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Method for reliable isolation of *Lactobacillus sakei* strains originating from Tunisian seafood and meat products

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Abstract

In Tunisia, several food products derived from meat or seafood are naturally processed, without any addition of bacterial starters. Such fermented, dried-cured, salted, or marinated products, as well as the raw meat or fish may thus provide a source to isolate the natural microflora colonizing such environments. We isolated lactic acid bacteria from a representative range of flesh-foods sold or manufactured in different parts of Tunisia, and selectively searched for *Lactobacillus sakei*, a lactic acid bacterium potentially useful as starter or protective culture. Eighty six (86) strains were isolated from various seafood (anchovy, sardine, sole, mullet, and octopus), or meat (pork, veal, beef, sheep, chicken, and turkey) products that were either fresh, or transformed by different traditional processes. Several methods were used in order to develop a rapid and reliable protocol for the direct identification of *L. sakei*. Amplified ribosomal DNA restriction analysis (ARDRA) classified the various isolates into 9 distinct groups. Search for the presence of the *L. sakei* specific *katA* gene indicated that all positive isolates were grouped in the same ARDRA group. Sequencing of 16S rDNA confirmed those isolates as *L. sakei*. Those 22 different *L. sakei* strains represent 25.6% of the total isolates, while other isolates found in the different ARDRA groups were tentatively ascribed to *Lactobacillus plantarum*, *Lactococcus lactis/garviae*, *Enterococcus avium*, *Streptococcus parauberis*, *Hafnia alvei*, *Pediococcus pentosaceus*, and *Lactobacillus curvatus* through 16S rDNA sequencing. A fast and reliable method to isolate and discriminate *L. sakei* from complex food environments is proposed.

Keywords: *Lactobacillus sakei*; Lactic acid bacteria; PCR

1. Introduction

Lactic acid bacteria (LAB) are commonly found in many food products and have a long history of use for food preservation—mainly through fermentations. Lactobacilli, lactococci, pediococci, and some streptococci are the main groups developed as starter cultures for the fermentation of a variety of dairy, vegetable, fish and meat products. *Lactobacillus sakei*, originally described in rice wine, is commonly associated with raw meat and is often used as a starter for dry sausage fermentation (Hammes et al., 1990; Champomier-Vergès et al., 2002). The use of *L. sakei* as a bio-protective agent for cooked meat products has also been proposed (Bredholt et al., 2001; Vermeiren et al., 2004) and lower numbers of the pathogen *Escherichia coli* O157:H7 have been observed when *L. sakei* is present in the endogenous flora of ground beef (Vold et al., 2000). The genome sequence of a strain isolated from dry sausage reveals several properties that may explain the adaptation of this species to meat and highlights its potential use as a bio-protective culture for fresh meat preservation (Chaillou et al., 2005). However, some *L. sakei* strains have been isolated that have been shown to be responsible for meat spoilage through ropy slime production (Mäkelä et al., 1992; Björkroth and Korkeala, 1997) and, although *L. sakei* has also been reported in fish products, it is sometimes considered a spoilage microorganism. For example, it has been associated with smoked salmon spoilage (Joffraud et al., 2001) and trout spoilage (Lyhs et al., 2002).

A large phenotypic diversity has been reported in *L. sakei* species. Consequently, classical identification methods for this

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species can be inaccurate and time consuming (Champomier et al., 1987; Montel et al., 1991). If ease of identification and selection of the best strains for food bio-protection is to be achieved, more efficient screening methods need to be developed.

Most of the *L. sakei* strains studied by the scientific community have been isolated from various countries: in North and South America, Asia, Europe, Australia, and New-Zealand. However, little work has been reported from the African continent. In Tunisia, many dry-cured, salted, or fermented products derived from meat, fish, or seafood are still prepared without the use of starter cultures. This provides the possibility of isolating endogenous strains of *L. sakei* from within the naturally seeded microflora of such products.

Dal Bello et al. (2003) showed that when investigating the microflora of complex environments, the media and incubation conditions used for plating bacterial samples may mask or enrich some species. These authors described media and incubation conditions allowing the isolation of *L. sakei* and other *Lactobacillus* species from human faeces and the oral cavity—although those bacteria may not represent the major populations in such environments (Dal Bello et al., 2003; Dal Bello and Hertel, 2006). The aim of the present study was to isolate lactic acid bacteria and to select *L. sakei* isolates in order to generate a non redundant collection of *L. sakei* strains representative of Tunisian meat- and fish-derived products. A rapid method is examined, the use of which should allow easy selection and characterization of *L. sakei* strains from complex environments.

2. Materials and methods

2.1. Bacterial samples

Bacterial isolates were sampled from different types of seafood (anchovy, sardine, sole, grey mullet, octopus) and meat (sheep, pork, veal, beef, turkey, chicken). The food products had been processed in a variety of ways (raw, fermented, salted, dry-cured, smoked, spiced or conserved in olive oil) originating from different regions of Tunisia. All products were commercially prepared exhibiting no evidence of spoilage and were thus considered fit for consumption. A 25 g sample of each food-type was ground under sterile conditions and homogenised by vortexing with 225 ml saline-peptone water (NaCl 8.5 $g \cdot l^{-1}$; bactopeptone 1 $g \cdot l^{-1}$) and incubated for 24 h at room temperature. Serial dilutions were then made in saline-peptone water and aliquots plated on to MRS (De Man et al., 1960) agar plates containing bromocresol green (25 $mg \cdot l^{-1}$) based on the method of Dal Bello and Hertel (2006). Plates were incubated anaerobically for 48 h at 30 °C and isolates selected from plates containing 100 to 150 total colonies. The morphology of colonies (colour, size) was recorded and, from each sample, one colony of each morphological type was selected for further examination. Selected isolates were purified on MRS plates and then kept at −70 °C in MRS broth containing 30% (v/v) glycerol. *L. sakei* Lb 674, a bacteriocin producer, was used as a reference strain (Holck et al., 1994).

2.2. DNA manipulation and PCR conditions

Chromosomal DNA of bacteria was prepared as described in Anderson and McKay (1983). Standard methods were used for electrophoresis and purification of PCR DNA fragments. The presence of specific PCR products was visualized after electrophoresis on 1.5% (w/v) agarose gels.

The 16S *rrn* genes were amplified as a 1.5 kb DNA fragment from each isolate by PCR with the two universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH* (5'-AAGGAGGTGATCCAGCCGCA-3') that hybridize near both ends of the 16S *rrn* bacterial genes (Broda et al., 1999). The PCR amplification was carried out in a PTC-200 thermocycler (MJ Research). The 50 µl PCR reaction mixture contained PCR buffer 1 X (Sigma), $MgCl_2$ 1.5 $mmol \cdot l^{-1}$, 0.2 $mmol^{-1}$ of each dNTP, 0.5 $\mu mol \cdot l^{-1}$ of each primer, 1 $\mu g \cdot ml^{-1}$ of chromosomal DNA, and 1 U of Taq DNA polymerase (Sigma). The program used was: 95 °C for 4 min; 35 cycles of [94 °C 45 s, 55 °C 45 s, 72 °C 1 min], and a final extension step at 72 °C for 5 min.

To detect the presence of the *katA* gene, encoding the haeme-dependent catalase, two primers specific for the *katA* gene of *L. sakei*: 702F (5'-AATTGCCTTCTTCCGTGTA, position 551–536 in the *katA* CDS) and 310-R (5'-AGTTGCGCACAATTATTTTC, position 127–139) (Ammor et al., 2005a), were used to amplify a 410 bp DNA fragment. PCR reactions were performed in a thermocycler (Bio-Rad MyCycler™) in a final volume of 25 µl containing PCR buffer 1 X (Fermentas), $MgCl_2$ 1.5 $mmol \cdot l^{-1}$, 0.2 $mmol \cdot l^{-1}$ of each dNTP, 0.8 $\mu mol \cdot l^{-1}$ of each primer, 2 U of Taq DNA polymerase (MBI Fermentas) and 1 $\mu g \cdot ml^{-1}$ of chromosomal DNA. Initial DNA denaturation step was performed at 94 °C for 5 min, followed by 30 cycles of [94 °C 1 min, 56 °C 1 min, 72 °C 1 min], a final extension step at 72 °C for 5 min. As an alternative method, FTA cards (Whatman) were used according to the manufacturer. Briefly,

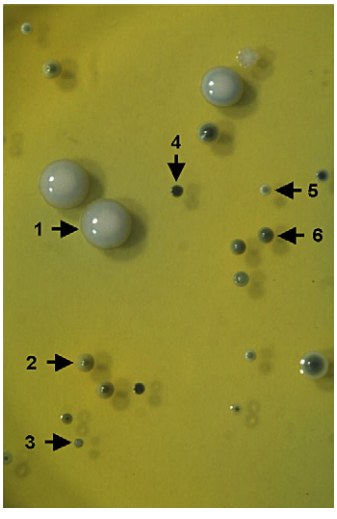

Fig. 1. Example of colony aspect after plating food sample dilutions. A mix of different isolates were plated on MRS plates containing 25 $mg \cdot l^{-1}$ bromocresol green. Different colony morphologies were observed. 1: white and large colony (BMG 96); 2: light green with green center (BMG 115); 3: grey colony (BMG 172); 4: dark green colony (BMG 92); 5: white with green center (BMG 95); 6: green colony (BMG 91). BMG 115 and BMG 95 were shown to be *L. sakei* isolates.

Table 1
Characterization of bacterial colonies obtained after sampling from various food products

Name	Isolated from	Colony morphology [a]	Population with same morphology cfu·g^{-1} [b]	ARDRA group	*katA*	Closest 16S rDNA sequence [c] (Accession number)
BMG 91	Anchovies (raw, salted and conserved in oil)	Green	8 10^6	G9	−	
BMG 92		Dark green	8 10^6	G9	−	
BMG 93		Grey	2 10^5	G7	−	
BMG 94		White	5 10^5	G8	−	
BMG 95		White+green center	5 10^5	G6	+	*Lactobacillus sakei* (EU80986)
BMG 96	Sardines (raw, spiced)	White and large	1 10^7	G9	−	*Lactobacillus plantarum*
BMG 97		Light green	7 10^5	G9	−	(EU801013)
BMG 98		White	4 10^5	G9	−	
BMG 99		Green	3 10^5	G9	−	
BMG 74	Sole (fresh)	Green	8 10^8	G4	−	*Pediococcus pentosaceus*
BMG 75		White	4 10^8	G1	−	(EU80993)
BMG 76		Grey	4 10^8	G7	−	
BMG 128	Grey mullet (fresh)	Green	4 10^8	G1	−	*Enterococcus avium* (EU801012)
BMG 129		White	4 10^8	G1	−	
BMG 163	Octopus (dried, salted)	Green and large	8 10^5	G3	−	
BMG 164		White+green center	4 10^5	G6	+	*Lactobacillus sakei* (EU801002)
BMG 165		Light green+green center	4 10^5	G6	+	*Lactobacillus sakei* (EU80992)
BMG 101	Turkey (packaged ham)	Light green+green center	8 10^5	G6	+	*Lactobacillus sakei* (EU801003)
BMG 102		White	2 10^6	G3	−	
BMG 103		White and small	5 10^6	G3	−	
BMG 104		Green and large	5 10^6	G3	−	
BMG 105		White+green center	8 10^5	G6	+	*Lactobacillus sakei* (EU80991)
BMG 106	Turkey (packaged sausage+garlic)	White and large + green center	6 10^6	G6	+	*Lactobacillus sakei* (EU80999)
BMG 107		White+green center	6 10^6	G6	+	*Lactobacillus sakei* (EU80995)
BMG 108		Green	3 10^6	G7	−	
BMG 109		Green and small	2 10^6	G3	−	
BMG 60	Turkey (packaged salami)	Light green and large	4 10^6	G4	−	*Pediococcus pentosaceus*
BMG 61		White	5 10^6	G1	−	(EU80990)
BMG 62		Grey	1 10^6	G1	−	
BMG 159	Turkey (packaged salami)	White and large	3 10^6	G9	−	
BMG 160		Green	3 10^6	G8	−	
BMG 161		White	1 10^6	G8	−	
BMG 162		Light green	3 10^6	G8	−	
BMG 151	Chicken meat (fresh)	White	4 10^6	G3	−	
BMG 152		White and large	5 10^6	G3	−	
BMG 153		Green and small	1 10^6	G8	−	
BMG 154		Green	1 10^5	G3	−	
BMG 130	Chicken meat (fresh)	White and large	4 10^6	G8	−	
BMG 131		White	4 10^6	G1	−	
BMG 132		Green	2 10^6	G1	−	
BMG 110	Beef (packaged smoked ham)	Green	8 10^5	G3	−	
BMG 111		White	2 10^6	G8	−	
BMG 112		White and small	5 10^6	G3	−	*Lactobacillus plantarum* (EU801011)
BMG 113		Green and large	5 10^6	G3	−	
BMG 114		Green and small	8 10^5	G3	−	*Lactobacillus plantarum* (EU801010)
BMG 124	Beef (packaged sausage)	Dark green	5 10^7	G7	−	*Hafnia alvei* (EU801015)
BMG 125		Green and small	5 10^7	G8	−	*Lactococcus lactis* (EU80989)
BMG 126		White+green center	5 10^7	G6	+	*Lactobacillus sakei* (EU801017)
BMG 127		Light green+green center	2 10^7	G6	+	*Lactobacillus sakei* (EU801005)
BMG 45	Beef (packaged sausage)	White+green center	8 10^7	G6	+	*Lactobacillus sakei* (EU80996)
BMG 46		Grey	8 10^7	G8	−	
BMG 40	Beef (packaged salami)	White+green center	7 10^6	G6	+	*Lactobacillus sakei* (EU801004)
BMG 41		Grey	3 10^6	G1	−	
BMG 147	Beef meat (fresh)	Green	5 10^5	G8	−	
BMG 148		White+green center	5 10^5	G6	+	*Lactobacillus sakei* (EU801001)
BMG 149		White	2 10^5	G5	−	*Streptococcus parauberis*
BMG 150		Green and small	5 10^5	G3	−	(EU801009)
BMG 70	Beef meat (ground)	Green	6 10^6	G9	−	
BMG 71		Grey	8 10^5	G8	−	
BMG 72		White	3 10^6	G3	−	
BMG 73		White+green center	5 10^6	G6	+	Lactobacillus sakei (EU80998)

Table 1 (*continued*)

Name	Isolated from	Colony morphology [a]	Population with same morphology cfu·g^{-1} [b]	ARDRA group	*katA*	Closest 16S rDNA sequence [c] (Accession number)
BMG 119	Beef meat (Merguez)	Green	4 10^7	G7	−	
BMG 120		Light green+green center	3 10^7	G6	+	*Lactobacillus sakei* (EU80987)
BMG 121		White	1 10^7	G7	−	
BMG 122		White and small	2 10^7	G9	−	
BMG 155	Veal meat (fresh)	White+green center	3 10^8	G3	−	
BMG 157		White	3 10^8	G2	−	*Lactobacillus curvatus* (EU801014)
BMG 158		Green and small	2 10^8	G8	−	
BMG 50	Sheep meat (fresh, South of Tunisia)	Grey	8 10^6	G1	−	
BMG 51		White + green center	8 10^6	G6	+	*Lactobacillus sakei* (EU80997)
BMG 37	Sheep meat (fresh, East of Tunisia)	White+green center	8 10^5	G6	+	*Lactobacillus sakei* (EU80988)
BMG 38		Grey	4 10^5	G1	−	
BMG 39		Green	4 10^5	G3	−	
BMG 169	Sheep meat (fresh, North of Tunisia)	Green	8 10^5	G7	−	
BMG 167		White+green center	5 10^5	G6	+	*Lactobacillus sakei* (EU801007)
BMG 168		Light green+green center	3 10^5	G6	+	*Lactobacillus sakei* (EU801008)
BMG 136	Sheep meat (fresh, West of Tunisia)	Light green	3 10^5	G6	+	*Lactobacillus sakei* (EU80994)
BMG 137		Light green	5 10^5	G8	−	
BMG 138		White+green center	5 10^5	G6	+	*Lactobacillus sakei* (EU801006)
BMG 115	Sheep meat (dried)	Light green+green center	2 10^7	G6	+	*Lactobacillus sakei* (EU80985)
BMG 116		White and small	3 10^7	G9	−	
BMG 117		Green and small	2 10^8	G9	−	
BMG 118		White	1 10^8	G1	−	
BMG 170	Pork meat (fresh)	White+green center	6 10^6	G6	+	*Lactobacillus sakei* (EU801000)
BMG 171		Green	3 10^6	G8	−	
BMG 172		Grey	5 10^6	G8	−	*Lactococcus garviae* (EU801016)

[a] Morphology (colour and size) of colonies after 48 h incubation on MRS agar (see also Fig. 1).
[b] Calculated cfu·g^{-1} for each morphology type in tested food product. Samples were incubated 24 h at 30 °C in peptone saline water before plating.
[c] 16S rDNA sequence was determined for various isolates of ARDRA groups and compared to databases to tentatively assign them to bacterial species.

15 µl of an overnight MRS culture were deposited on the membrane and let to dry for 24 h. Then 2 mm diameter punches were collected, rinsed twice with 200 µl TE buffer for 3 min, air dried for 1 h, and then used as DNA matrices for PCR experiments as described above. In this case the PCR reaction mixtures contained 50 µl.

2.3. ARDRA (amplified ribosomal DNA restriction analysis)

The 2 enzymes *Hinf*I and *Hind*III were chosen, on the basis of theoretical digestion with the Webcutter 2.0 tool (http://www.firstmarket.com/cutter/cut2) of the complete 16S *rrn* gene sequences of *L. sakei* deposited in databases. The 1.5 kb rDNA PCR products, obtained as described above were digested overnight at 37 °C in 20 µl reaction mixtures containing 8 µl of the PCR product solution, 2 µl of the commercially supplied incubation buffer, and 3 U of the restriction enzymes *Hinf*I or *Hind*III (Promega). DNA restriction patterns were analyzed by electrophoresis in 6% (w/v) polyacrylamide gel.

ARDRA banding patterns of each isolate, obtained after independent restriction digestion of the amplified 16S *rrn* gene with the two enzymes were analysed and profiles of each strain were clustered by the unweighted pair group method using the arithmetic averages (UPGMA) clustering algorithm and the Jacquard's correlation coefficient. Bacterial isolates showing the same pattern were assembled to form a group.

2.4. 16S rDNA sequencing

The 1.5 kb rDNA PCR products were treated with Shrimp alkaline phosphatase (USB) following the protocol recommended by the manufacturer and sequenced in both directions by using forward primer pA and reverse primer pH∗. The 16S rDNA sequences were compared to the databases (http://rdp.cme.msu.edu/) and analyzed using the BLAST Program and LALIGN software. The *L. sakei* genome database (http://migale.jouy.inra.fr/sakei/) was used for comparison to the *L. sakei* 16S rDNA genes. A phylogenetic tree based on 16S rRNA genes was also constructed to determinate the closest bacterial species by the neighbour-joining method (Saitou and Nei, 1987), using MEGA version 3.1 (Kumar et al., 2004).

3. Results

3.1. Selection of a LAB collection from Tunisian food products

Six different types of colony morphology were observed on plated samples (Fig. 1). In order to avoid redundancy in the strain collection, only one colony of each morphology was

selected from each sample for further study, leading to a total of 86 isolates, corresponding to 2–5 clones selected from each of the products that were analysed. Several methods were then used in order to define the most reliable and appropriate one allowing a specific selection of isolates belonging to the *L. sakei* species.

Table 1 summarizes the numbers of each type of colony isolated on MRS plates after sampling from various flesh-food products. Total counts on MRS plates, after incubation at 30 °C for 48 h, ranged from $1.3 \cdot 10^6$ to $1.6 \cdot 10^9$ cfu per g of food sample depending on the product. However, as plating of the diluted food samples were performed after 24 h incubation at room temperature in saline-peptone water, those counts may not reflect the exact population of the products at the time of sampling. As an indication, for seafood products, fresh fish showed the highest LAB population: $8 \cdot 10^8$ cfu/g were found on fresh grey mullet and $1.6 \cdot 10^9$ cfu per g on fresh sole, respectively. Interestingly, in processed seafood products the total LAB population diminished. Dry salted octopus showed the lowest LAB counts ($1.6\ 10^6$ cfu per g) whereas sardines and anchovies showed a LAB population around 10^7 cfu per g. However slightly more colony morphology types were observed from processed seafood compared to raw seafood, suggesting a higher population diversity after processing. Indeed, only 2 or 3 colony types were observed with clones isolated from raw fish, whereas 3 to 5 different types were noticed from samples isolated from processed seafood. The fresh beef and sheep-meat samples showed a low level of LAB

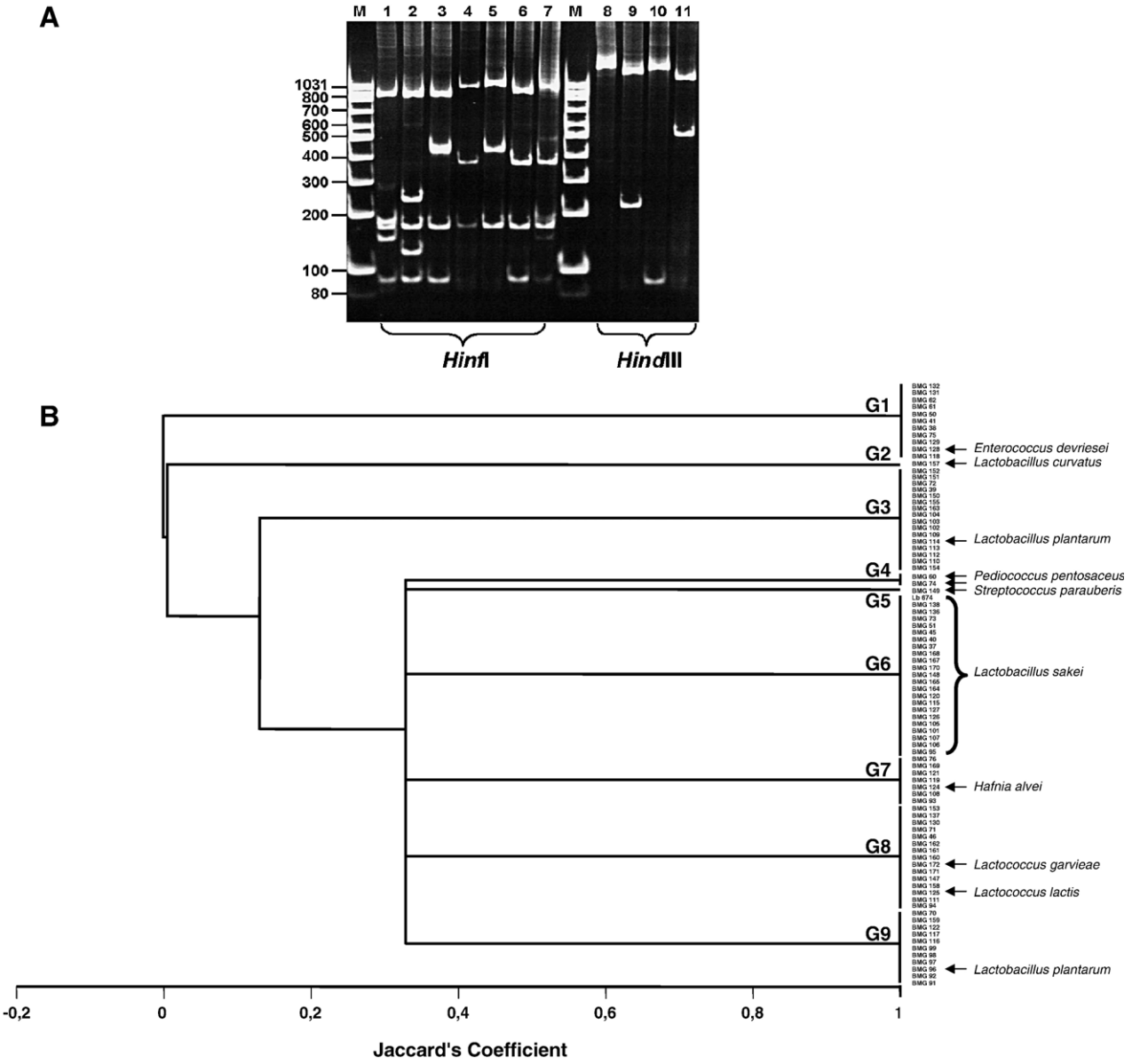

Fig. 2. ARDRA classification of the isolates. A) *Hind*III and *Hinf*I profiles. M) Molecular size ladder; 1) BMG 124; 2) BMG 125; 3) BMG 128; 4) BMG 95; 5) BMG 96; 6) BMG 157; 7) BMG 74; 8) BMG 95; 9) BMG 128; 10) BMG 157; 11) BMG 114. B) UPGMA clusterization of the 86 isolates into nine groups (G1 to G9). Tentative assignation to bacterial species is indicated when 16S rDNA was sequenced.

population (around 10^6 cfu per g), compared to the one to two higher log units found in other processed meat products. The traditional dried sheep-meat and a sample of fresh veal had the highest LAB population reaching respectively around 10^8 and 10^9 cfu per g.

3.2. Clusterization of the isolates by ARDRA

The total DNA was extracted for each of the 86 selected clones and an ARDRA profile was obtained after *Hind*III and *Hinf*I digestion of the PCR amplified *rrn* genes. Four distinct profiles were obtained from *Hind*III digestion, and 7 from *Hinf*I (Fig. 2A). After UPGMA clusterization 9 different groups could be distinguished, when combining both profiles, encompassing respectively 11, 1, 16, 2, 1, 22, 7, 15, and 11 isolates (Fig. 2B). Group G6, encompassing 22 isolates was assigned to *L. sakei,* as it also encompassed strain *L. sakei* Lb 674 used as a control. For each isolate, the ARDRA group is indicated in Table 1.

3.3. katA selective PCR

L. sakei possesses a haeme-dependent catalase encoded by the *katA* gene (Hertel et al., 1998). This gene is absent in many other lactic acid bacteria and, when present, shares only a partial identity with the *L. sakei* gene. For example the *Lactobacillus plantarum* and *Enterococcus faecalis* catalases are only respectively 68% and 61% identical to the one of *L. sakei* 23K. Thus, the use of *katA* specific primers to discriminate *L. sakei* isolates amongst other LAB presents a potentially useful discriminatory method. Indeed, we showed in a recent study

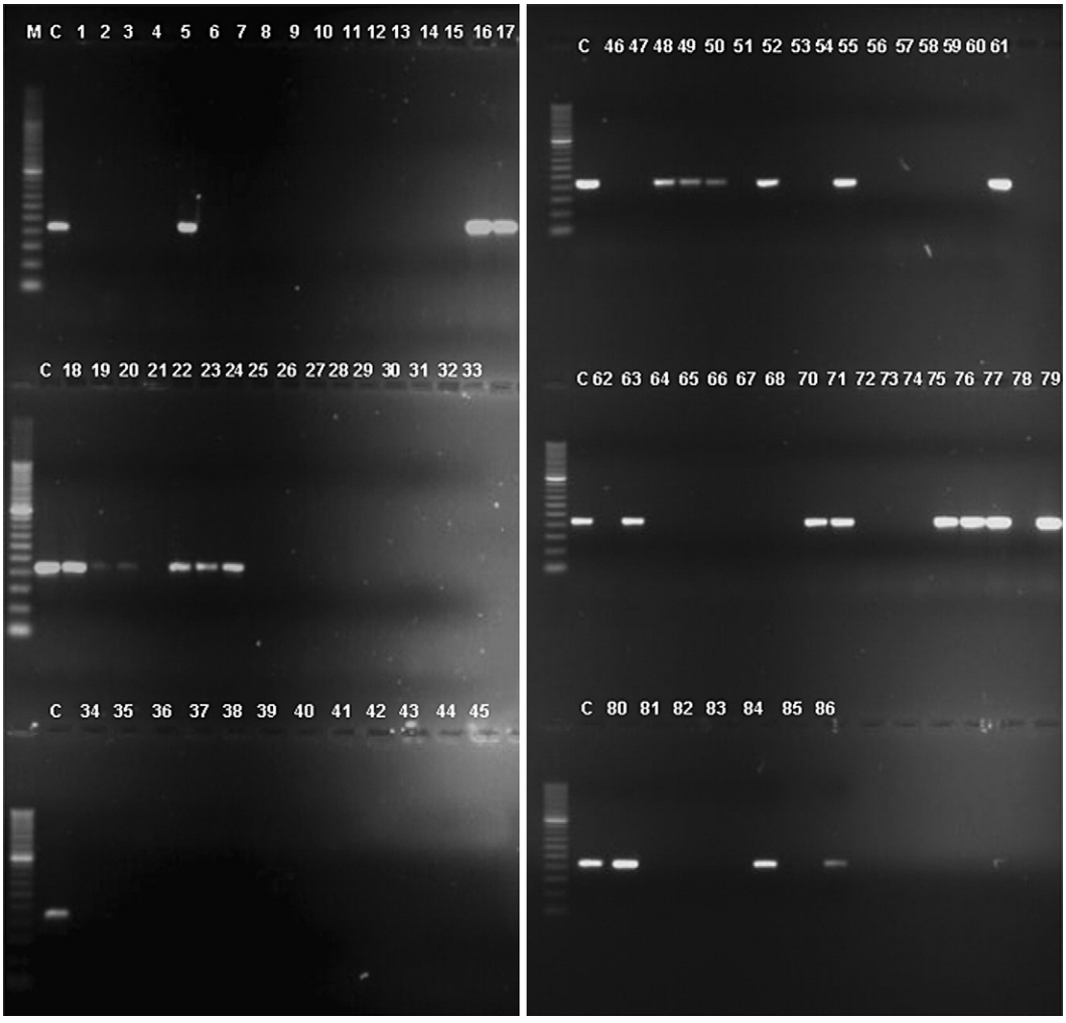

Fig. 3. PCR amplification of a 410 bp *L. sakei katA* specific fragment. (M) 100 bp molecular weight ladder, (C) *L. sakei* control strain. Strain order is in the same order as Table 1. 1: BMG 91, 2: BMG 92, 3: BMG 93, 4: BMG 94, 5: BMG 95, 6: BMG 96, 7: BMG 97, 8: BMG 98, 9: BMG 99, 10: BMG 74, 11: BMG 75, 12: BMG 76, 13: BMG 128, 14: BMG 129, 15: BMG 163, 16: BMG 164, 17: BMG 165, 18: BMG 101, 19: BMG 102, 20: BMG 103, 21: BMG 104, 22: BMG 105, 23: BMG 106, 24: BMG 107, 25: BMG 108, 26: BMG 109, 27: BMG 60, 28: BMG 61, 29: BMG 62, 30: BMG 159, 31: BMG 160, 32: BMG 161, 33: BMG 162, 34: BMG 151, 35: BMG 152, 36: BMG 153, 37: BMG 154, 38: BMG 130, 39: BMG 131, 40: BMG 132, 41: BMG 110, 42: BMG 111, 43: BMG 112, 44: BMG 113, 45: BMG 114, 46: BMG 124, 47: BMG 125, 48: BMG 126, 49: BMG 127, 50: BMG 45, 51: BMG 46, 52: BMG 40, 53: BMG 41, 54: BMG 147, 55: BMG 148, 56: BMG 149, 57: BMG 150, 58: BMG 70, 59: BMG 71, 60: BMG 72, 61: BMG 73, 62: BMG 119, 63: BMG 120, 64: BMG 121, 65: BMG 122, 66: BMG 155, 67: BMG 157, 68: BMG 158, 69: BMG 50, 70: BMG 51, 71: BMG 37, 72: BMG 38, 73: BMG 39, 74: BMG 169, 75: BMG 167, 76: BMG 168, 77: BMG 136, 78: BMG 137, 79: BMG 138, 80: BMG 115, 81: BMG 116, 82: BMG 117, 83: BMG 118, 84: BMG 170, 85: BMG 171, 86: BMG 172.

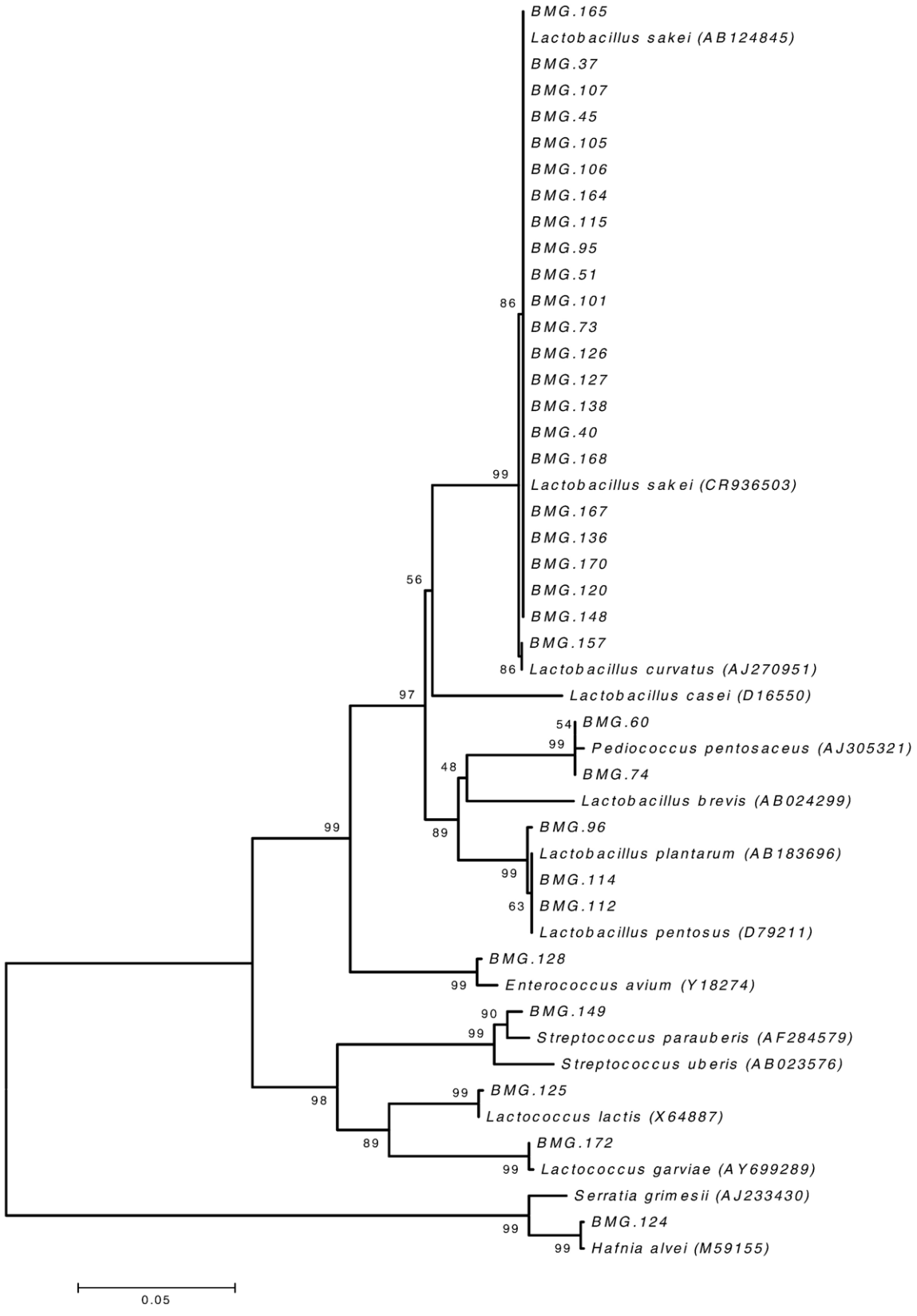

Fig. 4. Neighbor-joining phylogenetic tree of 16S rDNA sequences of 33 isolates and their closest phylogenetic relatives. Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1.

that on 36 isolates, suspected to belong to the *L. sakei* species, the PCR amplification of the *katA* gene was positive with 35 of those, whereas it was negative for all closely related species (Ammor et al., 2005a). Accordingly, the 86 isolates clustered by ARDRA were screened for the ability to amplify a 410 bp fragment with the two *katA* specific primers. All isolates from group G6, plus the control *L. sakei* strain resulted in the amplification of the expected fragment (Fig. 3). All the other isolates were all clearly negative except three strains (BMG 102, BMG 103, and BMG 172) which showed a faint band of the expected size (Fig. 3).

3.4. 16S rDNA sequencing

In order to verify that the isolates belonging to ARDRA group G6 were indeed *L. sakei* strains, and to tentatively assign the other isolates to bacterial species, the 16S rDNA amplified fragment (1500 bp) was sequenced. The 22 presumptive *L. sakei* strains and one isolate of each ARDRA group were used, as well as the *L. sakei* Lb 674 control strain. The DNA sequence was compared to the 16S rDNA sequence of the chromosome of *L. sakei* 23K (Chaillou et al., 2005), and to the Genbank database. To tentatively identify isolates of other groups than the *L. sakei* G6 ARDRA group, a 16S rRNA gene based phylogenetic tree was generated in order to determine the closest neighbors (Fig. 4). Analysis of the sequences showed that the 22 isolates of group G6 displayed indeed 100% identity with the 16S rDNA of *L. sakei*. The representative clone issued from ARDRA group G1 (BMG 128) encompassing 11 isolates showed a 16S sequence related to the *Enterococcus avium* group. The unique isolate BMG 157 of ARDRA group G2 was assigned to *Lactobacillus curvatus*. Its sequence was 99% identical to the 16S sequence of *L. curvatus* but also very similar to the one of *L. sakei* (99%). These two species are known to be closely related. However, as this clone did not harbour a *L. sakei katA* gene and belonged to an ARDRA group different from that of *L. sakei*, we thus considered this strain as an *L. curvatus* isolate. For ARDRA group G3, containing 16 isolates, the obtained 16S sequence of BMG 112 and BMG 114 was assigned to the *L. plantarum/pentosus* group. The two clones composing group G4 (BMG 60 and BMG 74) were tentatively assigned to *Pediococcus pentosaceus* (more than 99% identity to the database 16 S sequence). BMG 149, the unique strain belonging to ARDRA group G5 was considered close to *Streptococcus parauberis*. The clone selected from the ARDRA group G7 (BMG 124), had a 16S DNA sequence 99% identical to *Hafnia alvei* and was thus putatively belonging to this species. From the ARDRA group G8 encompassing 11 isolates, 2 were used for 16S sequencing. The closest neighbor for the BMG 125 sequence was *Lactococcus lactis*, whereas the sequence issued from BMG 172 was closer to *Lactococcus garvieae*. Finally the representative isolate of group G9, BMG 96, showed 99% identity to *L. plantarum*. These results are summarized in Table 1, Fig. 2B, and Fig. 4.

Thus, the DNA sequence of the 16S rDNA PCR fragments confirmed that the 22 isolates showing a typical *L. sakei* ARDRA profile and harbouring the presence of a *L. sakei katA* gene were indeed *L. sakei* strains. For the other ARDRA groups, the results could not clearly discriminate the strains as members of two different ARDRA groups (BMG114 and BMG 112 from G3 and BMG96 from G9) could be assigned to *L. plantarum* group, and in addition, from two isolates of the same ARDRA group G8 one might be identified as *L. lactis* and the second as *L. garvieae*.

4. Discussion

This study confirms that *L. sakei* is a bacterial species found in many meat products. It was found in all raw meat products tested except the veal and the two chicken samples, and on all processed meat products except smoked beef and turkey salami. In general, it is representing a large proportion of the LAB population. Interestingly, although *L. sakei* has been associated with the spoilage of processed fish products, such as smoked salmon and trout (Joffraud et al., 2001; Lyhs et al., 2002), in our study all the fish products tested did not show any evidence of spoilage. We observed *L. sakei* in two processed seafood products: marinated anchovies, where it represented 5% of the population counted on MRS plates; and dry salted octopus where two different strains represented half of the LAB population. Those two different processes correspond to anoxic or high osmolarity conditions, two stressing environments to which *L. sakei* is known to be well adapted (Chaillou et al., 2005).

Several other LAB species were also isolated in this study. Although their identification, based only on the 16S rDNA sequence, may require confirmation by additional methods, the species we tentatively identified have all been described in meat or fish related products. The presence of *L. plantarum*, *L. curvatus* and *P. pentosaceus* in meat or fish products is already well documented and those species are commonly used as starter to ferment many meat products. We noticed also the presence of undesirable microorganisms such as *L. garvieae*, *H. alvei*, *E. avium*, and *S. parauberis*. Those have also already been observed in meat or fish products (Ammor et al., 2005b; Rantsiou et al., 2005; Koort et al., 2006). *L. garvieae* is considered as an emerging zoonotic pathogen but its role in human infections is not clear (Blaiotta et al., 2002). *H. alvei* is mainly associated to different animal infections and is considered as an uncommon human pathogen (Janda and Abbott, 2006). *S. parauberis* is belonging to the pyogenic group of streptococci mostly associated to animal diseases (Hardie and Whiley, 1997). *E. avium*/*devriesei* members were first described in the oral cavity of horses and then observed as associated to animals (Collins et al., 2004; Svec et al., 2005). These undesirable bacterial species found in the Tunisian meat products may therefore have originated from animals.

Among the 86 LAB isolates collected on MRS plates, 22 could be identified as *L. sakei* therefore constituting a collection representative of Tunisian meat and seafood products. In addition, as only clones showing a different morphology on MRS plates containing 25 mg l^{-1} bromocresol green were

selected, this collection is yet considered as non redundant. However, as the couples of strains BMG164–BMG165, BMG106–BMG107, BMG126–BMG127, BMG167–BMG168, and BMG136–BMG138 were isolated from the same food sample, additional phenotypic and genotypic studies must be performed in order to prove that they are different.

After analysing the results obtained from the successive characterization steps, we noticed that the isolates belonging to *L. sakei* species all showed a similar colony morphology, which was almost never observed with the other bacterial isolates. Indeed, all the *L. sakei* strains but one (BMG136) showed small white or small light green colonies, with a dark green center. Only one isolate (BMG155) also showed a similar morphology, but had no amplifiable *katA* gene and was belonging to the ARDRA group G3. A careful observation of *L. sakei* clones on the bromocresol green medium revealed that clones were initially white, then developed a green colour starting from the centre of the colony to finally become all green. The white with a green centre morphology was observed after 48 h growth. We tested several growth conditions and we observed that this typical morphology was not depending on the aerobic/anaerobic incubation conditions (data not shown). This characteristic trait of the colonies, combined to the presence of the *katA* gene in the *L. sakei* species, could be used as a rapid method to identify *L. sakei* isolates in complex matrices. The first step would consist to select small white (or light green) clones with a dark green center, after incubating for 48 h on MRS plates containing 25 mg l^{-1} bromocresol green, then to detect by PCR the presence of the *katA* gene with specific primers deduced from the known *L. sakei* gene, and to confirm the species by amplifying and sequencing the 16S rDNA.

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