Propionibacterium olivae sp. nov. and Propionibacterium damnosum sp. nov., isolated from spoiled packaged Spanish-style green olives

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Five strains of Gram-stain-positive bacteria were isolated from anomalous fermentations occurring in post-packaging of sealed airtight food-grade plastic pouches of Spanish-style green olives. These isolates could be grouped into two sets, which showed a similarity in their respective 16S rRNA gene sequences of 98.40 and 98.44 % with *Propionibacterium acidipropionici* NCFB 563 and 98.33 and 98.11 % with *Propionibacterium microaerophilum* M5[T], respectively, and a similarity of 99.41 % between them. The 16S rRNA gene phylogeny revealed that the isolates grouped into two statistically well-supported clusters separate from *P. acidipropionici* NCFB 563 and *P. microaerophilum* M5[T]. Enzymic activity profiles as well as fermentation patterns differentiated these two novel bacteria from other members of the genus *Propionibacterium*. Finally, phenotypic, genotypic and phylogenetic data, supported the proposal of two novel species of the genus *Propionibacterium*, for which the names *Propionibacterium olivae* sp. nov. (type strain, IGBL1[T]=CECT 8061[T]=DSM 25436[T]) and *Propionibacterium damnosum* sp. nov. (type strain, IGBL13[T]=CECT 8062[T]=DSM 25450[T]) are proposed.

During the Spanish-style green-olive fermentations, there is an ecological succession of diverse micro-organism species. The first stage of the fermentation is characterized by the presence of *Enterobacteriaceae*, favoured by the high pH values reached after the alkaline treatment of the fruits that is characteristic of this olive preparation (Garrido-Fernández *et al.*, 1997; de Castro *et al.*, 2002). As the fermentation progresses, these micro-organisms disappear as consequence of the lowering of the pH due to the growth of lactic acid bacteria (LAB), mainly strains of *Lactobacillus pentosus*, which is characteristic of the second fermentation stage (de Castro *et al.*, 2002; Rejano *et al.*, 2010; Ruiz-Barba & Jiménez-Díaz, 2012). During the final, third stage of the fermentation, all fermentative substrates are exhausted and the LAB population declines steadily, thus starting the storage period. Values of pH below 4.0 and free acidities of 0.7–1.2 %, mainly as lactic acid, are considered indicative of a good fermentation so that these conditions, combined

with NaCl concentrations usually raised to 7–8 %, should guarantee the long-term preservation of the final product. However, during the storage period, if brine conditioning is not appropriate, or after the packaging of the fruits, if the product is not pasteurized and/or the storage conditions are not appropriate, an undesirable secondary fermentation, usually led by species of the genus *Propionibacterium*, could occur (Sánchez *et al.*, 2006). These bacteria increase the pH because of the production of acetic and propionic acids from the lactic acid either produced during the previous phase of active fermentation or added at the time of packaging (Sánchez *et al.*, 2006). This implies a considerable microbiological risk because such changes may facilitate further growth of spoilage or even pathogen microorganisms, such as those of the genus *Clostridium*, thus promoting different types of product spoilage (Kawatomari & Vaughn, 1956; Plastourgos & Vaughn, 1957; González Cancho *et al.*, 1973, 1980; Sánchez *et al.*, 2006). Three species of the genus *Propionibacterium*, namely '*Propionibacterium pentosaceum*', '*Propionibacterium zeae*' and *Propionibacterium acnes*, have been associated with 'zapatería' spoilage of olives (Plastourgos & Vaughn, 1957; González Cancho *et al.*, 1973, 1980). Propionibacteria are Gram-positive, catalase-positive, high G+C content, non-spore-forming

and non-motile bacteria, anaerobic or slightly aerotolerant and morphologically heterogeneous, that have a typical metabolism leading to the formation of propionic acid as the main end product of fermentation (Cummins & Johnson, 1986; Stackebrandt *et al.*, 2006). At the time of writing, the genus *Propionibacterium* consisted of 12 species and two subspecies (LPSN; http://www.bacterio.net/propionibacterium.html). Recently a novel species of the genus, 'Propionibacterium humerusii' (Butler-Wu *et al.*, 2011), has been described, but it has not yet been validated.

The genus *Propionibacterium* has been traditionally divided into 'dairy' (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum* and *P. thoenii*) and 'cutaneous' micro-organisms (*P. acidifaciens*, *P. acnes*, *P. australiense*, *P. avidum*, *P. granulosum* and *P. propionicum*) which mainly inhabit dairy/silage environments and the skin/intestine of human and animals, respectively. Dairy propionibacteria are generally recognized as safe microorganisms being valuable for both technological applications and health promotion as probiotics, whereas members of the cutaneous group have shown to be opportunistic pathogens in compromised hosts (Cousin *et al.*, 2011).

Here we report the isolation of two novel species of the genus *Propionibacterium* from Spanish-style green-olives packaged in sealed airtight food-grade plastic pouches filled with covering liquid (NaCl, citric acid, lactic acid, L-ascorbic acid; pH 3.2) that had suffered spoilage after two-year storage at room temperature (packaging is usually for three years). Spoilage consisted of abnormal turbidity and unpleasant odour, as well as a non-appropriated, elevated pH value of 4.5. Plastic pouches, frequently used as packing material, cannot be pasteurized as heat accelerates polymerization of ortho-diphenols and a progressive browning of the product (Sánchez *et al.*, 1991).

Samples of the covering liquids from the plastic pouches were aseptically taken, serially diluted and spread onto agar plates of de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.02 g bromophenol blue l^{-1} (AppliChem) and 0.05 % (w/v) L-cysteine (MRS-BPB; Lee & Lee, 2008), brain heart infusion (BHI; Biokar Diagnostics) supplemented with 0.05 % (w/v) L-cysteine, Reinforced Clostridial Medium (RCM; Biokar Diagnostics), Yeast glucose LEMCO (Naylor & Sharpe, 1958) and MacConkey Broth Purple (Biokar Diagnostics). Plates were incubated anaerobically at 30 °C for 72 h. For anaerobic incubations, a DG250 Anaerobic Workstation (Don Whitley Scientific) was used, with a gas mixture consisting of 10 % H_2/10 % CO_2/80 % N_2.

Preliminary identification of the isolates was done by partial 16S rRNA gene sequence analysis (~450 bp). For this, PCR amplification with primers plb16 (5′-AGAGTTTGATCC-TGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGT-AGTTAG-3′) was carried out (Kullen *et al.*, 2000).

The isolates were genotyped by Random Amplified Polymorphic DNA (RAPD) profiling, following the protocol of Ruiz-Barba *et al.* (2005). *P. acidipropionici* NCFB 563 and

P. microaerophilum DSM 13435^{T} (=M5^{T}) were also included in these analyses. The similarity of RAPD profiles was calculated using the Pearson's correlation coefficient, and Bionumerics 6.6 (Applied Maths) software was used to construct dendrograms based on UPGMA analysis.

To identify and determine the phylogenetic relatedness of the isolates, the 16S rRNA gene was amplified and sequenced (~1400 bp) with the primer pair 7for (5′-AGAGTTTGA-TYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTG-TTACGACTT-3′) (Lane, 1991). The identification of the isolates and their phylogenetic neighbours was carried out by the BLASTN program on the basis of 16S rRNA gene sequence data obtained (Altschul *et al.*, 1997) against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The calculation of pairwise sequence similarity was done using the global alignment algorithm (Myers & Miller, 1988), which was implemented at the EzTaxon-e server. The DNA sequences belonging to type strains of species of the genus *Propionibacterium* were obtained from the EzTaxon-e database. These sequences were aligned by using the CLUSTAL W method (Thompson *et al.*, 1994) with the MEGA 5 (version 5.2) software package (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed based on the neighbour-joining method (Saitou & Nei, 1987). Bootstrapping analysis (1000 replicates) was done to study the stability of the groupings.

The degree of DNA–DNA relatedness between strain IGBL1^{T}, strain IGBL13^{T}, *P. microaerophilum* DSM 13435^{T} and *P. acidipropionici* NCFB 563 were determined by the fluorometric method as described by Gonzalez & Saiz-Jimenez (2005). This method measures the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (DTm) using a real-time PCR thermocycler that obtains fluorescence determinations. Chromosomal DNA was extracted following the method described by Cathcart (1995). The results were expressed as mean percentage values based on three independent hybridization experiments. The G + C content of genomic DNA of strains IGBL1^{T} and IGBL13^{T} was determined by the fluorometric method described by Gonzalez & Saiz-Jimenez (2002).

Substrate utilization, the fermentation/oxidation profile, acid production and some other physiological characteristics were examined using the API 50 CHL fermentation kit (bioMérieux) after 48 h of incubation according to the manufacturer's instructions. Cell morphology was examined by light microscopy (400 ×) using cells from exponentially growing cultures. Growth at different temperatures, pH and NaCl concentrations was determined on Yeast Glucose LEMCO anaerobically. Catalase activity was determined by bubble production in a 10 % (v/v) H_2O_2 solution. Metabolic end products (organic acids) were monitored through HPLC analysis of the cell-free supernatants of 48 h cultures in Yeast Glucose LEMCO broth incubated at 30 °C both aerobically (through shaking

at 150 r.p.m. in a Gallenkamp orbital incubator) and anaerobically. Growth in lactate was checked in Yeast Extract-Sodium Lactate medium, both aerobically and anaerobically.

Five isolates showing a high degree of similarity (99 %) in the partial 16S rRNA gene sequence with species of the genus *Propionibacterium* were isolated from RCM, LEMCO or BHI agar plates. Three of the isolates (IGBL1^T, IGBL13^T and IGB34) were isolated from the brine of the spoiled plastic pouch of production batch 'B' and the other two (IGB32 and IGBL3) from the spoiled plastic pouch of production batch 'BC'. Both batches had been elaborated on the same date.

The RAPD profile analysis (Fig. 1) showed three well-separated clusters, one of them corresponding to strains IGBL1^T, IGBL3 and IGBL34, a second one corresponding to strains IGBL13^T and IGBL32 and a third one including the reference strains *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563. One representative isolate from each RAPD group (IGBL1^T and IGBL13^T) was selected for further analysis.

The BLASTN analysis of strains IGBL1^T and IGBL13^T showed 16S rRNA gene sequence similarities of 98.40 and 98.44 %, respectively, with *P. acidipropionici* NCFB 563, and 98.33 and 98.11 %, respectively, with *P. microaerophilum* M5^T, and a similarity of 99.41 % between the novel strains.

The phylogenetic analysis inferred from the 16S rRNA gene sequences using the neighbour-joining method, showed that the isolates IGBL1^T, IGBL3 and IGBL34, and IGBL13^T and IGBL32 formed two new sub-lines within the genus *Propionibacterium*. Bootstrap resampling values (94 %)

showed that branches of strains IGBL1^T and IGBL13^T were statistically significant (Fig. 2).

The difference in melting temperature between genomic DNA from strain IGBL1^T and that from strain IGBL13^T, *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563 were 9.0, 5.2 and 10.4 °C, respectively. In the same manner, the difference in melting temperature between genomic DNA from strain IGBL13^T and that from strain IGBL1^T, *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563 were 9.0, 7.2 and 7.0 °C. These values were all above the 5 °C ΔTm recommended as cut-off points for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that both strains IGBL1^T and IGBL13^T represent two novel species clearly differentiated from *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563. The G + C content of genomic DNA of strains IGBL1^T and IGBL13^T were 70.0 mol% (SD 1.0) and 67.5 mol% (SD 0.2), respectively. This range is in agreement with that described for other species of the genus *Propionibacterium* (Table 1).

The three analysed strains of the first novel species (IGBL1^T, IGBL3 and IGBL34) were shown to possess the same biochemical profile described in Table 1 for the type strain. In the same manner, the two strains of the second novel species (IGBL13^T and IGBL32) showed the same biochemical profile. Despite the high similarity at their 16S rRNA gene sequences, both novel species can be easily differentiated attending to their biochemical characteristics. Table 1 shows phenotypical tests that are useful for differentiation between the two novel species and from their closest phylogenetic relatives, i.e. *P. microaerophilum*
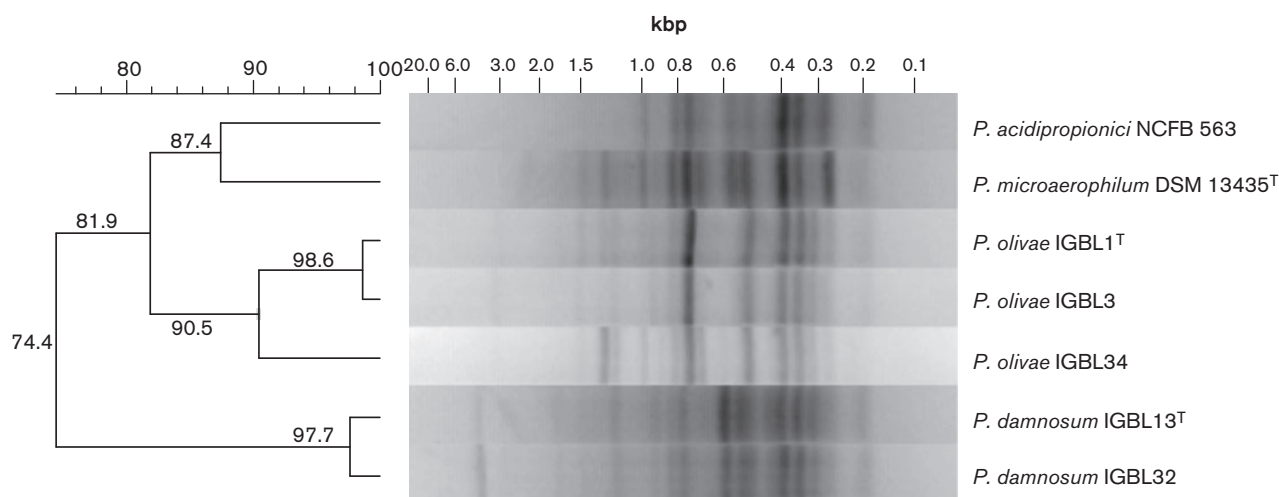

Fig. 1. RAPD profiles and dendrogram (UPGMA) representing genetic relationships among isolates of *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov. and the reference strains *P. acidipropionici* NCFB 563 and *P. microaerophilum* DSM 13435^T (=M5^T) based on genetic similarity matrix, calculated by using the pair-wise Pearson's correlation coefficient. Bar, similarity level (%).
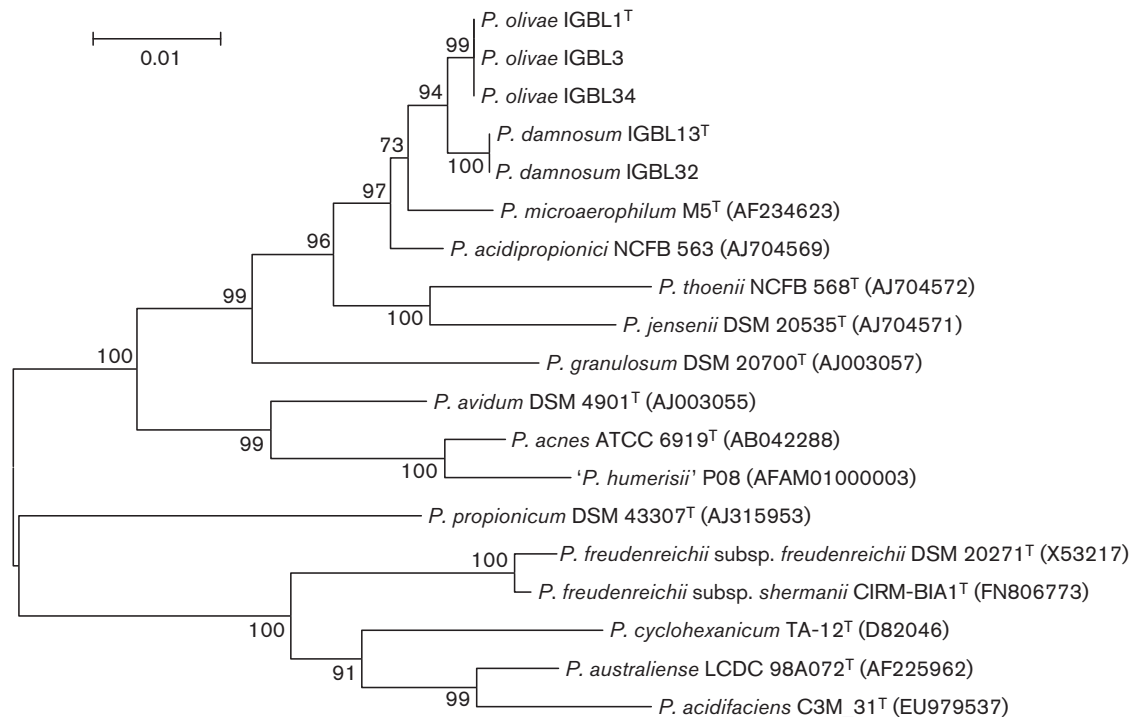
Fig. 2. Phylogenetic relationships of *P. olivae* sp. nov. IGBL1[T], IGBL3 and IGB34, and *P. damnosum* sp. nov. IGBL13[T] and IGB32 with strains of species of the genus *Propionibacterium* based on comparison of 16S rRNA gene sequences (1406 nt). GenBank accession numbers for the sequences used are given in parentheses. The tree was based on the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50 %. Bar, 0.01 nt changes per nucleotide position.

M5[T], *P. acidipropionici* NCFB 563, *P. jensenii* DSM 20535[T] and *P. thoenii* DSM 20276[T].

Strain IGBL1[T] grows at a pH range of 4.0 to 10.0, 4.0 % concentration of NaCl, in aerobic conditions at 30 °C, in the same manner as *P. microaerophilum* DSM 13435[T] and *P. acidipropionici* NCFB 563. Strain IGBL13[T], however, shows weak growth at pH 4, 4.0 % NaCl, and aerobic conditions at 30 °C, showing no growth at pH 9.0 or pH 10.0. Interestingly, *P. microaerophilum* DSM 13435[T], one of the closest taxa to these novel species was originally isolated from a similar environmental niche (olive mill wastewater; Koussémon *et al.*, 2001).

Cell morphology under the microscope was especially characteristic in strain IGBL13[T], which was able to form filaments, sometimes branched, of various lengths (5–30 µm). This characteristic has been previously observed in *P. propionicum* and strains of *P. acnes* phylotype III.

In conclusion, on the basis of DNA–DNA reassociation values and phenotypic, genotypic and phylogenetic characteristics, we suggest the existence of two novel species of the genus *Propionibacterium*, for which the names *Propionibacterium olivae* sp. nov. (type strain IGBL1[T]) and *Propionibacterium damnosum* sp. nov. (type strain IGBL13[T]) are proposed.

Description of *Propionibacterium olivae* sp. nov.

Propionibacterium olivae (o.li′vae L. gen. n. *olivae* of an olive, referring to the isolation of the strains from olives).

Cells are Gram-positive, facultatively anaerobic, aerotolerant, catalase-positive, non-motile, non-spore-forming, pleomorphic bacilli with rounded edges (0.7–0.8 × 1.4–4.0 µm) and arranged in pairs or in short chains in aged cultures (Fig. 3a). Colonies on Yeast Glucose LEMCO agar are circular with entire margins, highly convex, white to cream, opaque, creamy, and 3 mm in diameter. Growth in broth media produces a homogeneous turbidity. Positive for hydrolysis of aesculin but negative for starch. Cells ferment glycerol, erythritol, D- and L-arabinose, D-ribose, D-xylose, adonitol, D-glucose, D-galactose, D-fructose, D-mannose, inositol, mannitol, D-sorbitol, arbutin, salicin, maltose, cellobiose, trehalose, sucrose, xylitol, turanose, D- and L-arabitol. Grows in lactate, both aerobically and anaerobically. Does not ferment L-xylose, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, methyl α- D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, D-amygdalin, lactose, melibiose, inulin, melezitose, raffinose, glycogen, gentiobiose, D-lyxose, D-tagatose, D- or L-fucose, gluconate or 2- or 5-ketogluconate. Grows at a temperature range of 20 to 42 °C (with optimal growth at 30 °C), at a pH range of 4.0 to 10.0 and

Table 1. Biochemical characteristics that differentiate *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov. from other related propionibacteria

Strains: 1, *P. olivae* sp. nov. IGBL1[T]; 2, *P. damnosum* sp. nov. IGBL13[T]; 3, *P. microaerophilum* DSM 13435[T] (=M5[T]); 4, *P. acidipropionici* NCFB 563; 5, *P. jensenii* DSM 20535[T]; 6, *P. thoenii* DSM 20276[T]. The biochemical characteristics of taxa 1 and 2 were determined in this study, while those of taxa 3–6 were taken from Koussémon *et al.* (2001) and Kusano *et al.* (1997). +, Positive; –, negative; +/–, result depending on the authors; ND, no data available.

Characteristic	1	2	3	4	5	6
Production of acid from:						
D-Arabinose	+	–	–	+	–	–
L-Arabinose	+	+	+	+	–	–
D-Xylose	+	–	–	+	–	–
L-Sorbose	–	–	+	–	–	–
Rhamnose	–	+	+	+	–	–
N-Acetylglucosamine	–	+	+	+	–	+
Amygdalin	–	–	–	+/–	+	–
Arbutin	+	–	+	+	ND	ND
Cellobiose	+	+	+	+	ND	ND
Lactose	–	+	–	+	–	+
Melibiose	–	+	–	+	+	–
Melezitose	–	–	+	+	+	–
Raffinose	–	–	–	+/–	+	–
Starch	–	–	+	+	–	+
Glycogen	–	–	+	+/–	–	–
Gluconate	–	–	+	+	+	–
DNA G + C content (mol%)	70.0	67.5	67.1	67.5	66	66

in the presence of 4.0 % NaCl. No growth was observed after pasteurization at 80 °C for 10 min. Under anaerobic conditions, propionic and acetic acids are formed from glucose fermentation, while only acetic acid, plus a small amount of succinic acid are formed when grown aerobically.

The type strain, IGBL1[T] (=CECT 8061[T]=DSM 25436[T]), was isolated from the brines of plastic pouches of packaged, non-pasteurized, two-year-stored Spanish-style green olives that suffered spoilage consisting of abnormal turbidity and

unpleasant odour. The DNA G + C content of the type strain is 70.0 mol%.

Description of *Propionibacterium damnosum* sp. nov.

Propionibacterium damnosum (dam.no′sum. L. neut. adj. *damnosum* causing losses, referring to the isolation of the type strain from spoiled olives).

Cells are Gram-positive, facultatively anaerobic, aerotolerant, catalase-negative, non-motile, non-spore-forming, pleomorphic rods and are able to form filaments, sometimes branched, variable in length (0.6–0.7 × 5–30 μm) (Fig. 3b). Colonies on LEMCO agar are circular with irregular margins, white to cream, opaque, creamy, rough and 2–3 mm in diameter. In broth culture, the cells form aggregates having a granular appearance and being deposited at the bottom of the tubes. Hydrolyses aesculin but not starch. Cells ferment glycerol, erythritol, L-arabinose, D-ribose, adonitol, D-glucose, D-galactose, D-fructose, D-mannose, L-rhamnose, inositol, mannitol, D-sorbitol, *N*-acetylglucosamine, cellobiose, lactose, melibiose, sucrose, trehalose, xylitol, turanose and D- and L-arabitol. Grows in lactate, both aerobically and anaerobically. Does not ferment D-arabinose, D- or L-xylose, methyl β-D-xylopyranoside, L-sorbose, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-amygdalin, arbutin, salicin, maltose, inulin, melezitose, raffinose, glycogen, gentiobiose, D-lyxose, D-tagatose, D- or L-fucose, gluconate or 2- or 5-ketogluconate. Broth cultures grow well at a temperature range of 20 to 42 °C (with optimal growth at 30 °C), at a pH range of 4.5 to 8 and in the presence of 2.0 % NaCl, showing weak growth with 4.0 % NaCl. No growth was observed after pasteurization at 80 °C for 10 min. Under anaerobic conditions, propionic and acetic acids are formed from glucose fermentation, while only acetic acid is formed when grown aerobically.

The type strain, IGBL13[T] (=CECT 8062[T]=DSM 25450[T]), was isolated from the brines of plastic pouches of packaged, non-pasteurized, two-year-stored Spanish-style green olives that suffered spoilage consisting of abnormal turbidity and unpleasant odour. The DNA G + C content of the type strain is 67.5 mol%.
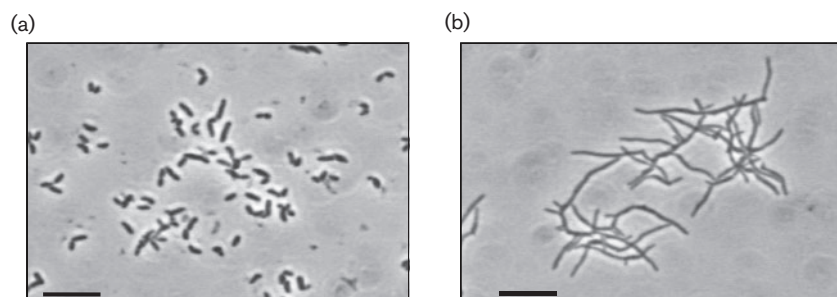
Fig. 3. Phase-contrast photomicrograph of *Propionibacterium olivae* sp. nov. IGBL1[T] (a) and *Propionibacterium damnosum* sp. nov. IGBL13[T] (b) from cultures in the stationary phase of growth. Bars, 10 μm.

Acknowledgements

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