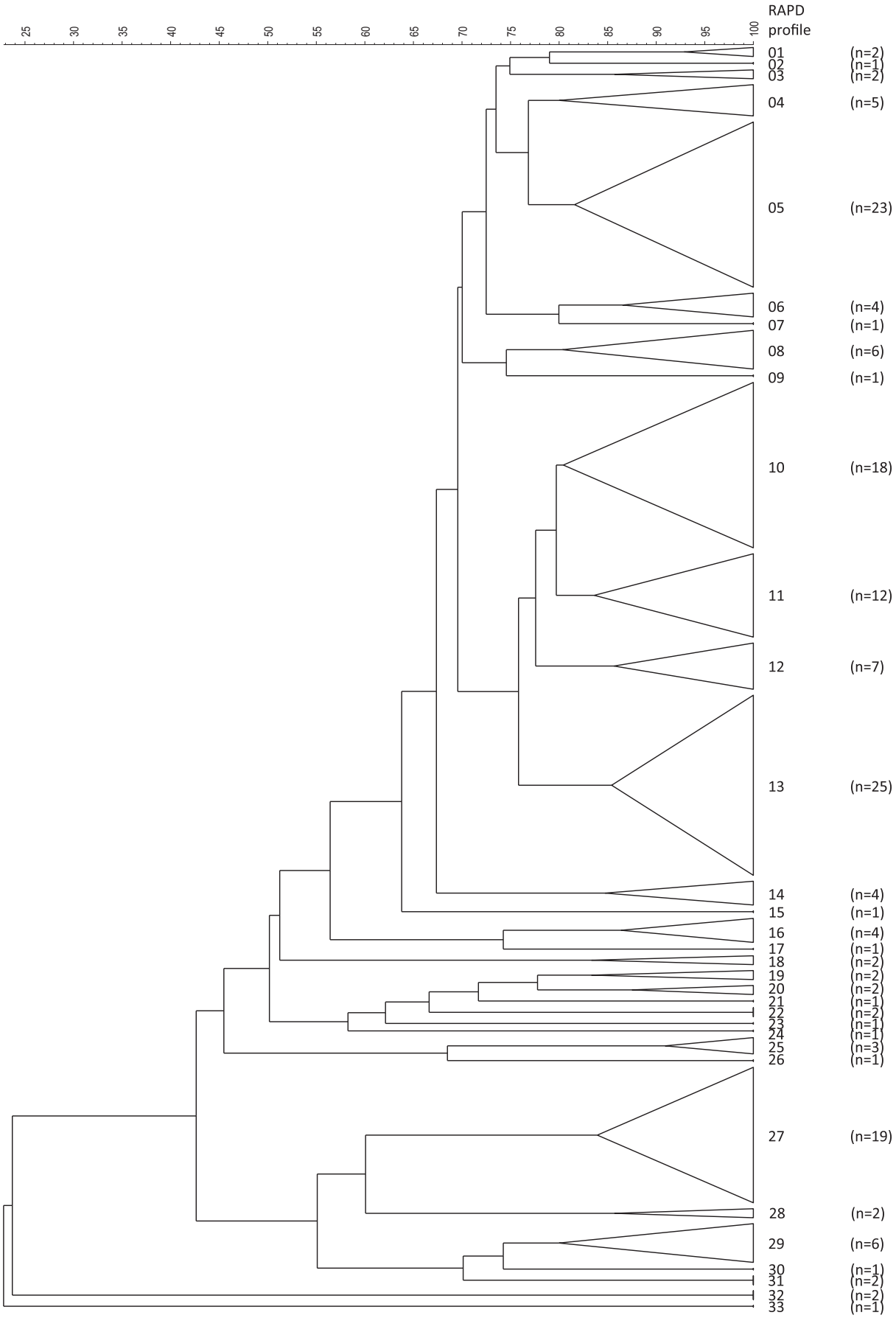

Fig. 2. Dendrogram obtained from RAPD-PCR patterns of presumptive *Bacillus* spp. The values between brackets refer to the number of isolates in each cluster. Upper line indicates the percentage of similarity.

Table 4
Molecular identification of spore forming bacteria.

Strain	Phenotypic cluster	RAPD cluster	GTG/BOXA1R cluster	Identification by 16S and BLAST search	Final identification by RFLP	Acc. No.	No. of isolates
ML101A	E	29	35	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692164	2
ML101B	D	25	20	*B. subtilis*/*B. mojavensis*	*B. subtilis*	KC692170	3
ML102B	D	28	41	*B. subtilis*/*B. mojavensis*	*B. subtilis*	KC692194	2
ML103A	F	32	33	*B. licheniformis*		KC692195	2
ML104B	F	18	1	*B. licheniformis*		KC692185	2
ML105	H	23	19	*B. pumilus*/*B. safensis*	*B. pumilus*	KC692196	1
ML106A	D	33	10	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692197	1
ML106B	D	19	28	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692198	2
ML208	E	29	35	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692193	4
ML211	H	5	23	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692172	5
ML215	D	1	27	*B. subtilis*/*B. mojavensis*	*B. subtilis*	KC692182	2
ML227	L	26	34	*P. polymyxa*		KC692186	1
ML233	E	5	24	*B. thuringiensis*/*B. cereus*	*B. thuringiensis*	KC692184	6
ML235	G	31	11	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692187	2
ML252	H	5	25	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692165	3
ML254	E	30	36	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692199	1
ML256	H	5	25	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692162	6
ML257	G	20	12	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692200	1
ML258	G	22	14	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692166	2
ML259	E	6	37	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692188	4
ML265	D	17	3	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692168	1
ML267	E	5	39	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692161	2
ML270	H	5	32	*B. pumilus*/*B. safensis*	*B. pumilus*	KC692158	1
ML272	E	15	21	*B. cereus*/*B. thuringiensis*	*B. cereus*	KC692201	1
ML274	D	11	6	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692159	11
ML275	G	21	13	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692202	1
ML323	D	16	7	*B. amyloliquefaciens*/*B. subtilis*	*B. amyloliquefaciens*	KC692203	4
ML345	D	10	3	*B. amyloliquefaciens*/*B. subtilis*	*B. amyloliquefaciens*	KC692167	2
ML353	H	4	22	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692160	5
ML361	D	13	5	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692163	25
ML374	H	3	30	*B. pumilus*		KC692204	2
ML376	D	10	40	*B. amyloliquefaciens*/*B. subtilis*	*B. amyloliquefaciens*	KC692189	2
ML384	I	11	26	*B. simplex*		KC692191	1
ML390	D	9	4	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692192	1
ML451	D	10	2	*B. amyloliquefaciens*/*B. subtilis*	*B. amyloliquefaciens*	KC692171	5
ML456	D	10	8	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692174	2
ML471	D	2	42	*B. amyloliquefaciens*/*B. subtilis*	*B. amyloliquefaciens*	KC692205	1
ML474	D	14	16	*B. subtilis*/*B. amyloliquefaciens*	*B. subtilis*	KC692190	4
ML477	H	8	17	*B. pumilus*/*B. safensis*	*B. pumilus*	KC692169	2
ML479	G	20	9	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692206	1
ML482	G	7	15	*B. megaterium*/*B. aryabhattai*	*B. megaterium*	KC692173	1
ML484	H	12	31	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692175	7
ML568	H	8	29	*B. pumilus*/*B. altitudinis*	*B. pumilus*	KC692176	4
ML581	D	10	3	*B. subtilis*/*B. amyloliquefaciens*	*B. amyloliquefaciens*	KC692179	7
ML582	D	27	38	*B. amyloliquefaciens*/*B. subtilis*	Not resolved	KC692180	19
ML618	H	24	18	*B. pumilus*/*B. safensis*	*B. pumilus*	KC692207	1

318 CFU/g as maximum level of concentration, a value close to that reported by other researchers (Gomes et al., 2011). Presumptive LAB were detected in twelve honeys and only two samples resulted positive for the presence of *Enterobacteriaceae*. Surprisingly, any bacterial growth was not detected on DWA medium, generally employed to reveal the osmophilic bacteria, probably because the composition of this medium is still too stressing to allow cell division. Clostridia, even though at low levels, were observed in about 40% of the samples analysed. Some works conducted on the search of *Clostridium* spp. reported negative results (Iurlina and Fritz, 2005; Gomes et al., 2011). However, some Italian honeys have been found to host SFB at levels comparable to those of our study (Piana et al., 1991), but higher concentrations, till two orders of magnitude, have been also reported for sugar rich matrix (Finola et al., 2007).

Except three samples, all other honeys and honeydew honeys were found positive for the presence of yeasts or moulds, with maximum concentrations of 18,600 and 27 CFU/g, respectively. The low value obtained for moulds confirmed that the growth of these organisms is limited in honey. The maximum yeast load was registered for the *Er. japonica* honey. This finding is not surprising because it is a product harvested in winter when the moisture content can be higher due to hygroscopic properties of honey (Zumla and Lulat, 1989). The other samples showed fungal levels closer to those found by other authors (Piana et al., 1991; Iurlina and Fritz, 2005).

All bacteria isolated were divided into three main groups after molecular identification. The Gram-negative isolates were identified as *Klebsiella pneumoniae*. LAB were grouped into three groups including one species each (*A. viridans*, *L. lactis* and *E. faecalis*). *K. pneumoniae* and *L. lactis* have been isolated from the digestive tract of *Apis cerana* (Nada et al., 2010; Ahn et al., 2012), but even though it is different from *A. mellifera*, it might be reasonably supposed that the same species may be associated to *A. mellifera* and, consequently, contaminate honey products. To our knowledge, no previous studies reported the presence of these species in honeydew honey and this is also the first finding of *K. pneumoniae*, *L. lactis*, *E. faecalis* and *A. viridans* from nectar honeys.

Seven groups of SFB were obtained and they included several species within *Bacillus* genus and *P. polymyxa*. *B. amyloliquefaciens*

Table 5
Molecular identification of yeasts.

RFLP profile	Isolate code	5.8S-ITS	Size of restriction fragments			Species	Acc. No.	Source			No. of isolates
		PCR	*Cfo*I	*Hae*III	*Hin*fI			TGY	No. of isolates	DWA	
I	ML403	600	200 + 180 + 110 + 60	450 + 150	300 + 175 + 125	*Aureobasidium pullulans*	KC692229			Miel (15)	2
II	ML114	620	320 + 300	550 + 70	350 + 270	*Cryptococcus uzbekistanensis*	KC692227	Miel (2,4)	2		
III	ML294	650	300 + 300 + 50	420 + 150 + 90	325 + 325	*Debaryomyces hansenii*	KC692228	Miel (7,26)	6	Miel (1)	8
IV	ML463	850	310 + 240 + 190 + 110	590 + 200 + 60	400 + 260 + 190	*Zygosaccharomyces mellis*	KC692231	Miel (3, 4, 7, 8, 9, 15, 16, 17, 19, 20, 21, 22, 25, 26, 27, 28)	111	Miel (3, 4, 7, 8, 9, 10, 16, 17, 19, 20, 21, 24, 25, 27, 28, 34, 35)	114
V	ML517	1000	400 + 390 + 140	700 + 160 + 60	530 + 260 + 130	*Zygosaccharomyces mellis*	KC692230	Miel (35)	2		
VI	ML281	900	320 + 190 + 130 + 60	400 + 270 + 170 + 60	490 + 270 + 140	*Zygosaccharomyces mellis*	KC692233	Miel (7, 17, 35)	10	Miel (28)	2
VII	ML343	820	300 + 220 + 190 + 110	820	400 + 250 + 170	*Zygosaccharomyces mellis*	KC692234	Miel (10)	4	Miel (19)	2
VIII	ML534	900	390 + 360 + 140	670 + 170 + 60	510 + 260 + 130	*Zygosaccharomyces mellis*	KC692235	Miel (13, 16, 19, 20, 34, 35)	26	Miel (13, 35)	6
IX	ML119	750	290 + 200 + 170 + 90	400 + 210 + 90	350 + 260 + 140	*Zygosaccharomyces rouxii*	KC692232	Miel (1)	10	Miel (1)	18

All values for the 5.8S-ITS PCR and restriction fragments are given in bp.
The number reported between brackets refers to sample code.

resulted the species with the highest number of isolates. Several studies reported the isolation of *Bacillus* species in honeys (Iurlina and Fritz, 2005; Alippi and Reynaldi, 2006) but this study represents the first report on the identification of *Bacillus* spp. in honeydew honeys. With the exception of two species (*Bacillus anthracis* and many *B. cereus* toxin-producer strains), *Bacillus* group is considered safe. So far, honey is not known to have been involved in disease caused by *B. cereus*, but López and Alippi (2010) considered honeys contaminated with *B. cereus* and *B. megaterium* as possible vectors of foodborne illnesses. However, thanks to the ability of several *Bacillus* strains to produce antibiotics, bacteriocins, or antifungal compounds, they find application to pursue agricultural and healthcare purposes (Alfonzo et al., 2012; Duc et al., 2004). Some strains of *P. polymyxa* (Lee et al., 2009), *B. subtilis* (Sabatè et al., 2009), *B. cereus*, *B. licheniformis* and *B. megaterium* (Alippi and Reynaldi, 2006), isolated from honey samples or different apiarian sources, were successfully tested against *Paenibacillus larvae*. *B. amyloliquefaciens* is considered able to inhibit *Ascosphaera apis* (Brittes Benitez et al., 2012). *P. larvae* and *A. apis* are two common pathogens causing American foulbrood and chalkbrood in honey bee colonies (Sabatè et al., 2009). Today diseases and parasites are considered one of the main reasons of the decrease in bee population (Genersch, 2010) and the selection of microbial strains with antagonistic effect represents an important strategy for the biological control of these dangerous organisms (Yoshiyama et al., 2013).

Nine yeast species were identified, but only *Z. rouxii* and *D. hansenii* were directly identified by 5.8S rRNA gene RFLP; for the other species the sequencing of the D1/D2 domain of the 26S rRNA gene was necessary, because atypical restriction profiles of 5.8S-ITS were registered. An atypical polymorphism for this region is not surprising, since many authors observed this behaviour in several yeasts (Solieri et al., 2007; Tofalo et al., 2009). The two osmotolerant *Z. mellis* and *Z. rouxii* are commonly associated with the honey environment (Deak, 2007; Carvalho et al., 2006), but no previous detection of *A. pullulans*, *C. uzbekistanensis* and *D. hansenii* in honey sources are available in literature. *Z. mellis* is a spoiling agent of high sugar foods and honeys (Wrent et al., 2010). This species is not able to use sucrose for growth (Kurtzman and Fell, 1998) and can easily survive in nectar and honey that are rich in glucose and fructose (Tysset and Rautlin de la Roy, 1974).

Filamentous fungi, on the basis of macroscopic, microscopic and molecular analysis were divided into 17 groups consisting of eight different genera (*Alternaria*, *Arthrinium*, *Aspergillus*, *Chaetonium*, *Cladosporium*, *Daldinia*, *Penicillium* and *Emericella*) with the prevalence of the species *Pe. corylophilum* and *As. niger* (50% and 32% of the samples, respectively). *Penicillium*, *Cladosporium*, *Alternaria* and *Aspergillus* genera are considered common contaminants of honey (Nasser, 2004; Kačániová et al., 2009), while the other species have not been reported yet. In particular, with the exception of *Da. concentrica*, a wood saprophyte fungus (Boddy et al., 1985), the other species belong to genera known as fungal allergens and mycotoxin producers (Griessler et al., 2010; Moss, 2002).

In conclusion, 35 microbial species associated with honeys and honeydew honeys were identified in this study, confirming that the stressing conditions of honey are highly selective. Thus, this matrix can be considered as a source of microorganisms useful to act in suboptimal conditions, e.g. to carry out the transformation of wastes from the food industry characterized by high concentration of sugars (e.g. molasses) in order to reduce the volume of untreated waste bulks and to valorise these substrates to obtain microbial metabolites with different applications.

The biodiversity indexes revealed some differences among samples within the same botanical origin. The microbiota of multifloral honeys showed the highest values for genotype richness

Table 6
Molecular identification of filamentous fungi.

RFLP	Isolate code	5.8S-ITS PCR	Size of restriction fragments		Specie (% identity[a])	Acc. No.	Source of isolation	No. of isolates
			*Cfo*I	*Hae*III				
I	ML356	620	360 + 140 + 120	470 + 150	*Alternaria alternata* (99)	KC692221	Miel 15	1
II	ML193	630	360 + 270	470 + 160	*Arthrinium* spp. (99)	KC692225	Miel (3, 4, 5, 7, 15)	9
III	ML168B	620	200 + 180 + 130 + 90	300 + 100 + 70 + 50	*Aspergillus niger* (99)	KC692215	Miel (3, 4, 5, 7, 8, 22, 24, 27, 29,32, 34, 38)	35
IV	ML280	620	330 + 270	290 + 170 + 85	*Aspergillus proliferans* (100)	KC692212	Miel 7	1
V	ML442	620	210 + 200 + 80 + 50	510 + 100	*Aspergillus spelunceus* (99)	KC692218	Miel 23	1
VI	ML176	620	280 + 190 + 150	250 + 130 + 80 + 60	*Chaetomium globosum* (99)	KC692213	Miel 2	1
VII	ML370	600	340 + 260	600	*Cladosporium cladosporioides* (99)	KC692219	Miel 11	1
VIII	ML286	600	180 + 160 + 130 + 80	600	*Daldinia concentrica* (99)	KC692211	Miel 8	1
IX	ML297	600	260 + 130 + 80	400 + 100 + 70	*Emericella discophora* (99)	KC692226	Miel 9	1
X	ML514	600	260 + 190 + 80	430 + 110 + 80	*Emericella qinqixianii* (99)	KC692210	Miel 33	1
XI	ML488	620	250 + 130 + 90 + 60	410 + 100 + 70	*Emericella* spp. (96)	KC692217	Miel 25	1
XII	ML369	630	180 + 90	260 + 90 + 70 + 55	*Penicillium corylophilum* (99)	KC692220	Miel (2, 3, 4,5, 6, 7, 9, 10, 11,12, 14, 22, 28, 29, 30, 32,33, 34, 36)	39
XIII	ML155	620	180 + 90	260 + 90 + 80 + 55	*Penicillium decumbens* (100)	KC692216	Miel (3, 4)	11
XIV	ML332	620	190 + 100	260 + 95 + 70	*Penicillium italicum* (99)	KC692222	Miel (10, 15)	3
XV	ML172	630	220 + 180 + 170 + 60	390 + 150 + 90 + 50	*Penicillium* spp. (95)	KC692214	Miel 5	1
XVI	ML329	630	180 + 170 + 140 + 90	260 + 95 + 70 + 50	*Penicillium polonicum* (99)	KC692223	Miel (2, 5, 6, 10, 11, 15)	9
XVII	ML291	620	170 + 90	250 + 100 + 70	*Penicillium echinulatum* (99)	KC692224	Miel 7	1

The number reported between brackets refers to the number code of samples.
[a] According to BlastN search of ITS1-5.8S-ITS2 rRNA gene sequences in NCBI database.

Table 7
Distribution of the microorganisms throughout honeys and honeydew honeys of different botanical origin.

Microorganisms	Species								
	Car. defloratus	*Cas. sativa*	*Citrus* spp.	*Euc .camaldulensis*	*Er. japonica*	*H. honey*	*Hed. coronarium*	Multifloral	*R. pseudoacaciae*
Bacteria:									
Bacillus amyloliquefaciens	x	x	x	x	x	x	x	x	
Bacillus cereus	x				x	x		x	
Bacillus licheniformis					x			x	
Bacillus megaterium	x		x	x		x	x	x	
Bacillus pumilus	x	x		x	x	x		x	
Bacillus simplex		x							
Bacillus subtilis		x			x		x	x	
Bacillus thuringiensis						x		x	x
Paenibacillus polymyxa								x	
Aerococcus viridans	x								
Lactococcus lactis							x		
Enterococcus faecalis			x			x		x	
Klebsiella pneumoniae			x				x		
Filamentous fungi:									
Alternaria alternata								x	
Arthrinium spp.			x					x	
Aspergillus niger			x	x		x	x	x	
Aspergillus proliferans			x						
Aspergillus spelunceus	x								
Chaetomium globosum								x	
Cladosporium cladosporioides									x
Daldinia concentrica								x	
Emericella discophora							x		
Emericella qinqixianii							x		
Emericella spp.				x					
Penicillium corylophilum			x	x		x	x	x	x
Penicillium italicum			x					x	
Penicillium polonicum			x					x	x
Penicillium spp.								x	
Penicillium decumbens			x					x	
Penicillium echinulatum			x						
Yeasts:									
Aureobasidium pullulans								x	
Cryptococcus uzbekistanensis			x						
Debaryomyces hansenii	x		x		x				
Zygosaccharomyces mellis	x		x	x		x	x	x	
Zygosaccharomyces rouxii					x				

Table 8
Biodiversity indexes.

Botanical source	Indexes		
	R	H′	D
Car. defloratus	2.15	1.72	0.24
Cas. sativa	1.67	1.33	0.28
Citrus spp.	2.98	1.97	0.22
E. camaldulensis	1.69	1.37	0.34
Er. japonica	1.67	1.26	0.43
H. honey	1.74	1.70	0.24
He. coronarium	2.73	1.87	0.22
Multifloral	3.91	2.35	0.15
R. pseudoacaciae	1.36	1.21	0.33

Indexes: R, Margalef's index of genotypes richness; H′, Shannon-Weaver's index of diversity; D, Simpson's index of dominance.

and diversity indexes, but contemporarily they were characterised by the lowest value of dominance. Some species were detected in almost all honeys and some strains, regardless on the origin of the product, were recognised more than once highlighting their strong adaptation to this matrix. For the species found occasionally, a correlation with the botanical or geographical origin cannot be found, but only an environmental contamination may be supposed. Furthermore, our findings are close to that reported by Snowdon and Cliver (1996) for industrial honey, probably thanks to the correct handling techniques applied by the beekeepers. Nevertheless, the presence of spores of potentially dangerous microorganisms evidenced the need to further study the microbial communities of honey for the hygienic safety of this product. Works are being prepared to evaluate the presence of viable but not cultivable populations in order to provide a more complete view of the microbial complexity of honeys.

Acknowledgements

The authors thank the beekeepers for kindly supply honey samples and the student Silvana Intile (University of Palermo) for her help with the microbiological analyses.

References

Ahn, J.H., Hong, I.P., Bok, J.I., Kim, B.Y., Song, J., Weon, H.Y., 2012. Pyrosequencing analysis of the bacteria communities in the guts of honey bees *Apis cerana* and *Apis mellifera* in Korea. J. Microbiol. 50 (5), 735–745.
Alfonzo, A., Lo Piccolo, S., Conigliaro, G., Ventorino, V., Burruano, S., Moschetti, G., 2012. Antifungal peptides produced by *Bacillus amyloliquefaciens* AG1 active against grapevine fungal pathogens. Ann. Microbiol. 62, 1593–1599.
Alippi, A.M., Reynaldi, F.J., 2006. Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources. J. Invertebr. Pathol. 91 (3), 141–146.
Arvanitoyannis, I., Krystallis, A., 2006. An empirical examination of the determinants of honey consumption in Romania. IJFST 41, 1164–1176.
Bardy, J., Slevin, N.J., Mais, K.L., Molassiotis, A., 2008. A systematic review of honey uses and its potential value within oncology care. J. Clin. Nurs. 17, 2604–2623.
Barnett, H.L., Hunter, B.B., 1998. Illustrated Genera of Imperfect Fungi, fourth ed. Burgess Publishing.
Beuchat, L., Frândberg, E., Deák, T., Alzamora, S., Chen, J., Guerrero, S., López–Malo, A., Ohlsson, I., 2001. Performance of mycological media in enumerating desiccated food spoilage yeasts: an interlaboratory study. Int. J. Food Microbiol. 70, 89–96.
Boddy, L., Gibbon, O.M., Grundy, M.A., 1985. Ecology of *Daldinia concentrica*: effect of abiotic variables on mycelial extension and interspecific interactions. T. Brit. Mycol. Soc. 85, 201–211.
Bogdanov, S., Jurendic, T., Sieber, R., Gallmann, P., 2008. Honey for nutrition and health: a review. J. Am. Coll. Nutr. 27 (6), 677–689.
Brittes Benitez, L., Voltolini Velho, R., De Souza Da Motta, A., Segalin, J., Brandelli, A., 2012. Antimicrobial factor from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American foulbrood. Arch. Microbiol. 194, 177–185.
Carvalho, M.C., Rocha, A., Estevinho, L., Choupina, A.B., 2006. Identification of honey yeast species based on RFLP analyses of the ITS region. Cienc. Tecnol. Alimentos. 5 (1), 11–17.
Ceauşi, C., Ţogoe, L., Tudor, L., Furnaris, F., 2009. The evaluation of microbiological quality of some bee honey sorts. Bull. UASVM Animal. Scie. Bio. 66 (1), 273–277.
Codex Alimentarius Commission, 2001. Revised Codex Standard for Honey. Codex STAN, 12–1981, Rev. 1 1987, Rev. 2.
Deak, T., 2007. Yeasts in specific types of foods. In: Deak, T. (Ed.), Handbook of Food Spoilage Yeasts, second ed. CRC Press, Boca Raton, pp. 117–201.
Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O., Cutting, S.M., 2004. Characterization of *Bacillus* probiotics available for human uses. Appl. Environ. Microb. 70, 2161–2171.
Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.
Farris, G.A., Fatichenti, F., Deina, P., Agostini, F., 1986. Aerobic and anaerobic spore-forming bacteria in sardinian honey. Annali della facoltà di Agraria dell'Università di Sassari 32, 173–179.
FDA BAM, 2006. Most Probable Number Determination from Serial Dilutions. In: Bacteriological Analytical Manual, Appendix 2.
Finola, M.S., Lasagno, M.C., Marioli, J.M., 2007. Microbiological and chemical characterization of honeys from central Argentina. Food Chem. 100 (4), 1649–1653.
Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.
Genersch, E., 2010. Honey bee pathology: current threats to honey bees and beekeeping. Appl. Microbiol. Biot. 87 (1), 87–97.
Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. Fems Microbiol. Lett. 205, 31–36.
Gomes, T., Feás, X., Iglesias, A., Estevinho, L.M., 2011. Study of organic honey from the northeast of Portugal. Molecules 16, 5374–5386.
Griessler, K., Rodrigues, I., Handl, J., Hofstetter, U., 2010. Occurrence of mycotoxins in Southern Europe. World Mycotoxin J. 3, 301–309.
Iurlina, M.O., Fritz, R., 2005. Characterization of microorganisms in Argentinean honeys from different sources. Int. J. Food Microbiol. 105, 297–304.
Jeyaram, K., Romi, W., Singh, T.A., Adewumi, G.A., Basanti, K., Oguntoyinbo, F.A., 2011. Distinct differentiation of closely related species of *Bacillus subtilis* group with industrial importance. J. Microbiol. Meth. 87 (2), 161–164.
Justè, M.S., Krause, B., Lievens, M., Klingeberg, C.W., Michiels, K., Willems, A., 2008. Protective effect of hop b-acids on microbial degradation of thick juice during storage. J. Appl. Microbiol. 104, 51–59.
Kačániová, M., Pavlicova, S., Hascik, P., Kociubinski, G., Knazovicka, V., Sudzina, M., Sudzinova, J., Fikselova, M., 2009. Microbial communities in bees, pollen and honey from Slovakia. Acta Microbiol. Immunol. Hung. 56 (3), 285–295.
Kurtzman, C.P., Fell, J.W., 1998. The Yeasts, a Taxonomic Study, fourth ed. Elsevier, Amsterdam, p. 1055.
Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73, 331–371.
Lavermicocca, P., Valerio, F., Visconti, A., 2003. Antifungal activity of phenyllactic acid against molds isolated from bakery products. Appl. Environ. Microb. 69 (1), 634–640.
Lay-Flurrie, K., 2008. Honey in wound care: effects, clinical application and patient benefit. Br. J. Nurs. 17, 32–36.
Lee, H., Churey, J.J., Worobo, R.W., 2009. Isolation and characterization of a protective bacterial culture isolated from honey active against American Foulbrood disease. FEMS Microbiol. Lett. 296 (1), 39–44.
López, A.C., Alippi, A.M., 2010. Enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus megaterium* isolates recovered from honey. Rev. Argent. Microbiol. 42 (3), 216–225.
Malika, N., Mohammed, F., Chakib, E.A., 2004. Antimicrobial activity of natural honey from aromatic and medicinal plants on antibiotic resistant strains of bacteria. Int. J. Agr. Biol. 6 (2), 289–293.
Moss, M.O., 2002. Mycotoxin review 1. *Aspergillus* and *Penicillium*. Mycologist 16, 116–119.
Nada, D.V., Vukovic, V., Nedic, N., 2010. Pathogenicity of some bacterial species isolated from the bee digestive tract. Acta Vet.-Beograd. 60 (1), 49–57.
Nasser, L.A., 2004. Isolation and characterization of fungi contaminating packaged honey commonly consumed in Saudi Arabia. Assiut Univ. Bull. Environ. Res. 7 (1), 1–7.
O'Donnell, K., Cigelnik, E., Nirenberg, H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.
Ohhira, I., Kuwaki, S., Morita, H., Suzuki, T., Tomita, S., Hisamatsu, S., Sonoki, S., Shinoda, S., 2004. Identification of 3-phenyllactic (*Galactites tomentosa* Moench) honey. J. Agric. Food Chem. 59, 364–369.
Piana, M.L., Poda, G., Cesaroni, D., Cuetti, L., Bucci, M.A., Gotti, P., 1991. Research on microbial characteristics of honey samples of Udine province. Riv. Soc. Ital. Sci. Aliment. 20, 293–301.
Rozanska, H., 2011. Microbiological quality of Polish honey. B. Vet. Pulawy 55 (3), 443–445.
Sabatè, D.C., Carrillo, L., Audisio, M.C., 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res. Microbiol. 160, 163–169.
Saraiva, M., Campos Cunha, I., Costa Bonito, C., Pena, C., Toscano, M., Teixeira Lopes, T., Sousa, I., Calhau, A., 2012. First case of infant botulism in Portugal. Food Control 26, 79–80.
Schwenninger, S.M., Lacroix, C., Truttmann, S., Jans, C., Sporndli, C., Bigler, L., Meile, L., 2008. Characterization of low-molecular-weight antiyeast metabolites

produced by a food-protective *Lactobacillus–Propionibacterium* coculture. J. Food Prot. 71, 2481–2487.
Snowdon, J.A., Cliver, D.O., 1996. Microorganisms in honey. Int. J. Food Microbiol. 31, 1–26.
Solieri, L., Cassanelli, S., Giudici, P., 2007. A new putative *Zygosaccharomyces* yeast species isolated from traditional balsamic vinegar. Yeast 24 (5), 403–417.
Stenlid, J., Karlsson, Jo, Hogberg, N., 1994. Intraspecific genetic-variation in *Heterobasidion annosum* revealed by amplification of minisatellite dna. Mycol. Res. 98 (1), 57–63.
Tofalo, R., Chaves–Lopez, C., Di Fabio, F., Schirone, M., Felis, G.E., Torriani, S., Paparella, A., Suzzi, G., 2009. Molecular identification and osmotolerant profile of wine yeasts that ferment a high sugar grape must. Int. J. Food Microbiol. 130 (3), 179–187.
Tysset, C., Rautlin de la Roy, Y., 1974. Study of osmophilic yeasts that ferment honeys produced in France. Bull. Assoc. Diplomes Microbiol. Fac. Pharm. Nancy 134, 1–26.
Tysset, C., Rousseau, M., 1981. Problem of microbes and hygiene of commercial honey. Rev. Med. Vet.-Toulouse 132, 591–600.
Ventorino, V., Chiurazzi, M., Aponte, M., Pepe, O., Moschetti, G., 2007. Genetic diversity of a natural population of *Rhizobium leguminosarum* bv. viciae nodulating plants of *Vicia faba* in the vesuvian area. Curr. Microbiol. 55, 512–517.
Versalovic, J., Schneider, M., De Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Method. Mol. Cell. Biol. 5, 25–40.
Voidarou, C., Alexopoulos, A., Plessas, S., Karapanou, A., Mantzourani, I., Stavropoulou, E., Fotou, K., Tzora, A., Skoufos, I., Bezirtzoglou, E., 2011. Antibacterial activity of different honeys against pathogenic bacteria. Anaerobe 17 (6), 375–379.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. PCR protocols: a guide to methods and applications. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. Academic Press, San Diego, pp. 315–322.
Wrent, P., Rivas, E.M., Peinado, J.M., De Silóniz, M.I., 2010. Strain typing of *Zygosaccharomyces* yeast species using a single molecular method based on polymorphism of the intergenic spacer region (IGS). Int. J. Food Microbiol. 142 (1–2), 89–96.
Yoshiyama, M., Wu, M., Sugimura, Y., Takaya, N., Kimoto-Nira, H., Suzuki, C., 2013. Inhibition of *Paenibacillus larvae* by lactic acid bacteria isolated from fermented materials. J. Invertebr. Pathol. 112 (1), 62–67.
Zumla, A., Lulat, A., 1989. Honey: a remedy rediscovered. J. R. Soc. Med. 82 (7), 384–385.