AN INNOVATIVE OLIGONUCLEOTIDE MICROARRAY TO DETECT SPOILAGE MICROORGANISMS IN WINE

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Abstract

The aim of this investigation has been the design and validation of an oligonucleotide microarray in order to detect 17 different wine-spoilage microorganisms, i.e. 9 yeasts, 5 lactic bacteria and 3 acetic acid bacteria species. Furthermore, several strains belonging to these species has been found to produce undesirable compounds for wine consumers. Oligonucleotide probes specific for each microorganism were designed to target the intergenic spacer regions (ISR) between18S-5.8S region for yeasts and 16S-ITS1 region for bacteria. Prior to hybridization the ISR were amplified by combining reverse transcriptase and polymerase chain reactions using a designed consensus primer. Each oligonucleotide-probes exclusively recognized its target without undesired aspecific cross-hybridizations. Under our experimental condition, the microarray assay analysis was able to detect the amount of DNA equivalent to 24 (*Saccharomyces cerevisiae*), 160 (*Lactobacillus brevis*) and 124 (*Gluconobacter oxydans*) cells, three species chosen as experimental models for the three studied microbial classes. Moreover, a novel procedure that allowed the extraction of genomic DNA from a mixture of eukaryotic and prokaryotic cells from contaminated wine was developed. The obtained results confirm that the microarray assay is able to detect specifically different spoilage microorganisms present in mixture in contaminated wines. For the first time the microarray methodology has been applied for the simultaneous identification of different mixed population of spoilage yeast and bacteria directly isolated from wine, thus indicating the practicability of oligonucleotide microarrays as a contamination control in wine industry.

Keywords: biotechnology; wine; wine spoilage; microarray; PCR.

1. Introduction

Yeasts and bacteria play important roles in winemaking such as catalysing the rapid, complete and efficient conversion of grape sugar into ethanol as well as reducing wine acidity, improving microbiological stability and enhancing wine aroma and flavour. However, under uncontrolled conditions, microbial activity may also be disadvantageous for wine quality (Bartowsky, 2009; M. Du Toit & Pretorius, 2000). Wine deterioration due to spoilage microorganisms is becoming a major problem for wine industry because can cause significant economic losses (Krisch, Chandrasekaran, Kadaikunnan, Alharbi, & Vágvölgyi, 2016; Luo, Schmid, Grbin, & Jiranek, 2012) also in the light of wine production increased scale all over the world (Mariani, Pomarici, & Boatto, 2012). Moreover, wine consumers, nowadays, demand milder processing, preservation and storage conditions that also contribute to increase wine spoilage drawback (Lockshin & Corsi, 2012). Microbial spoilage can occur at different stages during wine production or storage (Rankine, 1995; Tristezza et al., 2010). Many lactic acid bacteria genera, such as *Lactobacillus* and *Pediococcus*, are among the most concerning microbial contaminants and are well known for their capacity to depreciate wine (Bartowsky, 2009) as well as to produce undesirable compounds for wine consumers health such as biogenic ammines (Mateo, Torija, Mas, & Bartowsky, 2014; Russo et al., 2016). Also wine alterations due to activity and growth of contaminant yeasts in processed and bottled wines is a serious concern for wine industry (Krisch et al., 2016; Loureiro & Malfeito-Ferreira, 2003); wine spoilage yeasts belong to several genera including *Dekkera*/*Brettanomyces*, *Hanseniaspora*, *Candida*, *Pichia*, *Zygosaccharomyces* (Enrique et al., 2007; Loureiro & Malfeito-Ferreira, 2003). Furthermore, some strains belonging to these species were able to synthesize histamine and cadaverine during must fermentation (Tristezza et al., 2013). Even the species *Saccharomyces cerevisiae* might be considered as a spoilage organism when associated with re-fermentation of bottled wines (Deak, 2007; Loureiro & Malfeito-Ferreira, 2003; Tristezza et al., 2010).

Consequently, to prevent economical losses, it would be helpful to have tools able to simultaneously identify the undesirable microorganisms. Microarrays approach has been applied for microbial identification and detection in food stuffs (McLoughlin, 2011; Rasooly & Herold, 2008). Microarray technology based on species-specific sequences is rapid, sensitive and unambiguously allows identification of single species (Southern, 2001) into a mixed microbial community. For instance, the sensitive and specific detection and identification of ascomycetes has been carried out drawing primer pairs complementary to the highly conserved 18S and 5.8S regions of rRNA genes and using oligonucleotide capture probes complementary to the more variable ITS1 regions present in multiple copies in fungal and yeast genomes, that allow a discrimination of fungal and yeast species (Healy et al., 2004; Hsiao et al., 2005; Spiess et al., 2007). As far as bacterial detection is concerned, bacterial 16S rRNA genes, including nine "hyper-variable regions" (V1–V9), characterized by significant sequence diversity among different bacterial genera, have been utilized for species identification (Huws, Edwards, Kim, & Scollan, 2007).

Indeed, microarray applications could play an important role for safety and quality supervision, particularly in the food and beverage industries. DNA microarray tests have been developed for identification of food-borne bacterial pathogens in the environment (Call, Borucki, & Loge, 2003), in different food commodities (Wang et al., 2007) and also for the simultaneous detection of numerous pathogenic and non-pathogenic bacteria in raw milk (Giannino et al., 2009). Moreover, Weber and coworkers (2008) developed and applied an oligonucleotide microarray able to detect and identify viable bacterial species, belonging to the genera *Lactobacillus*, *Megasphaera*, *Pediococcus* and *Pectinatus*, recognized (Priest, 2006) as biological agents of beer spoilage. In general extensive studies have been carried out to optimize efficient molecular methods for the detection of wine spoilage microorganisms (Ivey & Phister, 2011), but none of them can ensure the simultaneous detection of numerous eukaryotic and prokaryotic undesired microorganisms.

The aim of the present study was to develop an alternative diagnostic method for the rapid and simultaneous detection of wine spoilage yeasts and bacteria directly extracted from contaminated

wines. A prototype oligonucleotide microarray, based on species-specific probes targeting rDNA-specific regions, was designed and assessed as able to detect 17 different wine-spoilage microorganisms, i.e. 9 yeasts, 5 lactic bacteria and 3 acetic acid bacteria species. To the best of our knowledge, this is the first report concerning a single microarray-based assay for the concurrent identification of different eukaryotic and prokaryotic microorganisms responsible for wine spoilage.

2. Materials and methods

2.1 Microbial strain cultures and DNA extraction

Yeast and bacterial strains used in this study (Table 1) were store at -80 °C in 50% glycerol. Diagnostic ability of the DNA microarray to detect microorganisms was determined using genomic DNAs extracted from test strains in laboratory media: YPD (1% yeast extract, 2% peptone, 2% glucose) for yeasts, MRS (Oxoid, Basingstoke, UK) for lactic acid bacteria and GY (5% glucose, 1% yeast extract) for acetic acid bacteria. Genomic DNAs from pure yeast and bacterial cultures were extracted using the methods respectively described by Tristezza et al. (2009) and Cappello et al. (2008). The concentration of the extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 DNA extraction from yeast/bacterial mixed cultures isolated from artificially infected wine

Genomic DNAs of mixed bacterial/yeast cells were directly extracted from artificially infected wine. The wine used was first micro filtrate and subsequently artificially contaminated with known amounts of microorganisms. The contaminated wines were centrifuged and the sediment was suspended in a suitably formulated suspension buffer. Briefly, one millilitre of artificially contaminated wine was centrifuged for 5 minutes at 8000 ×g and thereby the wine was removed. The pellet obtained was washed with 1 mL of Buffer A (60 mM Tris-HCl pH 7.4, 10 mM EDTA

pH 7.4), centrifuged for 5 minutes at 8000 ×g and the supernatant was discarded. The washed pellet was re-suspended using 8 mg of lysozyme (Sigma-Aldrich, Milan, Italy) + 0.8 mg lyticase (Sigma-Aldrich, Milan, Italy), in a final volume of 200 µL of Buffer A The slurry was mixed by vortex and incubated at 37°C for 1 hour. Then 400 µg of RNase (20 µL; Sigma-Aldrich, Milan, Italy) were added to the mixture and incubated for 2 minutes at 25°C. After a further addition of 400 µg of Proteinase K (20 µL) and 200 µL of Lysis solution [10 mM Tris (pH 8.0), 10 mM EDTA, and 2.0% SDS], the mixture was mixed by vortex and incubated at 55°C for 10 minutes. The lysate was added with 200 µL of absolute ethanol and the genomic DNAs were afterward extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions.

2.3 Primers and probes design

Primers and oligonucleotide probes used for identification of microorganisms, were designed using the reference sequences (18S-5.8S rRNA genes region for yeasts and 16S rRNA Gene-ITS1 [Internal Transcribed Spacer] region for bacteria) available in the GenBank database of the NCBI homepage (http://www.ncbi.nlm.nih.gov/). The selected sequences were compared with at least one sequence of the same species in the database and they were aligned with ClustalX implemented in BioEdit 7.0.5.2 software (Hall, 1999) for the selection of regions suitable for oligonucleotide probes design. The oligonucleotide probes were designed using Primer 3.0 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and the following parameters were applied: GC-content between 35 and 60%, maximum Tm set at 58°C and probe length between 20 and 30 bp. Probe sequences were tested for duplex and hairpin formation with the Oligo Analyzer 3.1 (http://www.idtdna.com) software. Each designed probe sequence was optimised by deleting or adding bases at both ends, according to melting temperature and duplex formation. Oligonucleotide probes were checked by BLAST analysis (http://www.ncbi. nml.nih.gov/BLAST/) against sequences from all available species within the database.

153 Oligonucleotide probes (Invitrogen) were synthesized adding at the 5' end 12 carbon residues as
154 spacer and a 5' NH_2 group.
155
156 *2.4 Construction of DNA-microarrays*
157 The oligonucleotide probes were modified by adding a sequence of 12 carbon atoms, linked to an
158 amino group, at 5' end. By this organic spacer the oligonucleotide probe is spaced out the slide
159 surface and fully exposed and available to bind target DNA. Interaction between the slide and the
160 oligonucleotide probes takes place by a covalent bond between the amino group of the
161 oligonucleotide and the epoxide coating the slide surface. The oligonucleotide probes were
162 deposited in duplicate on the epoxy slide either manually, according to the scheme reported in
163 Figure 1, or automatically, according to the scheme reported in Figure 4.
164 Probes were suspended in 2X saline-sodium citrate (SSC) buffer (1X SSC = 0.15 M sodium
165 chloride, 15mM trisodium citrate, pH 7) at a final concentration of 40 μM and distributed in a 96-
166 well plate. The oligonucleotide probes were spotted on the epoxy-coated glass slides (Nexterion®
167 Slide E) by contact printing using a robotic spotting SpotArrayTM 24 (Perkin Elmer) by the
168 following protocol: 55-60% humidity; pin contact time of 400 msec; deposition volume of 10 nL;
169 spot size diameter of 100 μm; distance between two spots of 400 μm. The improvement of
170 background and sensitivity of the spot fluorescence signals was achieved by preliminary study
171 using a manual contact printing MicroCaster^{TM} Arrayer (Whatman). This method allows a
172 deposition volume of 50-70 nL; spot size diameter of 400-700 μm; distance between two spots of
173 900-1300 μm. The variability of the spot size is due to a different pin contact time, performed by a
174 manual printing in order to allow the covalent bond between the epoxide group on the slide surface
175 and the amino group at 5' end of oligonucleotide probes. After deposition, the slide was incubated
176 in a humid chamber at room temperature for 2 hours and then stored at room temperature.
177
178 *2.5 DNA labelling*

The target DNA was labelled using one of the two primer, forward or reverse, labelled at the 5' end with the Cyanine 5 (Cy5) fluorochrome (Invitrogen™ Life Technologies, USA) by a Linear-After-The-Exponential-PCR (LATE-PCR). The LATE-PCR is an asymmetric PCR based on the amplification of a single strand of Cy5-labelled DNA at higher amount compared to the complementary strand, with predictable kinetics for many cycles beyond the exponential phase (Rice et al., 2007). LATE-PCR increases the number of strands labelled with cyanine in order to reduce the unlabelled complementary strands that, during the hybridization step on microarray, for competition effect is able to limit the binding with oligonucleotide probes immobilized on the array. The LATE-PCR method is a composed by two sequential steps that were carried out as following.

- Traditional Exponential-PCR. The base master mix consisted of 5 µL reaction buffer [10X, Euroclone; 160 mM $(NH_4)_2S0_4$, 670 mM TRIS HCl pH 8.8; 0.1% Tween-20], 3 mM $MgCl_2$ 50 (, 0.2 mM,dNTP mix (Invitrogen, USA), 0.2 µM of each genus primer (Cy5-primer and reverse or forward prime), 2 µL of DNA template, 2.5 units Taq polymerase (, Euroclone, Italy) and sterile water to 50 µl. Following an initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s. Amplification was followed by a final extension at 72°C for 5 min. 10 µL of product (1/5 of PCR reaction volume) was used for the subsequent Linear-PCR.
- Linear-PCR. Five µL of reaction buffer [10X, Euroclone; 160 mM $(NH_4)_2S0_4$, 670 mM TRIS HCl pH 8.8; 0.1% Tween-20], 3 mM $MgCl_2$ 50 mM, 0.2 mMdNTP mix (10, Invitrogen, USA), 0.2µM of Cy5-primer, 10 µL of the previously obtained PCR product (1/5 of Exponential-PCR reaction volume), 2.5 µL units Taq polymerase (Euroclone, Italy) and sterile water to 50 µL. Following an initial denaturation at 95°C for 2 min, products were amplified by 15 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 20 s and elongation at 72°C for 20 s. Amplification was followed by a final extension at 72°C for 1 min. The amplified Cy5-labelled DNA was purified by illustra MicroSpin G-50 Columns (GE Healthcare, USA) and diluted (1:2 v/v) with hybridization buffer for microarray analysis.

205

206 *2.6 Microarray hybridization*

207 Before hybridizations, the spotted slide was incubated twice for 2 min in a solution of 1 mM HCl,
208 then 10 min in a solution of 100 mM KCl, washed twice in sterile water and blocked for 15 min at
209 50°C with Blocking solution [50mM ethanolamine; 0.1% SDS, 0.1M Tris, pH 9], in order to
210 inactivate residual reactive epoxy groups. After two washing steps with sterile water, the slide was
211 dried by centrifugation for 5 min at 200 ×g and placed into the hybridization chamber.
212 The Cy5-labelled DNA diluted (1:2 v/v) with hybridization buffer (3X SCC; 0,1% SDS; 30%
213 deionised form amide, Sigma), was denatured at 95°C for 3 min and then immediately applied into
214 the well of the hybridization chamber (Nexterion® IC-16, Schott, Germany). Wells were covered
215 with a plastic layer to avoid evaporation during hybridization and incubated for 4 hours (or
216 overnight) at 42°C. After hybridization, the slide was removed from hybridization chamber and
217 washed in 4X SSC for 1 min, twice in 2X SSC with 0.1% SDS for 5 min, in 0.2X SSC for 1 min
218 and finally in 0.1X SSC for 1 min. After the washing steps, the microarray was dried by
219 centrifugation for 4 min at 200 ×g and analyzed at the laser scanner.

220

221 *2.7 Scanning and data analysis*

222 The fluorescence signal for Cy5 was determined at 633 nm by using a ScanArray Express laser
223 scanner (Perkin-Elmer, Foster City, CA, USA). Slides were scanned with a resolution of 10 μm and
224 at the same laser power and sensitivity level of the photomultiplier. The draw fluorescence data
225 acquired were stored as image files in TIFF format and analyzed quantitatively by ScanArray
226 Express software (Perkin-Elmer, USA). The fluorescence signal of each spot was calculated as the
227 difference between the mean of pixel intensities and the mean of background fluorescence signals,
228 defined by surrounding pixel intensity according to Heiskanen et al. (2000).

229

230 **3. Results**

3.1 Bioinformatic analysis and design of oligonucleotide probes for microarray construction

The bioinformatic analysis of rDNA cistron sequences (18S-5.8S rRNA genes region for yeasts and 16S rRNA gene-ITS1 region for bacteria) belonging to different strains of each of the 17 species (Table 2) has produced three separate multiple alignments, deriving respectively from yeast, lactic and acetic bacteria rDNA sequences. Each output file allowed to highlight both conserved regions (on which the primer pair used for the preparation of the target DNA has been built) and non-common regions (on which the oligonucleotide probes to be immobilized on the microarray slides have been constructed). For yeasts, the forward primer has been identified on the 18S region and the reverse primer on the 5.8S region, whereas for bacteria the forward primer has been identified on the 16S region and the reverse primer on the ITS1 region (Table 3). Size of the different specific fragments is indicated in Table S1 and the obtained amplicons are shown in Figure S1. In the case of lactic acid bacteria, a 300 bp long amplicon was obtained. The forward primer was used in the preparation of the each of the three specific-target DNAs by LATE-PCR assay (Table 3). A species-specific oligonucleotide probe for each microorganism was designed in the region between the two sequences used to draw the two primers. Each primer was constructed to be 20 nucleotides long and with hybridization temperature (Tm) of 58-60°C (Table 4) and their ability to exclusively recognized its species-specific target was confirmed by separately submitting each primer sequence to BLAST analysis (Figure S2).

Seventeen oligonucleotide probes were designed in order to specifically recognize and hybridize with the target DNA of the corresponding microorganism, in particular 9 oligonucleotide probes for the nine species of yeasts and 8 oligonucleotide probes for the acetic acid and lactic acid bacteria species were constructed, which were immobilized on the epoxy slide.

3.2 Labelling of the target DNA and microarray hybridization

Preparation of the target DNA was carried out by PCR using, in each amplification, the forward primer labelled with the Cy5 fluorescent tag. In order to obtain a more evident signal, the target DNA synthesis was carried out by using the Linear-After-The-Exponential (LATE)-PCR, which allowed to obtain an increased signal with a lower background noise. The Figure 1 shows the results obtained hybridizing separately the 17 target DNAs with the DNA microarray. In all assays, a very low background noise was obtained. Furthermore, the experimental conditions used produced a high intensity fluorescence signal strictly corresponding to the specific oligonucleotide probe immobilized on the epoxy glass slide. This indicates the absence of aspecific cross-hybridization signals. In fact, each of the 17 oligonucleotide probes exclusively recognized its target not hybridizing with any target of the other yeast or bacteria species.

3.3 Microarray sensitivity assessment

To assess sensitivity limit of the microarray, the minimal detectable concentration of target DNA was determined. The sensitivity test was carried out using three model microorganisms, namely *Saccharomyces cerevisiae* (yeast), *Gluconobacter oxydans* (acetic acid bacteria) and *Lactobacillus brevis* (lactic acid bacteria). Different solutions containing decreasing amounts of DNA of the three model microorganisms (i.e. 50 pg, 10 pg, 2 pg and 0.4 pg) were prepared and used as template in LATE-PCR reactions using respectively the primer pairs Liev_For_Cy5/Liev_Rev, Acet_For_Cy5/Acet_Rev, Latt_For_Cy5/Latt_Rev. The electrophoretic analysis of LATE-PCR products indicate that the expected amplicons are visible when 50 and 10 pg of template DNA were used, while no products are observed when using 2 and 0.4 pg of template DNA (Figures S3).

When the LATE-PCR products of the three model microorganisms DNAs (at the four different concentrations) were utilized for hybridization of the microarray slide, the hybridization signal is present in all samples. Moreover, a very low level of background noise and no cross-reactions were observed, thus confirming the high specificity of each target DNA (Figure 2). Under the experimental condition used, the microarray was able to detect target DNA obtained from LATE-

PCR performed with 0.4 pg of template, that means the amount of DNA corresponding to 24 (*S. cerevisiae*), 160 (*L. brevis*) and 124 (*G. oxydans*) cells.

3.4 Simultaneous detection of microorganisms from DNA mixtures

A further step in the optimization of the microarray was the simultaneous amplification of target DNAs deriving from a mixture of different microorganism in order to verify the specific production of the expected target DNAs and the absence of undesired non-specific amplification products. Thus we developed a procedure for extracting genomic DNA from a mixture of prokaryotic and eukaryotic microbes directly from contaminated wine by the concurrent addition of lysozyme and lyticase enzymes, able to respectively degrade the cellular wall of bacteria and yeasts.

Four separate amplification reactions were set up using simultaneously the three pairs of primers Liev_For_Cy5/Liev_Rev (yeasts), Latt_For_Cy5/Latt_Rev (lactic acid bacteria) and Acet_For_Cy5/Acet_Rev (acetic acid bacteria) and, as substrate, the following mixtures of genomic DNAs, at the concentration of 20 pg/µL each: Mix 1, *S. cerevisiae* and *Schizosaccharomyces pombe;* Mix 2, *S. cerevisiae, Pichia membranifaciens* and *L. brevis;* Mix 3, *S. cerevisiae, Candida stellata, L. brevis* and *G. oxydans;* Mix 4, *S. cerevisiae, Pichia anomala, P membranifaciens, L. brevis* and *G. oxydans* (Figure S4). The four different target DNA preparations were used to hybridize separately four identical arrays. Figure 3 shows the results obtained after the four independent hybridizations carried out using the above-described four mixture of target DNAs. In all the performed experiments a highly specific fluorescence signal was observed. A very low level of background noise and no undesired cross-hybridization signal were obtained. The results obtained clearly indicate that the microarray is useful to identify specifically the DNA of different microorganisms (yeasts, lactic acid and acetic acid bacteria) present in the mixture and to assess that the contemporary presence of different target DNAs in the hybridization mixture does not cause any interference among the different amplified targets.

3.5 Detection of microorganisms from spoiled wine

In order to detect simultaneously one or more microorganisms directly from spoiled wines, a procedure was set up that allowed the extraction of genomic DNA from a mixture of eukaryotic and prokaryotic cells. The wine used was first micro filtered and then artificially contaminated using a mixture containing known cell concentration of model microorganisms, representative of the three classes of spoilers, *S. cerevisiae* (yeasts), *L. brevis* (lactic acid bacteria) and *A. aceti* (acetic bacteria), mixed in the following proportions:

A) *S. cerevisiae*: 10^6 CFU/mL; *L. brevis*: 10^6 CFU/mL; *A. aceti*: 10^6 CFU/mL.

B) *S. cerevisiae*: 10^5 CFU/mL; *L. brevis*: 10^5 CFU/mL; *A. aceti*: 10^5 CFU/mL

C) *S. cerevisiae*: 10^4 CFU/mL; *L. brevis*: 10^4 CFU/mL; *A. aceti*: 10^4 CFU/mL

D*) S. cerevisiae*: 10^3 CFU/mL; *L. brevis*: 10^3 CFU/mL; *A. aceti*: 10^3 CFU/mL

After incubation in wine, the four microorganisms mixtures were concentrated by centrifugation and each sediment was separately re-suspended in the suspension buffer formulated *ad hoc* during this work. In particular the optimization of two enzymatic reactions carried out simultaneously was achieved by adding to the aforementioned buffer the optimal amount of lysozyme and lyticase that are respectively able to degrade the cell wall of bacteria and yeasts. Genomic DNA released in the lysate was purified by chromatography on a silica gel column. Reproducible amplification of the expected products was obtained by using as substrate the DNA extracted from all the mixtures except that from mixture D. Target DNAs amplified from the genomic template extracted from Mixture C were used in the hybridization reaction with the microarray (Figure 4). The experimental conditions adopted have produced a high intensity fluorescence signal corresponding to the specific oligonucleotide probe for *A. aceti, L. brevis* and *S. cerevisiae*, thus indicating that each DNA target recognizes only its specific oligonucleotide probe without cross-interference and background noise. The above described procedure was validated by artificially contaminating sterile wine with 4 different combination of mixed microorganisms, at the above established minimal-detectable concentration each (10^4 CFU/mL) i.e. Mix A: *S. cerevisiae*, *P. membranifaciens*; Mix B: *S.*

cerevisiae, *L. brevis, P. membranifaciens*; Mix C: *S. cerevisiae, L. brevis, G. oxydans, C. stellata*; Mix D: *S. cerevisiae*, *L. brevis, P. membranifaciens*, *P. anomala, G. oxydans*. The DNAs extracted from each mixture were used as substrate for LATE-PCR reactions and the obtained amplicons were used to separately hybridize the microarrays (Figure 5). The results obtained confirm that the microarray allows in a specific manner the clear and specific detection of different spoilage microorganisms directly from contaminated wines.

4. Discussion

Commonly, microbial species present in wine are identified using conventional microbiological approaches based on cultivation methods (Bester, Cameron, Toit, D, & Witthuhn, 2010). Unluckily, cultivation is time-consuming and labour intensive (Fleet, 1993; Kopke et al., 2000) whereas morphological and physiological tests are not always useful to identify and classify different microorganisms (Hernán-Gómez, Espinosa, & Ubeda, 2000; Muyzer, 1999). Traditional culture methods, based on biochemical and physiological characteristics, often lead to disappointing results and misidentification (Van Der Vossen & Hofstra, 1996), whereas methods based on molecular detection and identification are fast and reliable (Krisch et al., 2016).Many culture-independent molecular methods allow analysis of total microbial DNA, isolated from mixed microbial populations, in order to detect and identify single microbes in food ecosystems (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013; Ivey & Phister, 2011). Genetic fingerprinting of complex microbial populations is, at present, used broadly to investigate the microbial ecology of grape must fermentations (Nisiotou, Spiropoulos, & Nychas, 2007; Rantsiou et al., 2013; Urso et al., 2008). Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) assay has been also employed, because of its capability to detect, identify individual species and produce the overall profile of microbial populations (Cocolin et al., 2013). Although the above methods demonstrated to be able in specifically detect several wine spoilage microbes, the availability of

quick and sensitive methods to simultaneously monitor the presence of both prokaryotic and eukaryotic contaminant microorganisms is of crucial importance to reduce economical losses and to ensure wine safety.

Even though the DNA microarray technology still detains for its application some cons, such as the needing of extensive bioinformatic analysis, this methodology has several pros when compared to other molecular approaches. DNA microarray is a molecular identification method by which DNA probes, grouped and arrayed on a slide, allow simultaneous molecular identification and characterization of many specific sequences in a single step (Southern, 2001). The detection system of the signal provides that each DNA fragment in the sample specifically hybridize with the oligonucleotide probes spotted on the slide in a known position. The power of this technology lies mainly in the ability to analyze simultaneously a large number of DNA sequences in a single sample and a high number of samples in a compact and relatively cheap device.

When analyzing food for microbial contamination, this approach provides the opportunity to obtain detailed information about the presence of contaminant species (Rasooly & Herold, 2008). Considering the high number of species of bacteria and ascomycetes that could potentially be responsible for wine alteration (Bartowsky, 2009; Krisch et al., 2016), a broad-spectrum detection system as microarray technology might be very useful.

The purposes of this research was to develop a method based on the application of bioinformatic, biochemical and molecular protocol and to validate the use of a DNA microarray, produced during this work, for the simultaneous detection and identification of spoilage yeast and bacteria after the isolation of their DNAs directly from wine. Wine is a co-culture of many different microorganisms, either prokaryotic and eukaryotic, for this reason we also checked whether the microarray could identify multiple targets in a mixed sample. To achieve this goal, it was essential to develop a protocol for the extraction of genomic DNA from mixtures of eukaryotes and prokaryotes from wine. Total DNA isolated from complex food matrices contains large amounts of DNA from different microbial groups (bacteria and yeasts) that have the potential to interfere with specific

amplification of particular DNA sequences (Chen, Wang, & Chen, 2008). The few protocols available in literature are poorly applicable for the extraction of genomic DNA from wine due to the presence of high concentrations of polyphenolic compounds, which severely interfere with the subsequent enzymatic reactions of PCR gene amplification (García-Beneytez, Moreno-Arribas, Borrego, Polo, & Ibáñez, 2002; Siret, Boursiquot, Merle, Cabanis, & This, 2000). For these reasons, it was very important to optimize a protocol of genomic DNA extraction from wine with the aim of: i) extracting in a single step genomic DNA from mixtures of eukaryotic and prokaryotic cells, ii) achieving DNA yields sufficient to realize subsequent reactions of gene amplification, iii) obtaining preparations of good quality genomic DNA.

Polymorphisms of sequences coding for ribosomal RNA (rDNA) were selected as barcode for the identification of bacterial species. In prokaryotes, the *locus* encoding rRNA contains the highly conserved three genes, 16S, 23S and 5S, separated by highly variable regions known as "internal transcribed spacers" or ITS (Ludwig & Schleifer, 1994).

The rDNA *locus* has been widely used for the identification of bacterial (Lebonah et al., 2014) and fungal (Das & Deb, 2015) species because: i) its products are abundant (up 80% of total cellular RNA), can be isolated and identified easily, ii) the rRNA genes sequences are highly conserved facilitating amplification by PCR, iii) the presence of highly variable regions allows discrimination of the different species (Olsen, Lane, Giovannoni, Pace, & Stahl, 1986); moreover the rDNA sequences of many bacterial species are available in data banks. The spacer region 16S-23S of rDNA has been widely used also for the identification of *Bacillus anthracis* (Nübel et al., 2004) and *Campylobacter* (Keramas et al., 2003) by microarray. Yeasts characterization was achieved by designing the oligonucleotide probes considering variations in the ITS region sequences according to Leinberger and coworkers (2005).

In general, the DNA microarray designed in this study allows the identification of five species of lactic acid bacteria (belonging to the genera *Lactobacillus* and *Pediococcus*) and three species of acetic acid (belonging to genera *Acetobacter* and *Gluconobacter)* as well as nine species of yeasts,

412 all together representing the 'etiological cause' of major alterations in the wine industry (Comi,
413 2005). The data produced by this work have shown that: i) an efficient procedure to obtain good
414 quality DNA preparations, to be used as PCR-template form microbial mixture, was developed, ii)
415 the oligonucleotide probes, specific for each considered microorganism, recognize only their
416 specific target, with the exception of the *L hilgardi* oligo that had also a 100% match with *L*
417 *buchneri* and also with the wine-unrelated species L. *parabuchneri*, *L. keferi* and *L. rapi*; iii) the
418 microarray is able to detect the presence of yeasts, lactic and acetic acid bacteria at very low
419 concentrations (10^4 CFU/mL). The probes produced are suitable to distinguish their own target
420 DNAs from other target DNAs present on the microarray (Liu, Mirzabekov, & Stahl, 2001, Liu et
421 al. 2001) giving signal of high intensity and absence of background noise. Our findings indicate that
422 the probes used are characterized by a discrimination capacity better than those previously reported
423 (Drobyshev et al., 1997; Yershov et al., 1996; Zheng, Alm, Stahl, & Raskin, 1996). However, to
424 discriminate two closely related species like *L hilgardii* and *L. buchneri* it will be important to test
425 additional probes that could target other regions of rDNA, such as that between 23S and 5S pre-
426 rRNA. Other possible strategies to obtain increased specificity and sensitivity could consider the
427 use of PNA (peptide nucleic acids) as an alternative to DNA as probes (Weiler, Gausepohl, Hauser,
428 Jensen, & Hoheisel, 1997) or the preparation of longer probes (Relógio, Schwager, Richter,
429 Ansorge, & Valcárcel, 2002).
430 In conclusion, in this study for the first time the microarray methodology was applied for the
431 simultaneous identification of different species of yeasts and bacteria directly from wine. The
432 microarray developed is a novel tool, which not only allows the identification of the most
433 representative species of the microbial community responsible for wine spoilage but also the
434 investigation of population dynamics of indigenous wine yeast and bacteria populations. However, the
435 number of possible secondary wine spoilage agents is higher than the microbial species considered
436 in this investigation and it is likely to increase in the future, because of the identification of new

spoilage microorganisms. Further studies will be required in order to expand progressively the specific target range by adding other oligonucleotide probes specific for novel microbial species.

Acknowledgements

This research was partially supported by the Apulia Region in the framework of the Projects NEWINE (Bando "Ricerca e sperimentazione in Agricoltura"; Project code PRS_042), BIOTECA" (Bando "Aiuti a Sostegno Cluster Tecnologici Regionali"; Project code QCBRAJ6) and IPROVISP (Bando "Aiuti a Sostegno Cluster Tecnologici Regionali"; Project code VJBKVF4). Vittorio Capozzi is supported by Fondo di Sviluppo e Coesione 2007-2013 – APQ Ricerca Regione Puglia "Programma regionale a sostegno della specializzazione intelligente e della sostenibilità sociale ed ambientale - FutureInResearch". The authors thank Mr. Giovanni Colella for the valuable technical help and Prof. J. Smith for proofreading and valuable linguistic advice.

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Captions to figures

Figure 1. Microarray analyses carried out using a specific target-DNA for each array.The oligonucleotide probes were deposited by a manual contact printing MicroCaster™ Arrayer (Whatman, Maidstone, UK). The schematic representation of the array used is reported.

Figure 2. Microarray analyses carried out using for each array a target-DNA specific to the organism designated at the indicated concentrations of DNA template. The oligonucleotide probes were deposited by a manual contact printing MicroCaster™ Arrayer (Whatman, Maidstone, UK).

Figure 3. Microarray analysis performed using for each array target-DNAs specific for different organisms in the following mixtures: (A) *S. cerevisiae*, *S. pombe*; (B) *S. cerevisiae*, *P. membranifaciens*, *L. brevis*; (C) *S. cerevisiae*, *C. stellata*, *L. brevis*, *G. oxydans*; (D) *S. cerevisiae*, *P. anomala*, *P. membranifaciens*, *L. brevis*, *G. oxydans*. The oligonucleotide probes were deposited by a manual contact printing MicroCaster™ Arrayer (Whatman, Maidstone, UK).The schematic representation of the array used is reported.

Figure 4. Microarray analyses performed using: (A) genomic DNA extracted from wine artificially inoculated with a mixture of the following microorganisms: *A. aceti* LMG1261, *L. brevis* LMG11435, *S. cerevisiae* CBS1171; (B) not inoculated wine. The oligonucleotide probes were deposited by robotic spotting SpotArray™24 (Perkin Elmer, *Waltham, USA*). *The schematic representation of the array used is reported.*

Figure 5. Microarray analysis performed using genomic DNA extracted from wine artificially inoculated with a mixture of the following microorganisms: (A) *S. cerevisiae*, *P. membranifaciens*; (B) *S. cerevisiae, L. brevis, P. membranifaciens*; (C) *S. cerevisiae, L. brevis, G. oxydans, C. stellata*; (D), S. *cerevisiae, L. brevis, P. membranifaciens, P. anomala, G. oxydans*; (E) not inoculated wine. The oligonucleotide probes were deposited by robotic spotting SpotArrayTM 24 (Perkin Elmer, Waltham, USA). The schematic representation of the array used is reported.

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674 **Table 1.** Microorganism strains utilized in this study.
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Organism	Strain
YEASTS	
Saccharomyces cerevisiae	S288c
Zygosaccharomyces rouxii	CBS 732
Zygosaccharomyces bailii	GK02
Brettanomyces bruxellensis	CBS 72
Schizosaccharomyces pombe	972
Pichia membranifaciens	CBS 107
Pichia anomala	CBS 5759
Candida stellata	CBS 157
Hanseniaspora vineae	CBS 2171
LACTIC BACTERIA	
Lactobacillus plantarum	WCFS1
Lactobacillus brevis	ATCC 367
Lactobacillus hilgardii	ATCC 8290
Pediococcus damnosus	ATCC 29358
Pediococcus pentosaceus	SL4
ACETIC BACTERIA	
Gluconobacter oxydans	621H
Acetobacter aceti	DSM3508
Acetobacter pasteurianus	ATCC33445

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678 Table 2. Accession numbers of the sequences utilized to design primers and probes.

Organism	Sequence Acc. Nr.
YEASTS	
Saccharomyces cerevisiae	NC_001144.5, MF118616.1, MF118614.1, MF118613.1 , MF118612.1, MF118611.1 , MF118610.1 , MF118609.1, MF118608.1, MF118606.1, F118605.1, MF118604.1, LC269189.1, KY693710.1, KY693708.1, KY315926.1 , KY962551.1, KY962550.1, KY962549.1, KX434761.1, Y794751.1, LC215450.1, KY488348.1, CP011466.1, KY794729.1, X859535.1
Zygosaccharomyces rouxii	KY106065, KY106071.1, KY106069.1, KY106068.1, KY106066.1, KY106065.1, KY106064.1, KY106063.1, KY106062.1, KY106061.1, KX539236.1, KX539235.1, KX539234.1, KX539233.1, KJ507666.1, KM249341.1, LN849134.1
Zygosaccharomyces bailii	KJ433981.1, KY106027.1, KY106026.1, KY106023.1, KY106022.1, KY106020.1, KY076624.1, NR_138201.1, LN849135.1, KP241898.1, KP132936.1, JX458104.1 , JX458102.1, JX458100.1
Brettanomyces bruxellensis	KY103308.1, KY103322.1, KY103321.1, KY103320.1, KY103319.1, KY103318.1, KY103316.1, KY103315.1, KY103313.1, KY103312.1, KY103311.1, KY103309.1, KY103307.1, KU729031.1
Schizosaccharomyces pombe	CU329672, KY105378.1, NR_121563.1, JQ726610.1, EU916982.1, AY251633.1, V01361.1, AB054041.1, Z19578.1
Pichia membranifaciens	KY104614.1, KY104631.1, KY104630.1, KY104628.1, KY104627.1, KY104625.1, KY104624.1, KY104622.1, KY104621.1, KY104620.1, KY104619.1, KY104618.1, KY104617.1, KY104616.1, KY104615.1, KY104613.1, KY104611.1, KY104610.1, KY104609.1, Y104608.1
Pichia anomala	KY105894.1, KY105896.1, KY105895.1, KY105893.1, KY105892.1, KY105890.1, KY105889.1, KY105888.1, KY105887.1, KY105886.1, KY105883.1, KY105882.1, KY105880.1, KY105877.1, KY105876.1, KY105875.1, KY105874.1, KY105873.1, KY105872.1, KY105871.1, KY105870.1, KY105867.1, KY105865.1
Candida stellata	KY102416.1, AY160766.1, AY188852.1
Hanseniaspora vineae	KY103580.1, KY693711.1, KY103584.1, KY103583.1, KY103582.1, KY103581.1, KY076611.1, NR_138203.1, KM384180.1, KM384177.1, KM384176.1, KM384175.1, FJ231441.1, FJ231440.1
LACTIC BACTERIA	
Lactobacillus plantarum	NC_004567, CP021501.1, CP017379.1, CP017374.1, CP017363.1, CP017354.1, CP018209.1, CP020816.1, CP020861.1, CP019348.1, CP019722.1, CP017406.1, CP018324.1, CP013149.1, CP017954.1, CP015308.1, CP013753.1, CP013749.1, CP016071.1, CP015857.1
Lactobacillus brevis	CP000416, CP005977.1, CP015398.1, AP012167.1, JN383920.1, JN368473.1, JN368472.1, JN368471.1, EF412991.1, EF412994.1, EF412993.1, EF412992.1, AY582720.1, AB102858.1, AY821851.1, AY839298.1, AF429617.1, AF429584.1, AF429583.1, AF429547. , AF429542.1, AF405353.1, X74221.1
Lactobacillus hilgardii	NZ_GG670001.1, U161617.1, EF536365.1, EF536366.1, AJ616222.1, KU922755.1
Pediococcus damnosus	AF405365, AJ318414, CP012294.1, CP012288.1, CP012283.1, CP012275.1, CP012269.1, AF405385.1, AF405366.1, AF405376.1, AF405367.1
Pediococcus pentosaceus	NC_022780, CP015918.1, CP021474.1, CP006854.1, KC767943.1, JN696685.1, JN696705.1, CP000422.1
ACETIC BACTERIA	
Gluconobacter oxydans	CP000009, CP003926.1, CP004373.1, AB163823.1, AB163824.1, AB163830.1, AB163833.1, CP016328.1, AB163865.1, AB163861.1, AB163859.1, AB163841.1, AB163825.1
Acetobacter aceti	X74066, AB111902.1, AJ007831.1, AB161358.1, CP014692.1
Acetobacter pasteurianus	X71863, AJ007834, AJ007834.1, AB086017.1, AP014881.1, HF677570.1, AP011170.1, AP011163.1, AP011156.1, AP011149.1, AP011142.1, AP011135.1, AP011128.1, AP011121.1, AM049398.1

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681
682 **Table 3.** Primer pairs for the specific amplification of the target
683 sequence of yeasts, lactic and acetic bacteria.
684

Primer name	Primer sequence	Tm (°C)
YEASTS		
Liev_For	CAAGGTTTCCGTAGGTGAAC	58
Liev_Rev	CCAAGAGATCCRTTGYTGAA	58
LACTIC BACTERIA		
Latt_For	AACAAGGTAGCCGTAGGAGA	58
Latt_Rev	GTTAGTCCCGTCCTTCATCG	60
ACETIC BACTERIA		
Acet_For	TCGTAACAAGGTAGCCGTAG	58
Acet_Rev	CAAGCGTGTGCTCTAACCAA	60

685

686

687
688 **Table 4**. Oligo probes immobilized onto the epoxydated surface of the glass slide.
689

Microorganism	Oligo sequence	Lenght	TM (°C)
YEASTS			
S. cerevisiae	ACTCTCCATCTCTTGTCTTCTTGCCCAG	28	70
Z. bailii	GAACACAACTACTCCAGACTCGTCAATC	28	68
Z. rouxii	CCCTCCAACACTTTGAGAGAACTCCGT	27	70
B. bruxellensis	TTATCCTTGCTTATCCACGTGTCTGCAC	28	68
S. pombe	TTCACAGAAAGGTAAATGGATAAGAGAAGAAA	32	66
P. membranifaciens	TGACGTGTGTATACTCCAGGTTTAGGTGTTT	31	70
P. anomala	TGTTTAGACCTTTGGGCAGTAAGCCAG	27	68
C. stellata	GACCGAAGTCTTGGCTGTTCACAGTGG	27	71
H. vineae	CGCGCAAACTACAGCCAATAGCAAGAAC	28	70
LACTIC BACTERIA			
L. plantarum	AACGGTAAATGCGATTAATGAGTTTAGCGATAA	33	68
L. brevis	TCAACAAGTATGTGTAGCCTCCGTATATTCCTT	33	70
L. hilgardii	GTTAACAAACTCAAAATAACGCGGTGTTCTCG	32	70
P. damnosus	CGACATATGTGTAGGTTTCCGTTTCTAAATATCC	34	70
P. pentosaceus	CCTACGGTAAAGTGATTAATTGAGTTTAGCG	31	68
ACETIC BACTERIA			
G. oxydans	AAATTATAGGAAGGGATATGTTGACGGCG	29	67
A. aceti	CAAACCCAGTCCAATCTGTGAGTTGAAA	28	67
A. pasteurianus	AAACCCGACTGAATAACCTAGACAATACAT	30	67

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Figure

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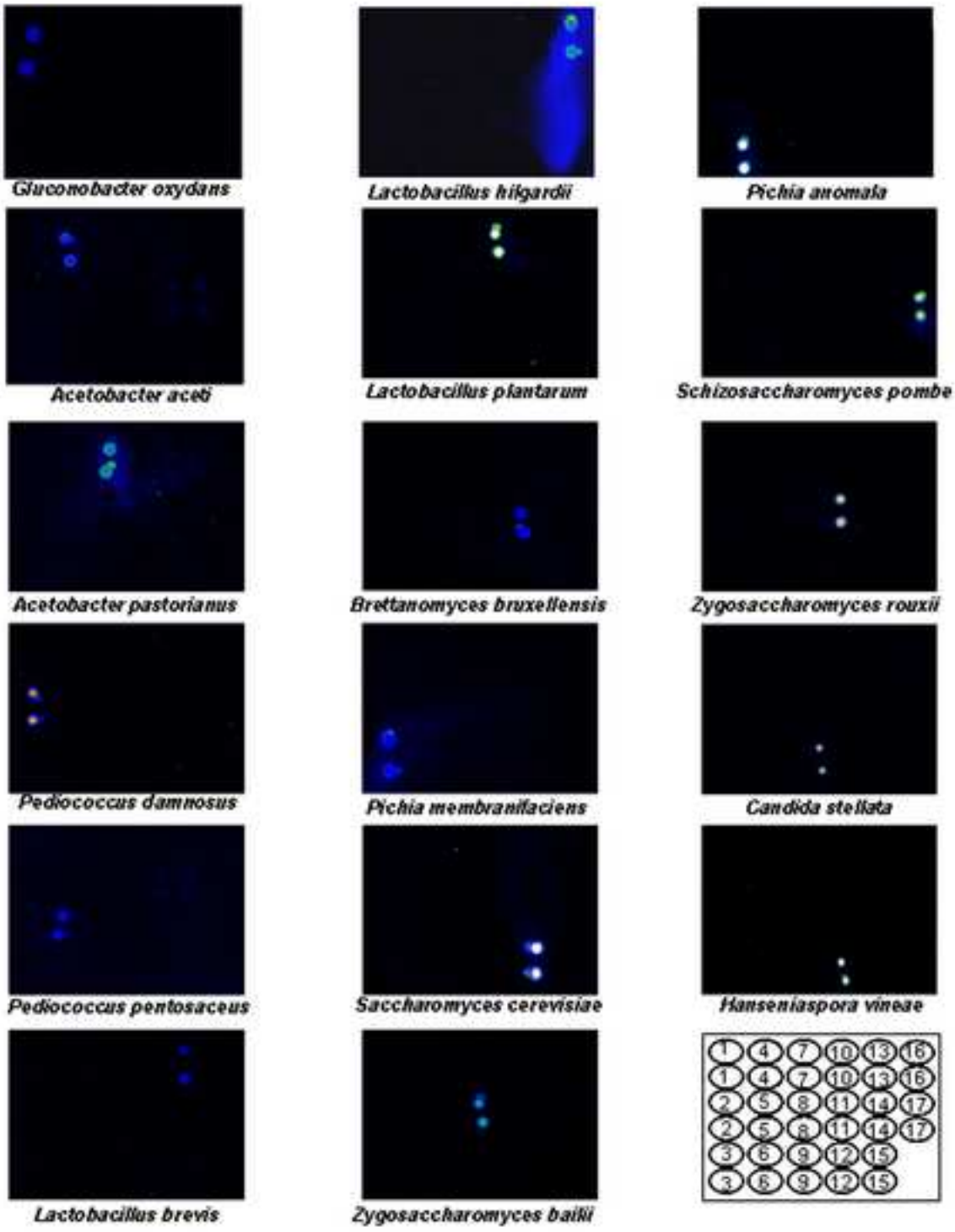

1 *Gluconobacter oxydans*
2 *Pediococcus damnosus*
3 *Pichia membranifaciens*
4 *Acetobacter aceti*
5 *Pediococcus pentosaceus*
6 *Pichia anomala*
7 *Acetobacter pasteurianus*
8 *Zygosaccharomyces bailii*
9 *Candida stellata*
10 *Lactobacillus plantarum*
11 *Zygosaccharomyces rouxii*
12 *Hanseniaspora vineae*
13 *Lactobacillus brevis*
14 *Brettanomyces bruxellensis*
15 *Saccharomyces cerevisiae*
16 *Lactobacillus hilgardii*
17 *Schizosaccharomyces pombe*

Figure 1

Figure 2

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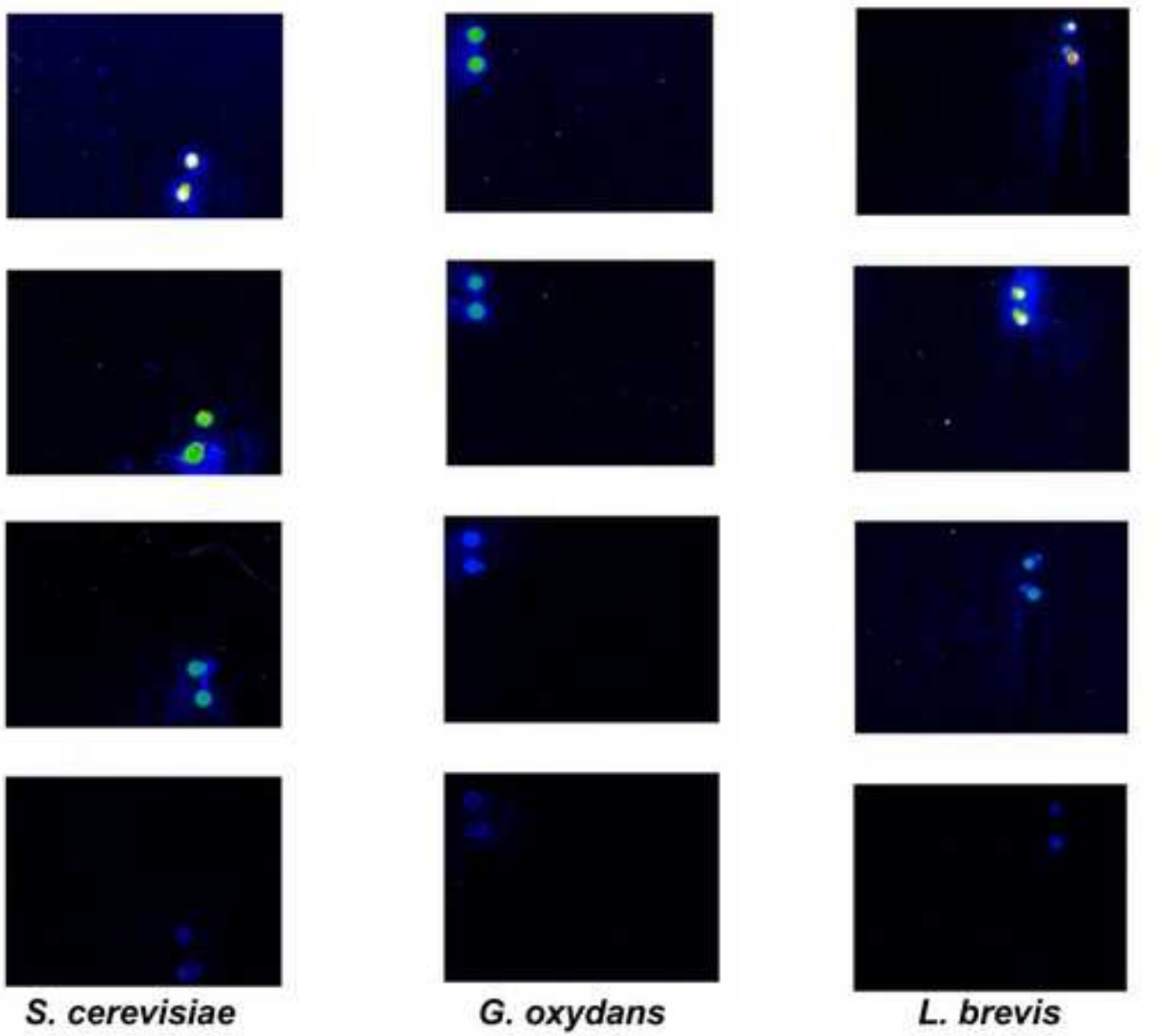

Figure 2

Figure 3

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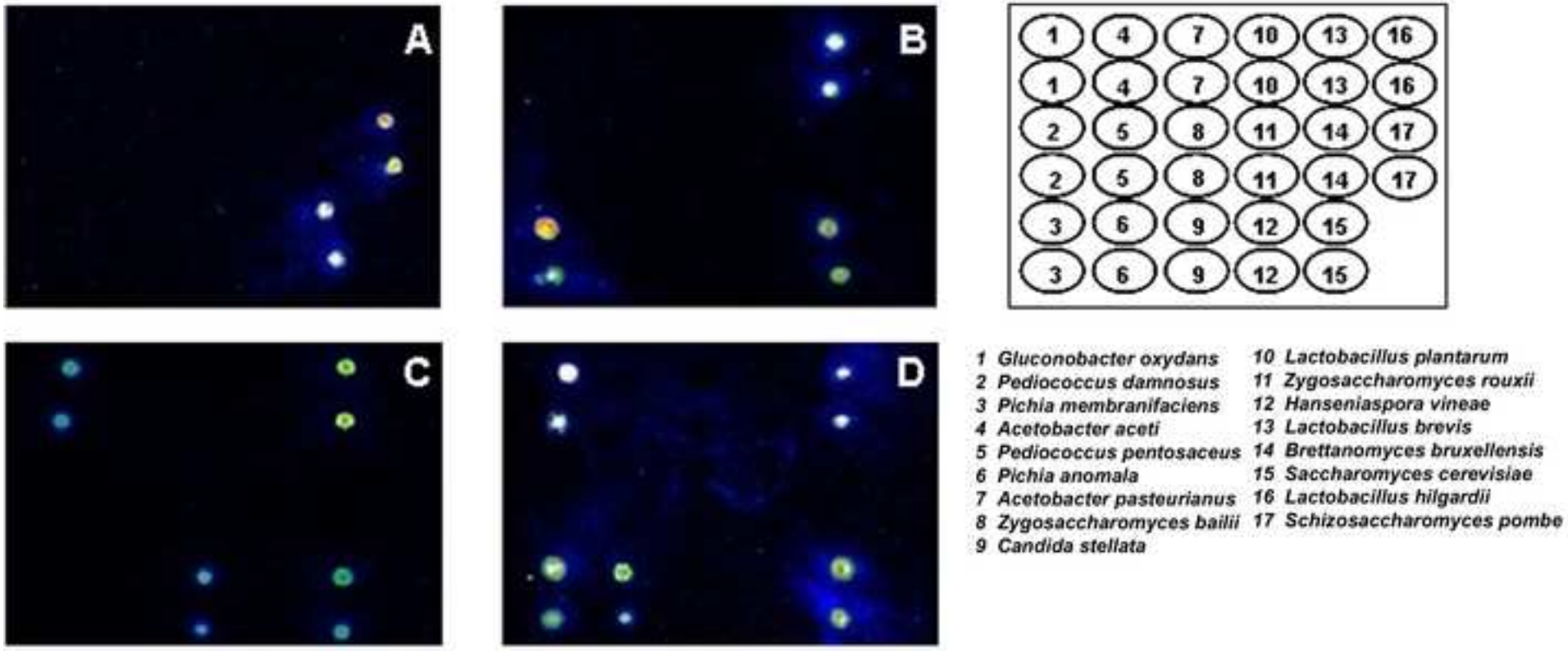

Figure 3

Figure 4
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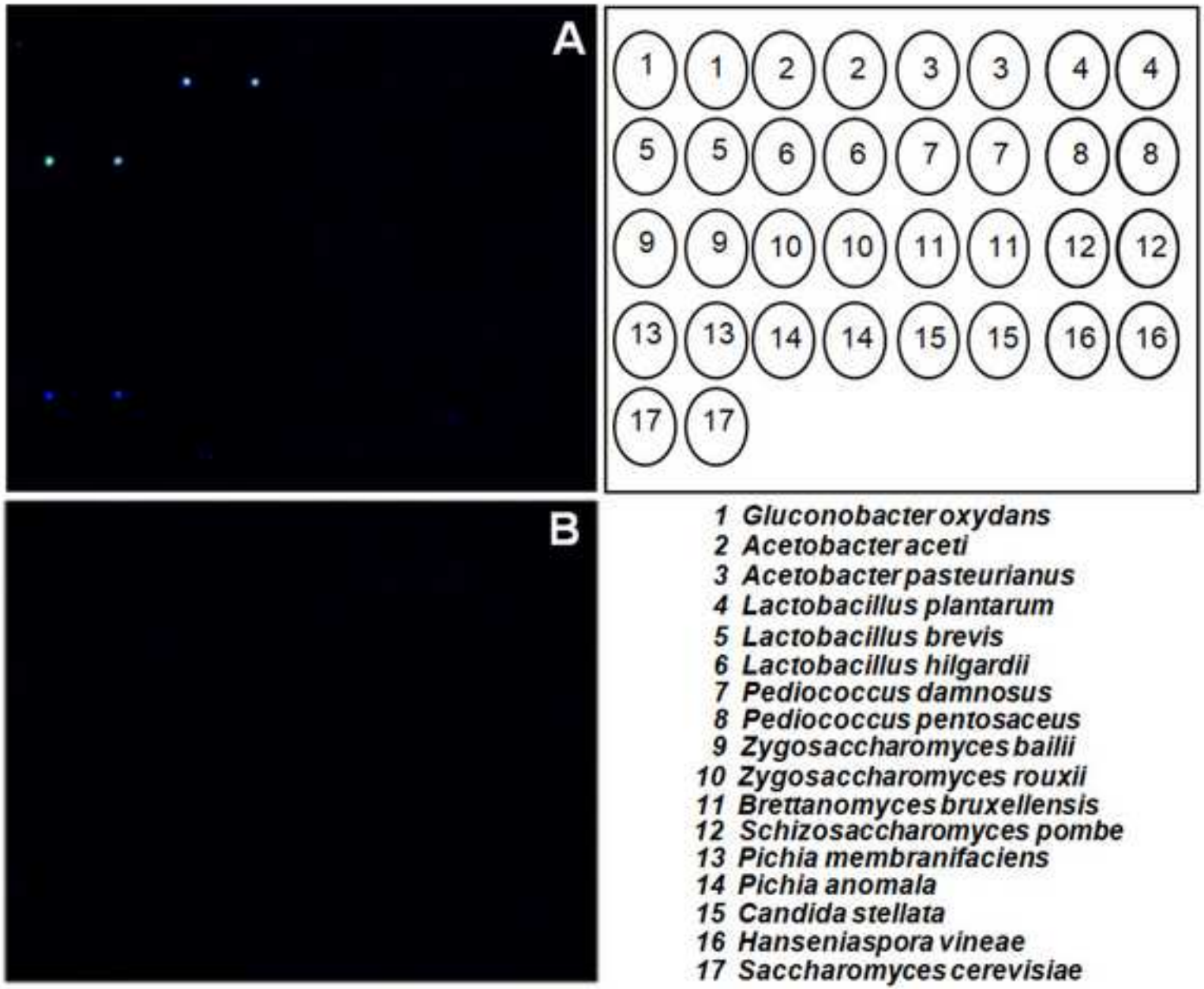

Figure 4

Figure 5

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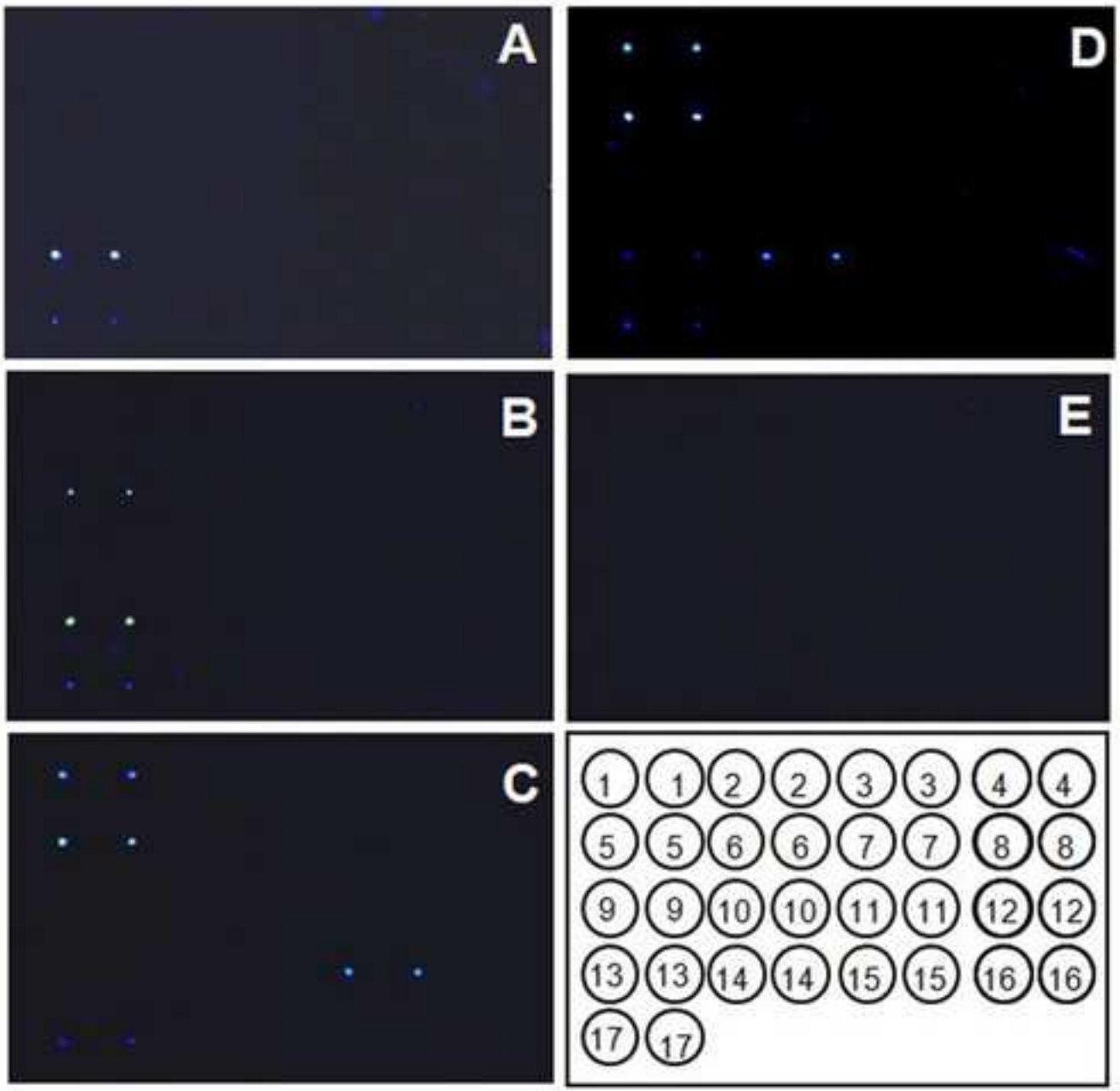

1 *Gluconobacter oxydans*
2 *Acetobacter aceti*
3 *Acetobacter pasteurianus*
4 *Lactobacillus plantarum*
5 *Lactobacillus brevis*
6 *Lactobacillus hilgardii*
7 *Pediococcus damnosus*
8 *Pediococcus pentosaceus*
9 *Zygosaccharomyces bailii*
10 *Zygosaccharomyces rouxii*
11 *Brettanomyces bruxellensis*
12 *Schizosaccharomyces pombe*
13 *Pichia membranifaciens*
14 *Pichia anomala*
15 *Candida stellata*
16 *Hanseniaspora vineae*
17 *Saccharomyces cerevisiae*

Figure 5

Supplementary Materials

AN INNOVATIVE OLIGONUCLEOTIDE MICROARRAY TO DETECT SPOILAGE MICROORGANISMS IN WINE

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[†] Both authors contributed equally to this work

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Patrizia Rampino, Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100 Lecce, Italy. Phone: +390832298688; Fax: +390832298858; Email: patrizia.rampino@unisalento.it

Table S1. Length of the amplicons produced after PCR assay respectively using the Liev_For/ Liev_Rev, Latt_For/ Latt_ Rev and Acet_For/Acet_Rev primer primes on yeasts, lactic and acetic bacteria genomic DNA templates.

Microorganims	Lenght (bp)
YEASTS	
Saccharomyces cerevisiae	423
Zygosaccharomyces rouxii	287
Zygosaccharomyces bailii	426
Brettanomyces bruxellensis	154
Schizosaccharomyces pombe	483
Pichia membranifaciens	150
Pichia anomala	244
Candida stellata	187
Hanseniaspora vineae	350
LACTIC BACTERIA	
Lactobacillus plantarum	315
Lactobacillus brevis	326
Lactobacillus hilgardii	334
Pediococcus damnosus	342
Pediococcus pentosaceus	327
ACETIC BACTERIA	
Gluconobacter oxydans	212
Acetobacter aceti	280
Acetobacter pasteurianus	298

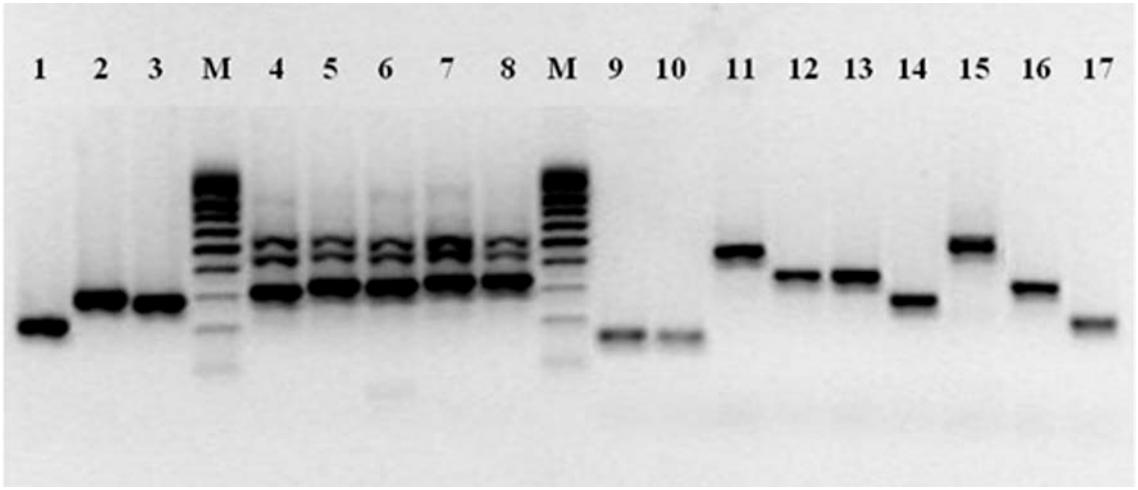
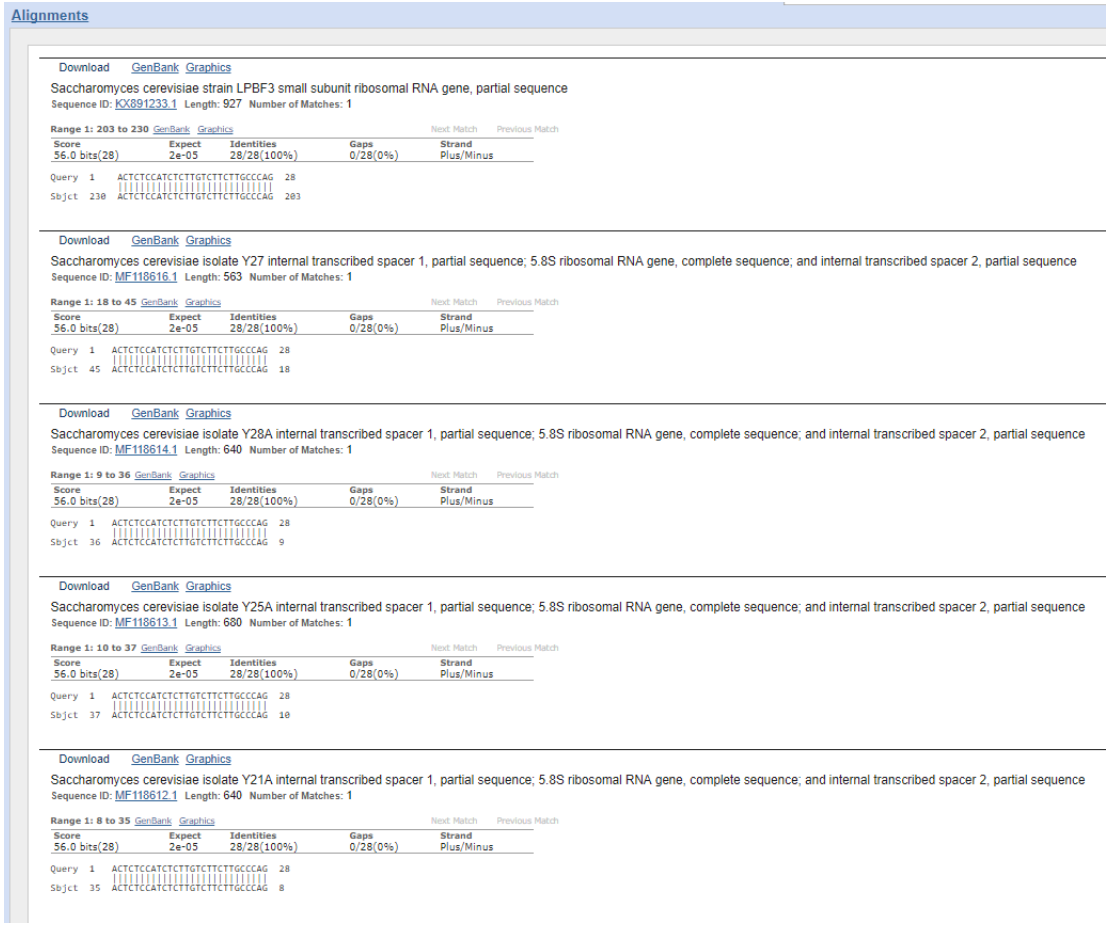


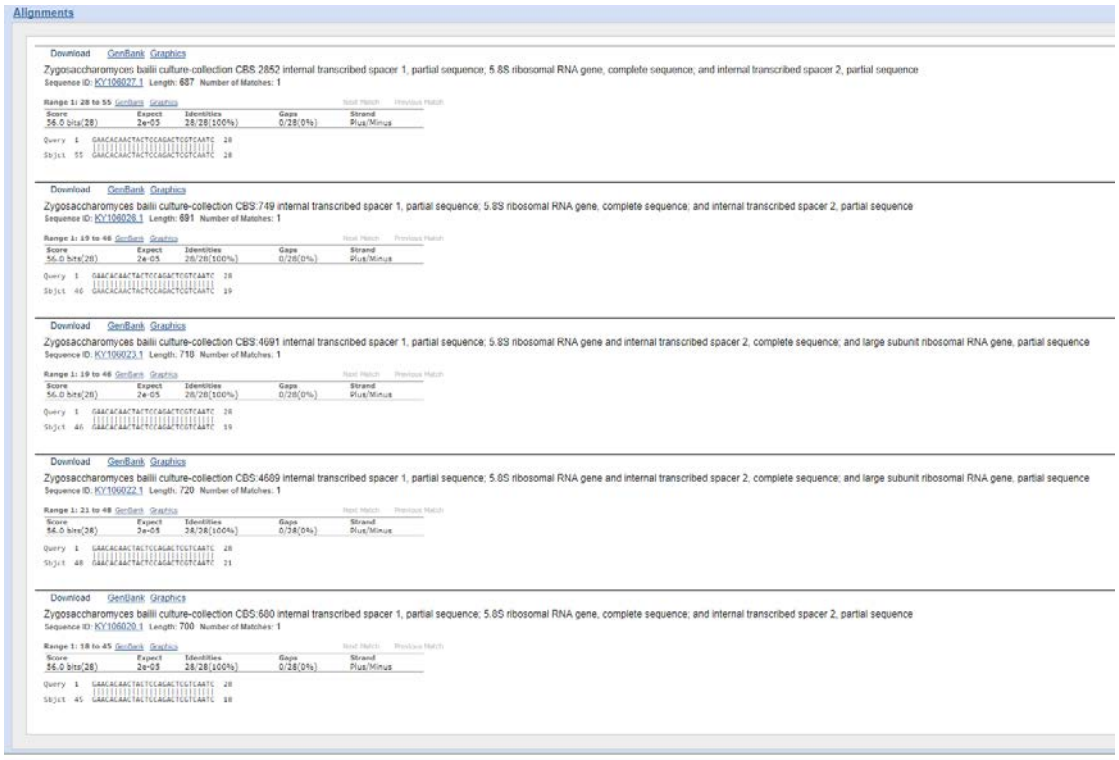
Figura S1. Electrophoretic profiles of amplification products of the chromosomal region corresponding to the gene cluster encoding the ribosomal RNA of bacteria (16S-ITS1) and yeasts (18S-5.8). The amplification was performed using the primers pairs *Acet_For/Acet_Rev* for acetic acid bacteria, *Latt_For/Latt_Rev* lactic acid bacteria and *Liev_For/Liev_Rev* for yeasts. Lane 1, *Gluconobacter oxydans*; lane 2, *Acetobacter pasteurianus*; lane 3, *A. aceti;* lane 4, *Lactobacillus plantarum*; lane 5, *L. hilgardii*; lane 6, *L. brevis*; lane 7, *Pediococus damnosus*; lane 8, *P. pentosaceus*; lane 9, *Brettanomyces bruxellensis*; lane 10, *Pichia membranifaciens*; lane 11, *Saccharomyces cerevisiae*; lane 12, *Zygosaccharomyces bailii*; lane 13, *Hanseniaspora vineae*; lane 14, *Pichia anomala*; lane 15, *Schizosaccharomyces pombe*; lane 16, *Z. rouxii*; lane 17, *Candida stellata;* lane M, DNA Ladder 100bp (Euroclone).

Figure S2.

S. cerevisiae ACTCTCCATCTCTTGTCTTCTTGCCCAG

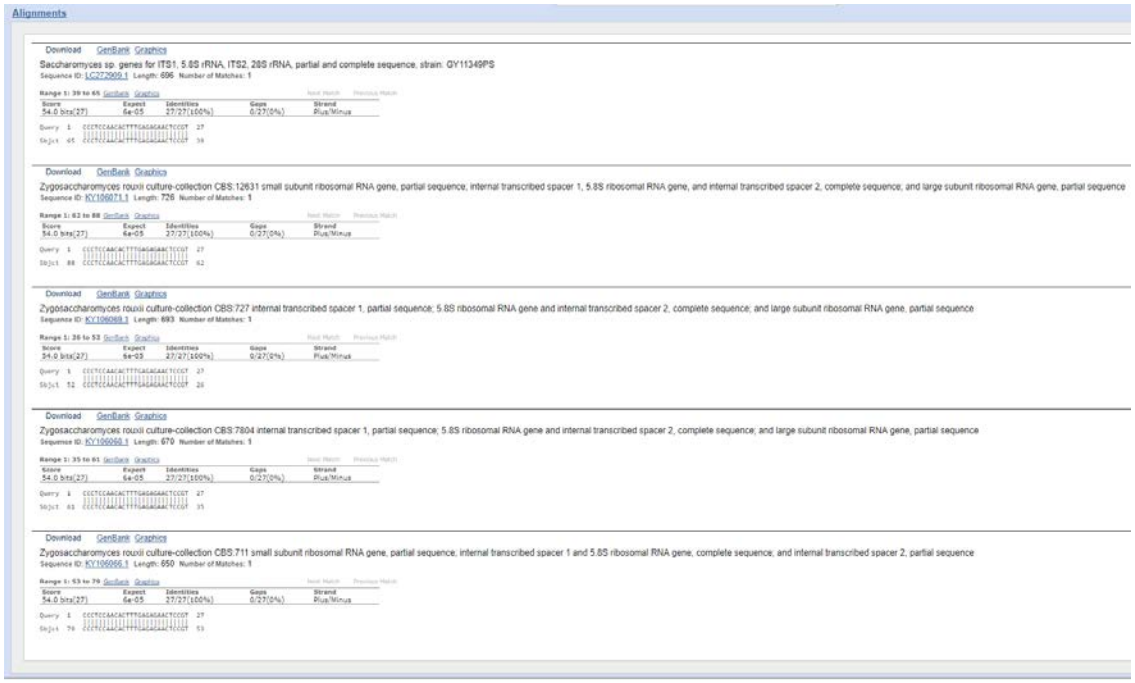


Z. bailii GAACACAACTACTCCAGACTCGTCAATC



56

Z. rouxii CCCTCCAACACTTTGAGAGAACTCCGT



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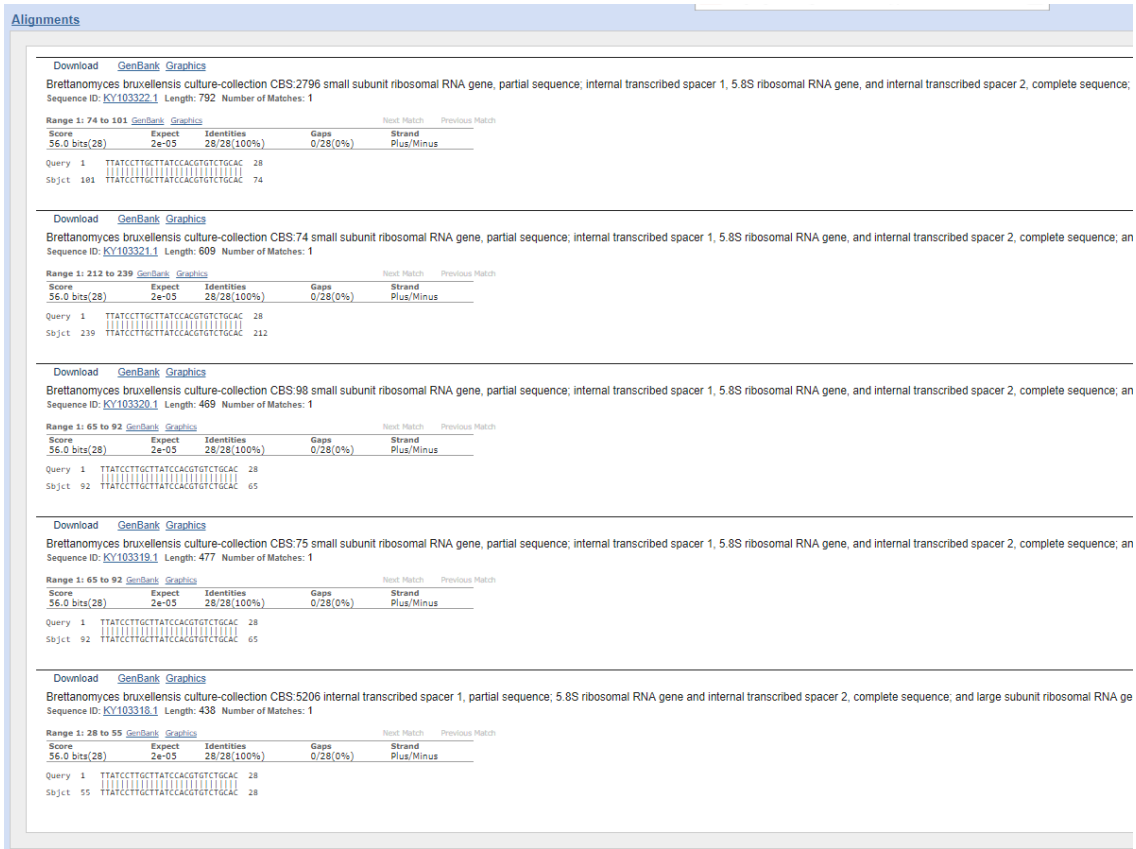
D. bruxellensis TTATCCTTGCTTATCCACGTGTCTGCAC



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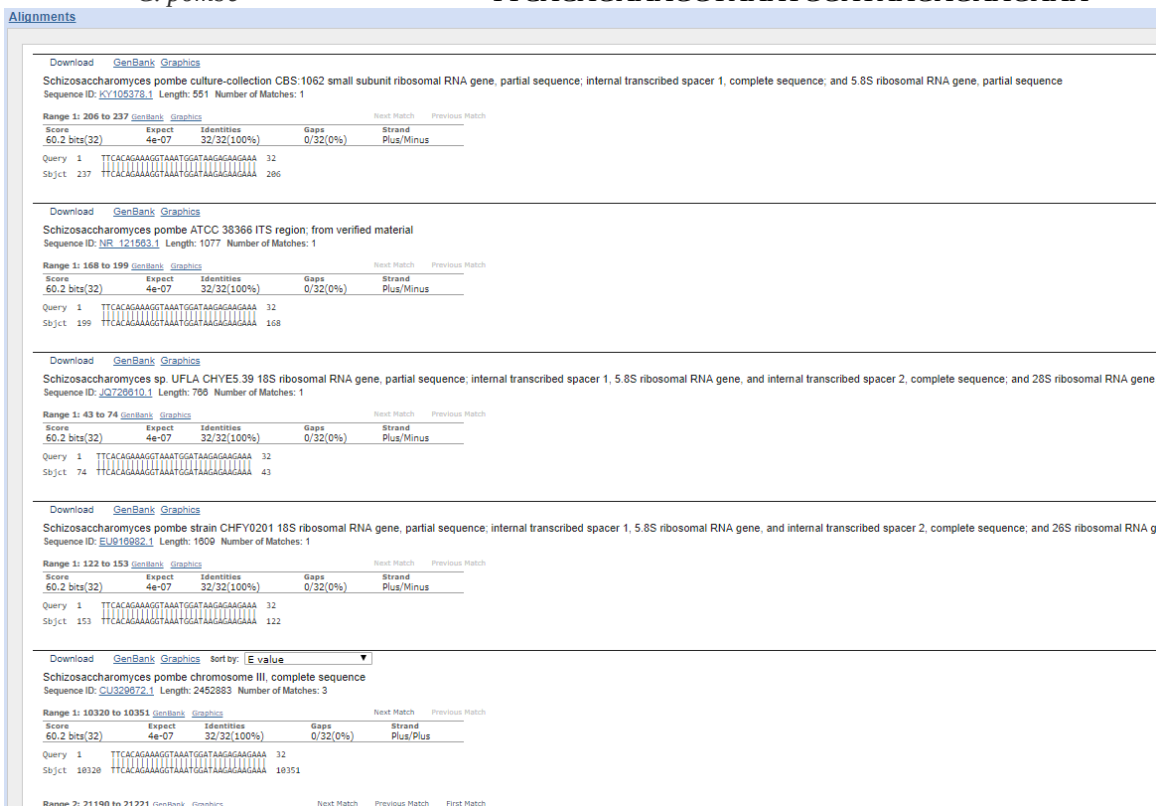
C. pombe TTCACAGAAAGGTAAATGGATAAGAGAAGAAA



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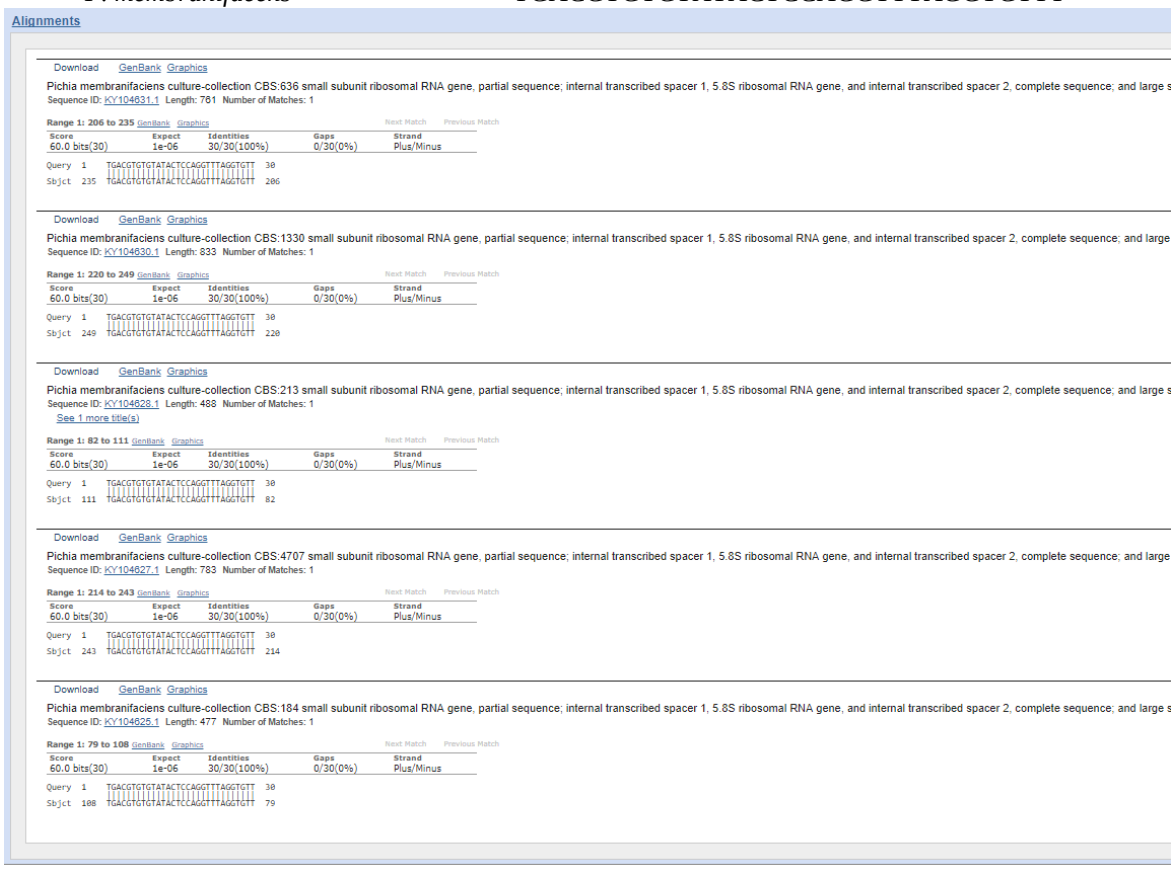
P. membranifacens TGACGTGTGTATACTCCAGGTTTAGGTGTTT



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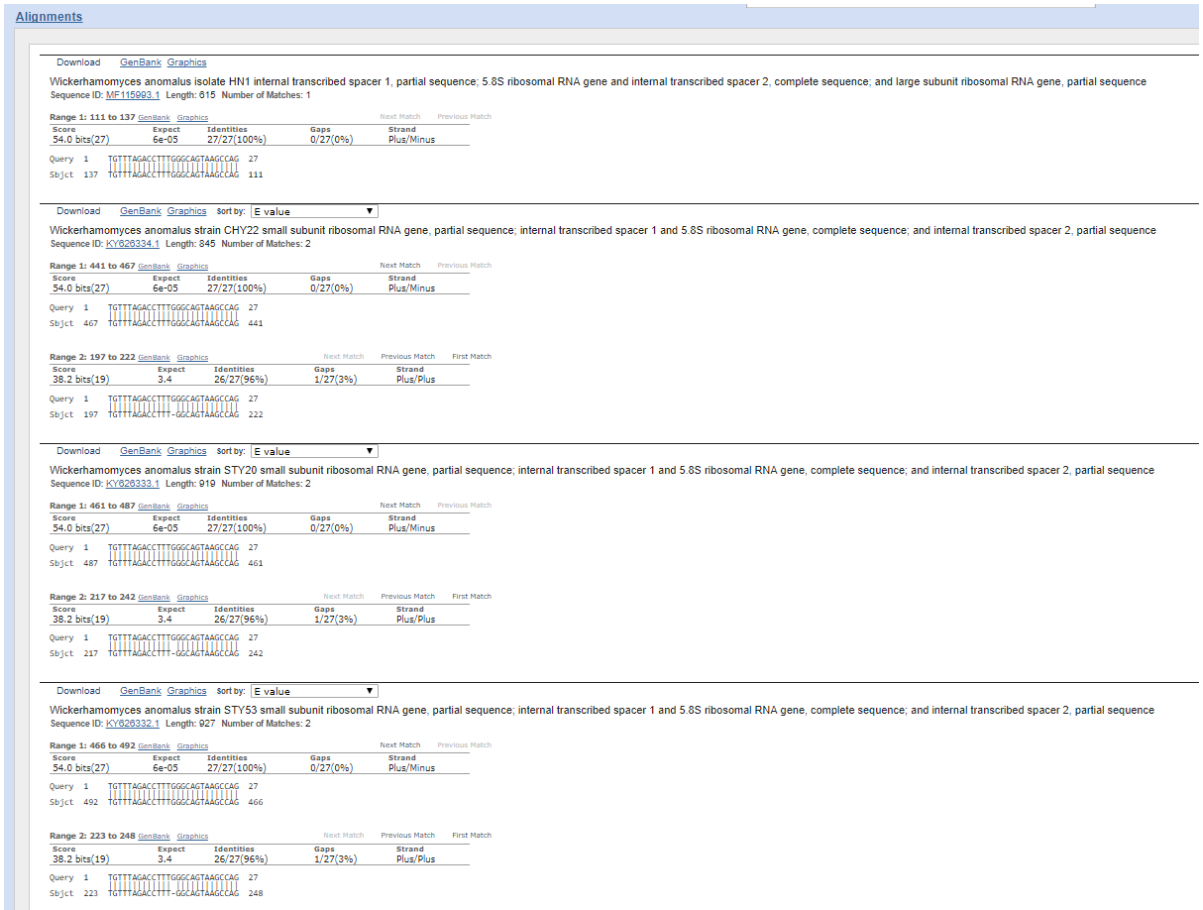
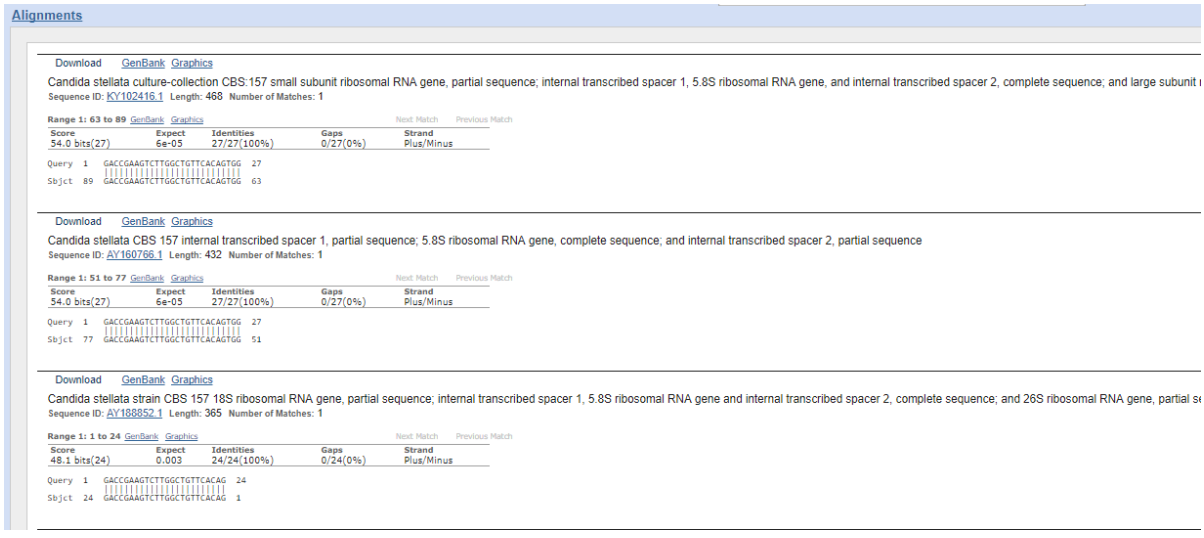
P. anomala TGTTTAGACCTTTGGGCAGTAAGCCAG



C. stellata GACCGAAGTCTTGGCTGTTCACAGTGG



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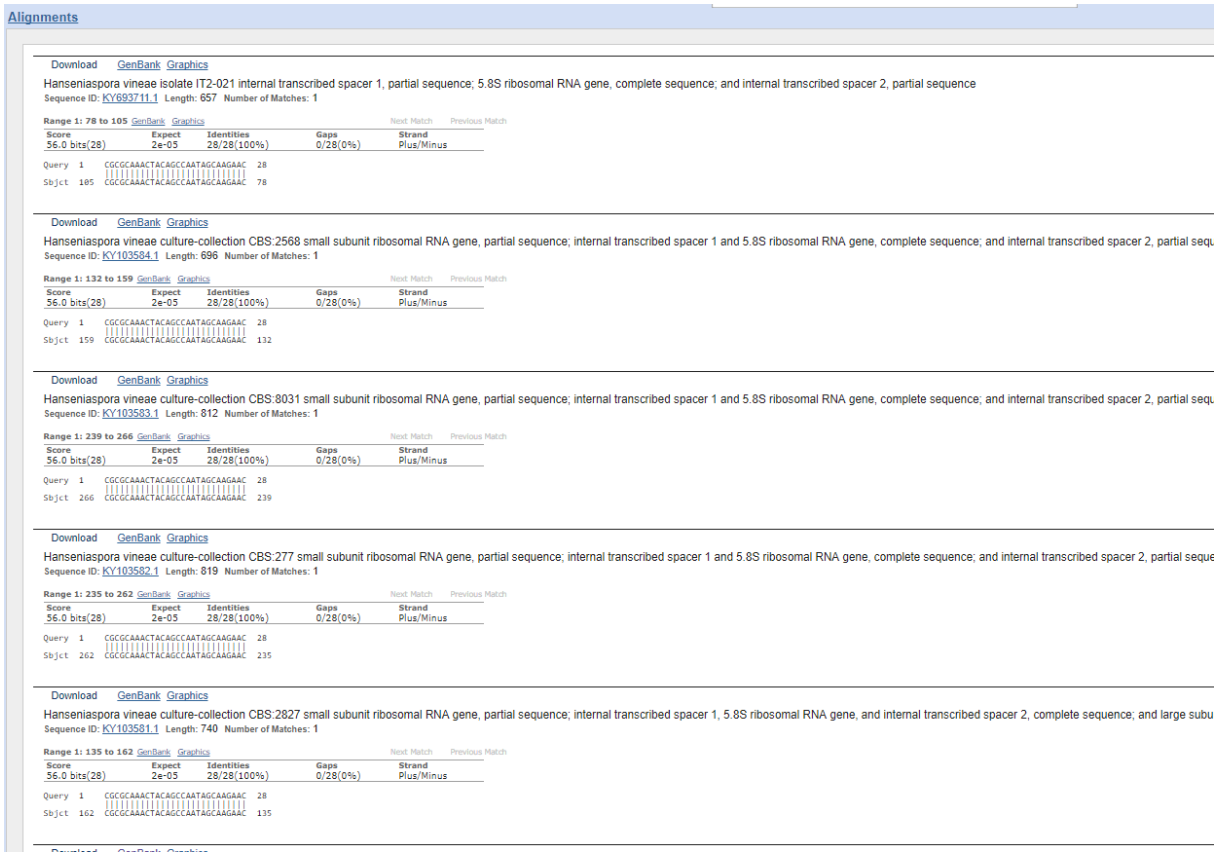
H. vineae CGCGCAAACTACAGCCAATAGCAAGAAC



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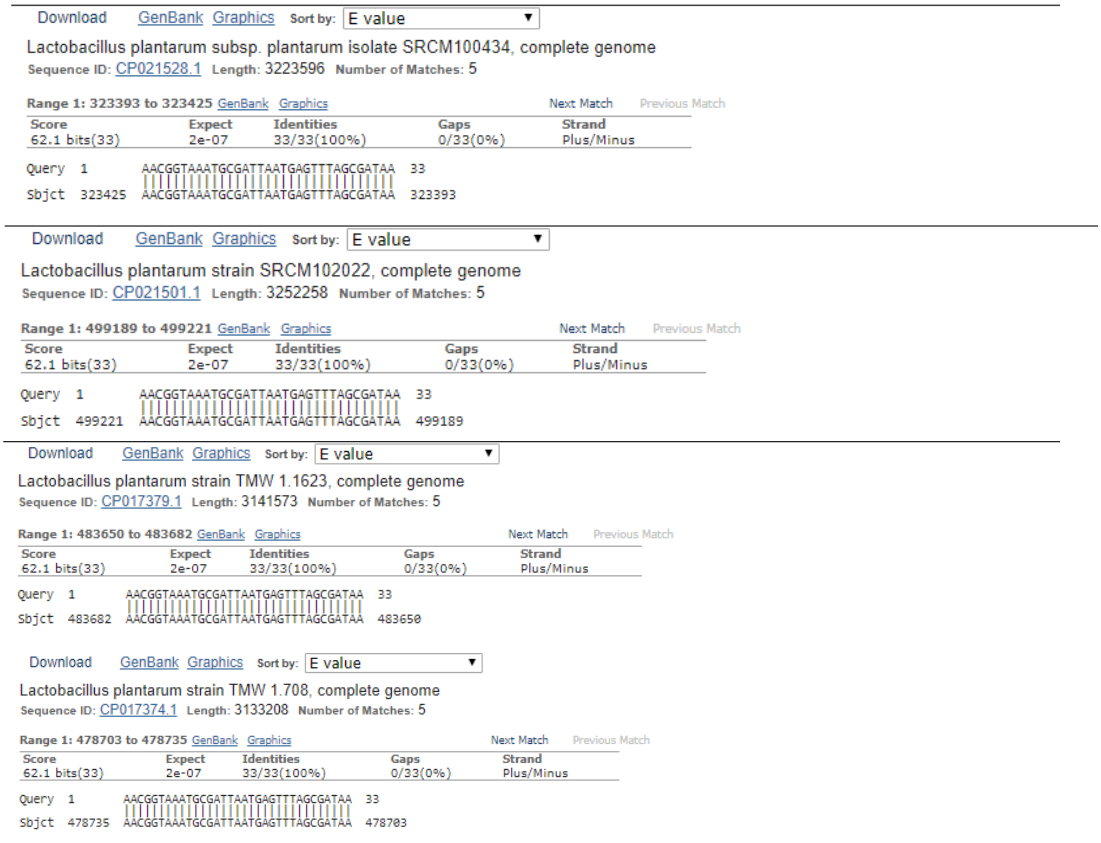
L. plantarum AACGGTAAATGCGATTAATGAGTTTAGCGATAA



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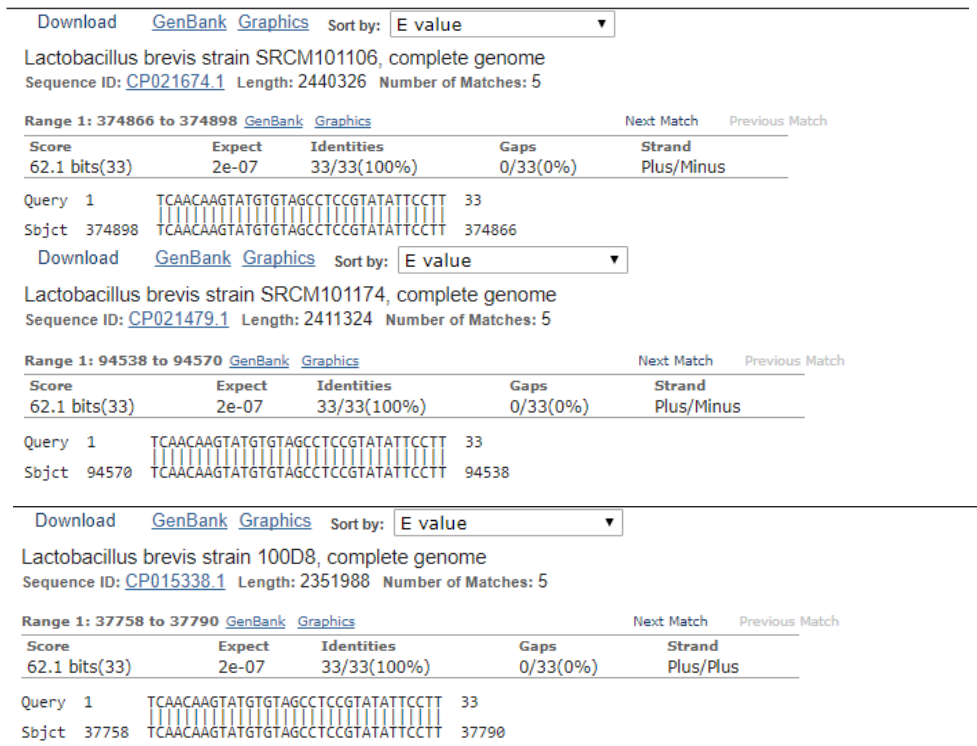
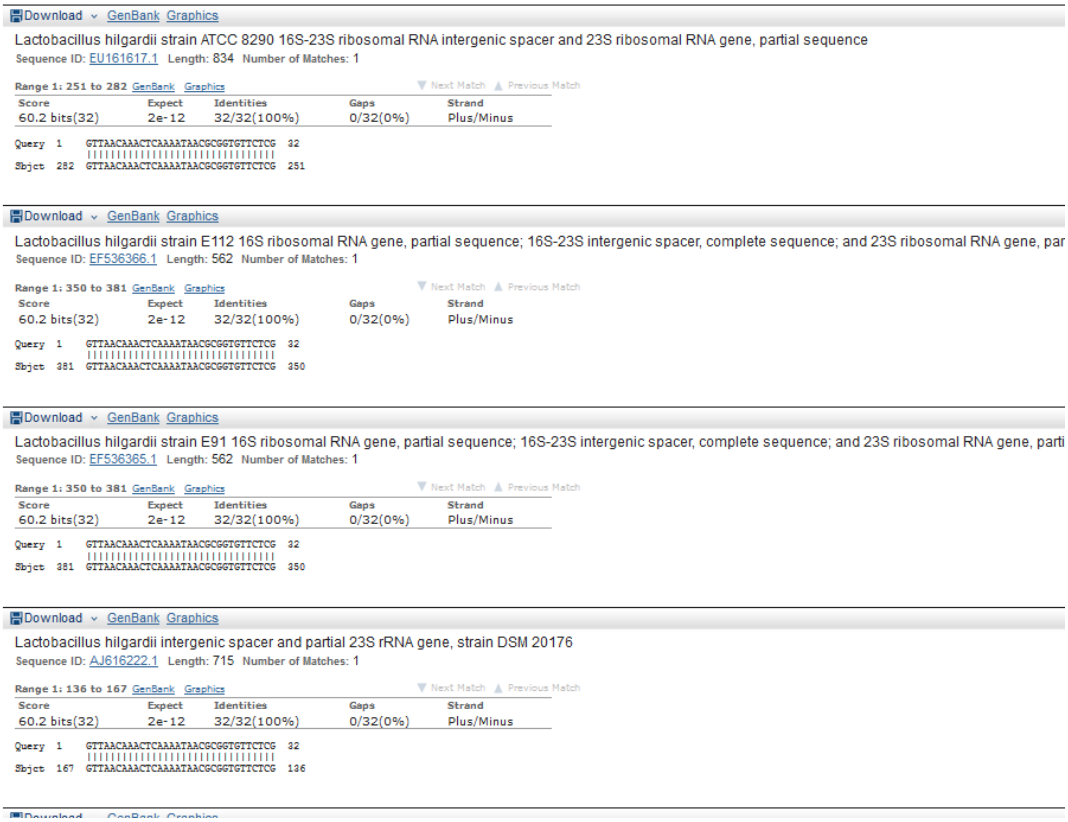
L. brevis TCAACAAGTATGTGTAGCCTCCGTATATTCCTT



L. hilgardii GTTAACAAACTCAAAATAACGCGGTGTTCTCG



84

P. damnosus CGACATATGTGTAGGTTTCCGTTTCTAAATATCC

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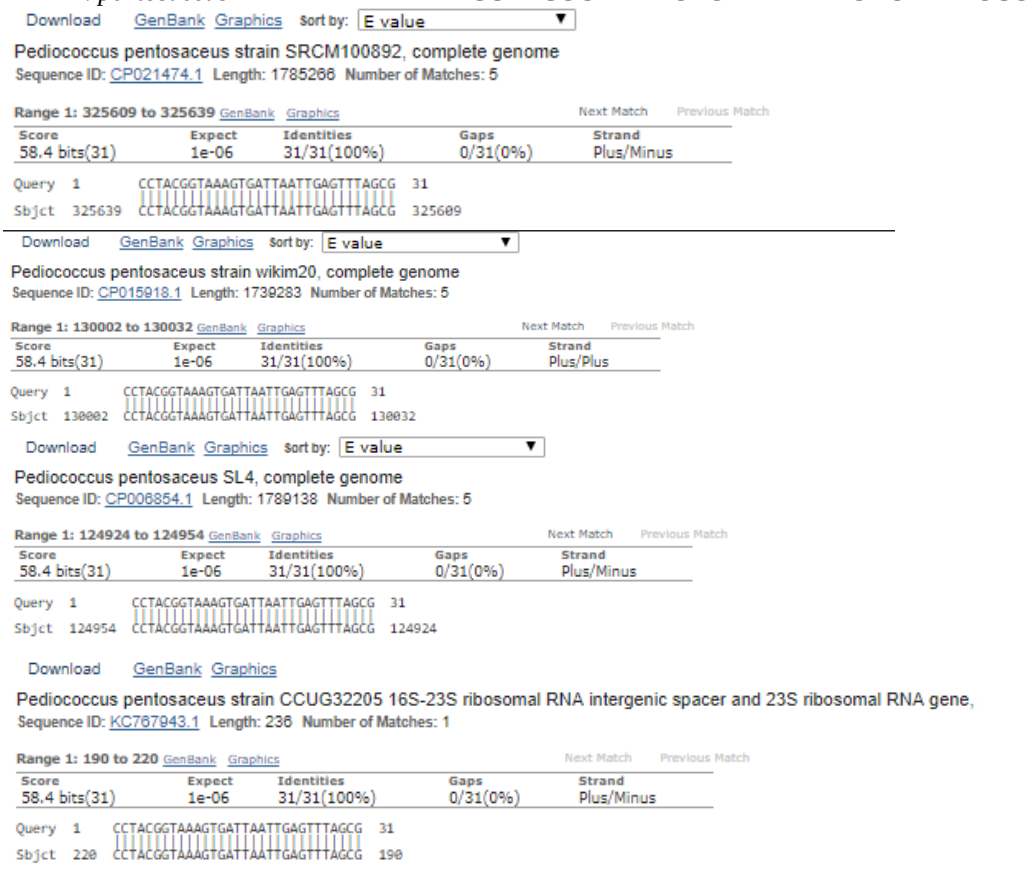
P. pentosaceus CCTACGGTAAAGTGATTAATTGAGTTTAGCG



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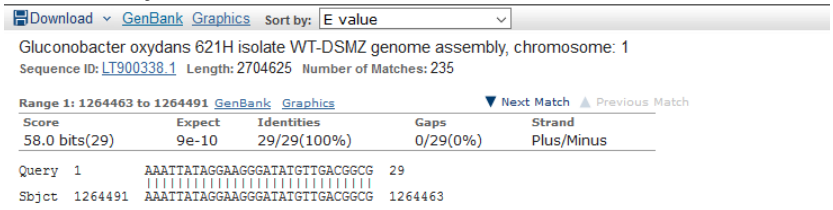
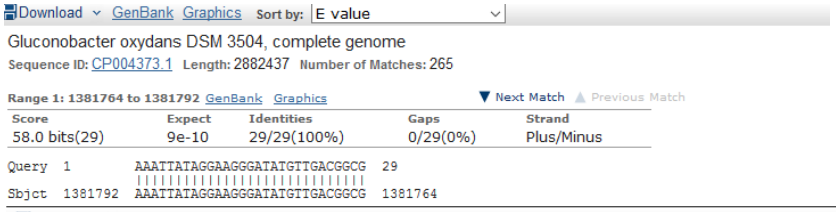
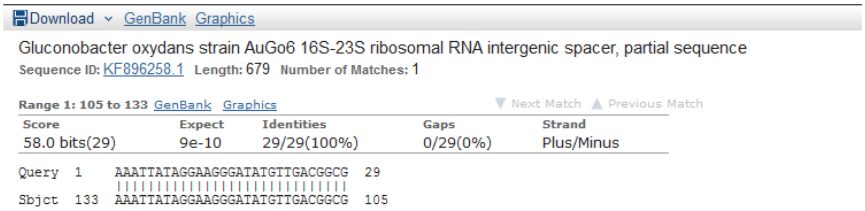
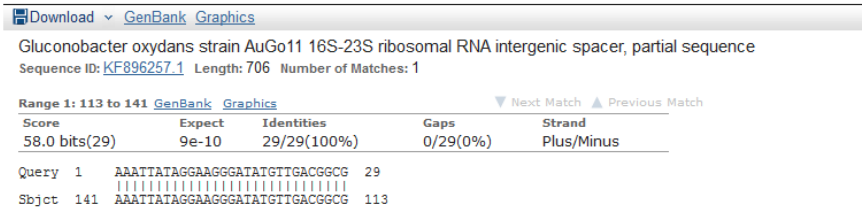
G. oxydans AAATTATAGGAAGGGATATGTTGACGGCG

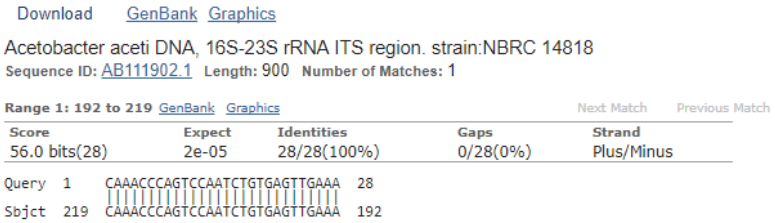


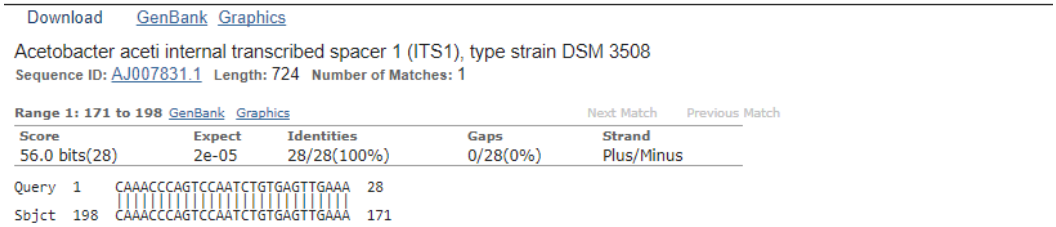


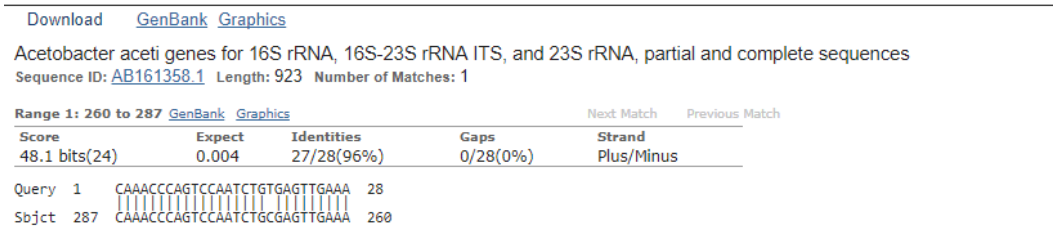




A. aceti CAAACCCAGTCCAATCTGTGAGTTGAAA







A. pasteurianus AAACCCGACTGAATAACCTAGACAATACAT

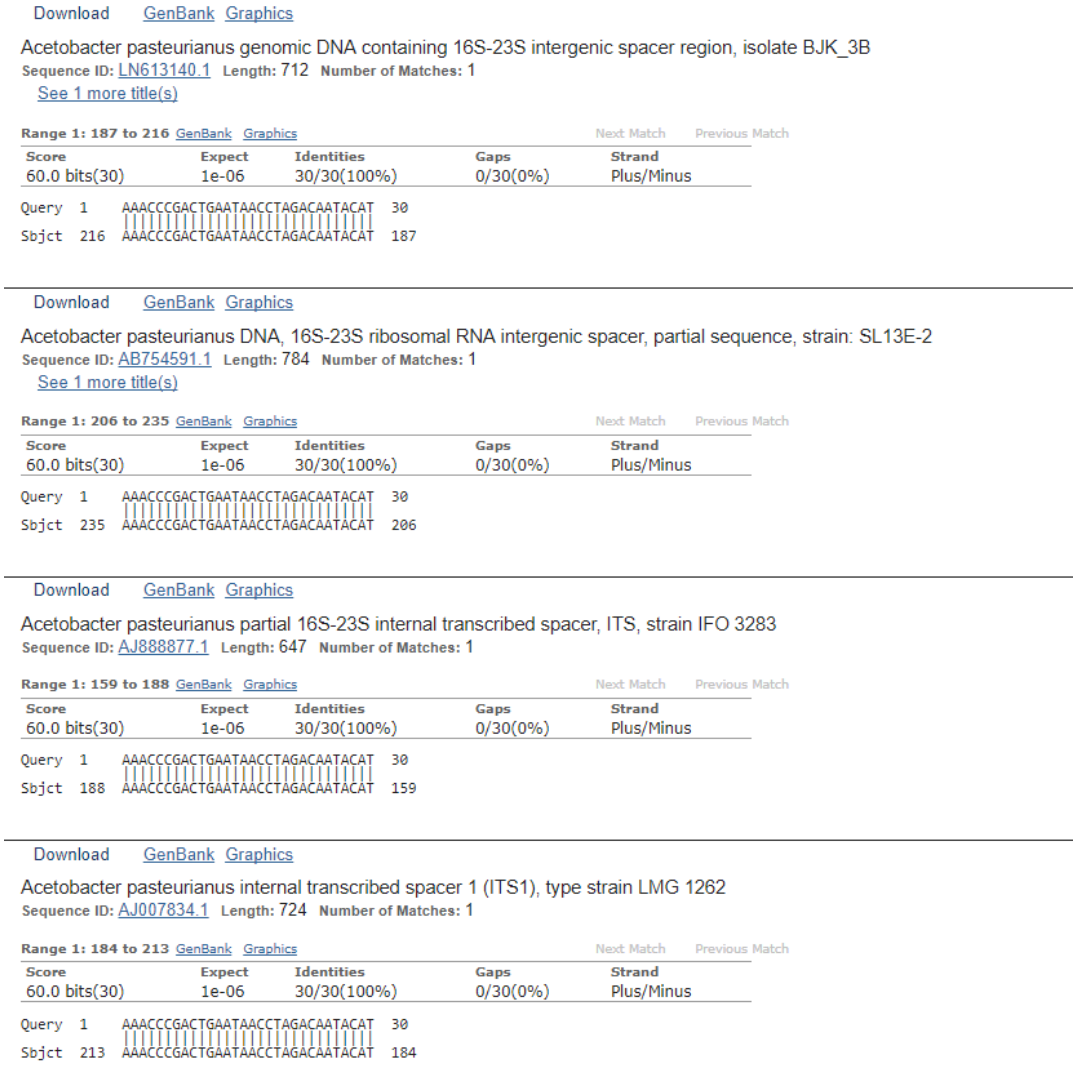


Figure S2. Evaluation of the specificity of the 17 species-specific oligoprobes by sequence alignment and similarity search carries out by BLAST. Each primer sequence and the source organism are indicated.

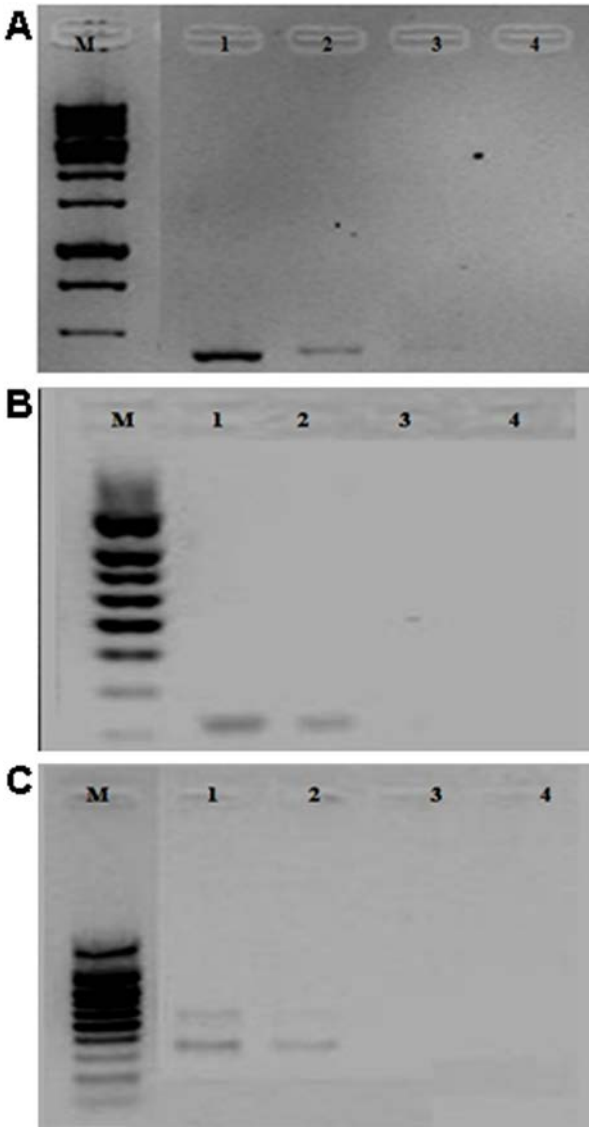


Figure S2. Panel A. Electrophoretic profiles of amplification products of rDNA region 18S-5.8S from *S. cerevisiae*. The amplification was performed using the pair of primers *Liev_For_Cy5/Liev_Rev* and different amounts of target-DNA: lane 1, 50 pg; lane 2, 10 pg; lane 3, 2 pg; lane 4, 0.4 pg; M, DNA Ladder 100 bp (Euroclone). **Panel B.** Electrophoretic profiles of amplification products of rDNA region 16S-ITS1 from *G. oxydans*. The amplification was performed using the pair of primers *Acet_For_Cy5/Acet_Rev*. and different amounts of target-DNA: lane 1, 50 pg; lane 2, 10 pg; lane 3, 2 pg; lane 4, 0.4 pg; M, DNA Ladder 100 bp (Euroclone). **Panel C.** Electrophoretic profiles of amplification products of rDNA region 16S-ITS1 from *L. brevis*. The amplification was performed using the pair of primers *Latt_For_Cy5/Latt_Rev*. and different amounts of target-DNA: lane 1, 50 pg; lane 2, 10 pg; lane 3, 2 pg; lane 4, 0.4 pg; M, DNA Ladder 100 bp (Euroclone).

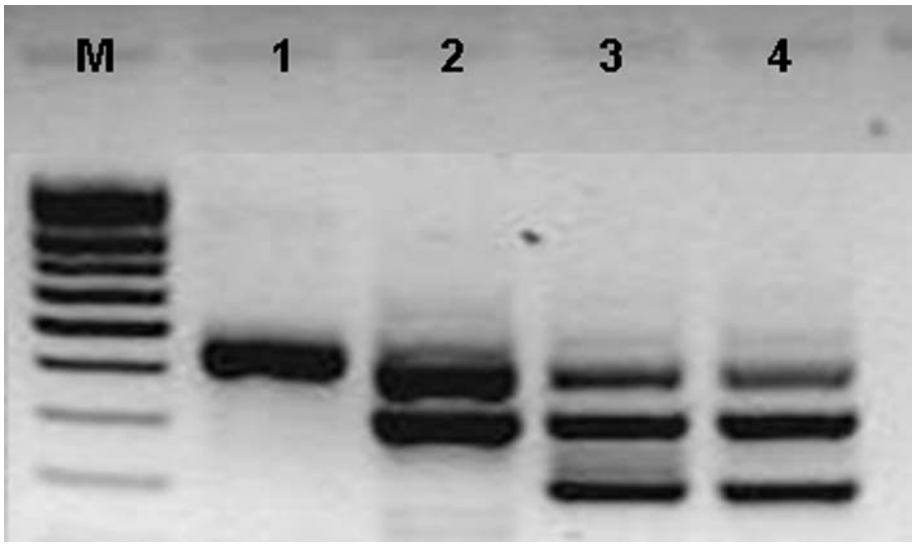


Figure S3. Electrophoretic profiles of amplification products obtained by PCR multiplex of the chromosomal region corresponding to the gene cluster encoding the ribosomal RNA of bacteria (16S-ITS1) and yeasts (18S-5.8). The amplification was performed using the pairs of primers *Acet_For/Acet_Rev* for acetic acid bacteria, *Latt_For/Latt_Rev* lactic acid bacteria and *Liev_For/Liev_Rev* for yeasts. Lane 1, *S. cerevisiae*, *S. pombe*; lane 2, *S. cerevisiae*, *P. membranifaciens*, *L. brevis*; lane, *S. cerevisiae*, *C. stellata*, *L. brevis*, *G. oxydans*; lane 4, *S. cerevisiae*, *P. anomala*, *P. membranifaciens*, *L. brevis*, *G. oxydans*; lane M, DNA Ladder 100 bp (Euroclone).