Food Anal. Methods (2011) 4:173–185
DOI 10.1007/s12161-010-9152-6

Development of a DNA Array for the Simultaneous Detection and Identification of Sugar Thick Juice Bacterial Contaminants

Annelies Justé · Bart Lievens · Ingeborg Frans · Michael Klingeberg · Chris W. Michiels · Kris A. Willems

Received: 25 January 2010 / Accepted: 18 May 2010 / Published online: 30 June 2010

Abstract Despite the use of generally accepted good storage practices, sugar thick juice degradation caused by microbiological contamination occasionally occurs, causing considerable financial loss. In this study, a DNA array was developed for simultaneous detection and identification of the most prominent microflora present during thick juice storage, which may cause degradation of the thick juice. Specific oligonucleotides were developed for several bacterial taxa, including the genera *Bacillus*, *Kocuria*, *Staphylococcus* and *Tetragenococcus* and the species *Aerococcus viridans*, *Leuconostoc mesenteroides* and *Tetragenococcus halophilus*. The DNA array was validated using both pure cultures and industrial samples. In addition, comparisons were made between the developed array, PCR assays specifically targeting the thick juice contaminants and classical microbial platings. The array was found to be reliable and sensitive enough to detect and identify the target bacteria. In addition, the array was used to monitor the target microbial populations in thick juice during long-term storage and degradation. Results are discussed in relation to DNA stability in thick juice.

Keywords Multiplex · *Tetragenococcus halophilus* · Thick Juice Degradation · DNA Stability · Monitoring

Introduction

Storing sugar extracts as thick juice, a form of sucrose syrup, is common practice in the sugar industry. Earlier research and industrial practice have demonstrated that thick juice stability is best managed by controlling parameters such as solids content, pH and temperature (Asadi 2007). Nevertheless, even under good storage practices (Justé 2008), microbio-

A. Justé and B. Lievens equally contributed to this work

A. Justé · B. Lievens · I. Frans · K. A. Willems
Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), Consortium for Industrial Microbiology and Biotechnology (CIMB),
Department of Microbial and Molecular Systems (M2S),
KULeuven Association, Lessius University College,
De Nayer Campus,
B-2860 Sint-Katelijne-Waver, Belgium

A. Justé · B. Lievens · I. Frans · K. A. Willems
Scientia Terrae Research Institute,
B-2860 Sint-Katelijne-Waver, Belgium

A. Justé · B. Lievens (✉) · I. Frans · C. W. Michiels · K. A. Willems
Leuven Food Science and Nutrition Research Centre (LFoRCe),
B-3001 Leuven, Belgium
e-mail: bli@scientiaterrae.org

M. Klingeberg
Südzucker AG, Mannheim/Ochsenfurt,
ZAFES,
67283 Obrigheim/Pfalz, Germany

C. W. Michiels
Center for Food and Microbial Technology,
Department of Microbial and Molecular Systems, KULeuven,
B-3001 Leuven, Belgium

logical problems may occur resulting in degradation of the thick juice (Sargent et al. 1997; Justé et al. 2008a). The most prominent symptoms of this degradation are a pronounced drop in pH (from pH 9 to pH 5 or 6) and a marked rise in reducing sugars (Sargent et al. 1997; Willems et al. 2003), resulting in reduced sucrose yield and financial loss.

In an ecological study, *Tetragenococcus halophilus* was identified as the major organism responsible for thick juice degradation, but some less abundant microbial populations of the thick juice flora were also suggested to contribute to the degradation process (Justé et al. 2008a). These populations include various strains of *Bacillus*, and *Staphylococcus* species as well as strains of *Tetragenococcus* species other than *T. halophilus.* Other contaminating species, including *Aerococcus viridans*, *Leuconostoc mesenteroides* and *Kocuria* species may also be found in thick juice (Willems et al. 2003; Samaraweera et al. 1995; Justé et al. 2008b).

In order to prevent or control such microflora-related problems during thick juice storage, timely detection, and accurate identification of all potential spoiling contaminants is required as well as a better understanding of the microbial dynamics during thick juice storage. This microbiological information could be used by the sugar industry to determine which tanks will be emptied first. In case of problematic microbiological results, a fast evaporation of the specified tank will follow to minimize the economic losses.

Currently, DNA array technology is the most suitable technique for rapid detection and identification of several targets in a single assay (Lievens et al. 2005a; Justé et al. 2008c), even when different species can only be discriminated by a single-nucleotide polymorphism in the target gene (Lievens et al. 2006). Since hybridization signals are proportional to the amount of target DNA (Lievens et al. 2005b), this technology has the potential to allow the study of target microbe dynamics (Lievens et al. 2007). With this technique, specific detector oligonucleotides are immobilized on a solid support and used for target microorganism identification. Generally speaking, the target DNA is polymerase chain reaction (PCR)-amplified and simultaneously labeled using universal primers spanning a genomic region harboring target-specific sequences. Labeled amplicons are then hybridized to the array (Rudi et al. 2002; Volokhov et al. 2002; Lievens et al. 2003; Eom et al. 2007). As a result, DNA array technology combines nucleic acid amplification with the theoretically unlimited screening capacity of DNA arrays, resulting in high sensitivity, specificity and throughput capacity.

The main objective of this study was to design and develop a DNA array to specifically detect and identify the most prominent bacteria present during storage of thick juice and which may contribute to thick juice degradation, i.e. the genera *Bacillus*, *Kocuria*, *Staphylococcus* and *Tetragenococcus*, and the species *A. viridans*, *L. mesenteroides* and *T. halophilus*. The developed tool has been briefly announced previously (Justé et al. 2008d) and has now been validated using both pure cultures and industrial samples. In addition, we attempted to monitor the microbial populations in thick juice during storage and degradation using the DNA array.

Materials and Methods

Study Samples and DNA Extraction

A collection of 59 bacterial isolates was used in this study, encompassing the most prominent thick juice bacterial contaminants (Justé et al. 2008b) and their phylogenetic relatives (Table 1). All isolates were aerobically cultured on Tryptone Soya Agar (TSA) at 30 or 37 °C. Genomic DNA was extracted from 5-day-old cultures as described previously (Lievens et al. 2003). When thick juice samples were investigated, genomic DNA was extracted from 20 mL filtrated samples according to the same protocol and diluted 1:10. DNA yield and purity was determined spectrophotometrically.

Design of Target-specific Oligonucleotides

Specific oligonucleotides were developed from the 16S rRNA gene to identify the most relevant thick juice contaminants, including the genera *Bacillus*, *Kocuria*, *Staphylococcus* and *Tetragenococcus* and the species *A. viridans*, *L. mesenteroides* and *T. halophilus*. 16S rRNA gene sequences from the target species, as well as from their closest phylogenetic relatives, were retrieved from GenBank. The design of the genus-specific oligonucleotides was based on at least eight sequences of phylogenetic divergent species belonging to the target genus (Takahashi et al. 1999; Xu and Côté 2003), and at least two sequences from strains belonging to a phylogenetically related genus. Species specific oligonucleotides were designed based on all available sequences of the target species in GenBank and a set of closely related sequences. Following sequence alignment, unique polymorphisms were localized manually and used to design target-specific oligonucleotides. The length of these oligonucleotides was adjusted to obtain detector sequences with a similar melting temperature of 60 °C±4 °C as calculated using Wallace's rule. Since all oligonucleotides are hybridized at a fixed temperature of 54 °C, differences in melting temperature may have an impact on the sensitivity and/or specificity of the oligonucleotide-based assay. Oligonucleotide sequences were submitted for a BLAST search against the GenBank database to determine their in silico specificity. Suitable sequences were selected for further experiments. In addition to the target-specific oligonucleotides, a 3′-digoxigenin-labeled control oligonucleotide (Dig1) with no homology to a known sequence was used as a reference for detection (Lievens et al. 2003). Furthermore, two oligonucleotides (O341F and O516R) based on the

Table 1 Bacterial isolates used in this study

Species	Isolate	Species	Isolate
Aerococcus christensenii	LMG 19525^T	*Leuconostoc mesenteroides* subsp. *dextranicum*	LMG 6908^T
Aerococcus urinae	LMG 19526	*Leuconostoc pseudomesenteroides*	LMG 11482^T
Aerococcus urinaequi	LMG 13989^T	*Listeria ivanovii*	LMG 11388^T
Aerococcus viridans	LMG 17931^T	*Macrococcus hajeki*	LMG 21711^T
Aerococcus viridans	A82	*Micrococccus luteus*	LMG 4050^T
Arthrobacter globiformis	LMG 3813	*Micrococccus luteus*	B326
Bacillus sp.	Z78	*Micrococccus lylae*	LMG 14192
Bacillus cereus	LMG 2098	*Pediococcus parvulus*	LMG 11486^T
Bacillus cereus	B156	*Pediococcus pentosaceus*	LMG 11488^T
Bacillus flexus	LMG 11155^T	*Pseudomonas* sp.	B384
Bacillus flexus	B236	*Pseudomonas syringae*	LMG 5046
Carnobacterium divergens	LMG 9199^T	*Shewanella baltica*	LMG 2250^T
Enterococcus faecalis	LMG 19434^T	*Staphylococcus* sp.	A27
Kocuria kristinae	LMG 14216	*Staphylococcus epidermis*	LMG 10273
Kocuria palustris	LMG 19167^T	*Staphylococcus equorum*	LMG 19116
Kocuria rhizophila	LMG 8816	*Staphylococcus lugdunensis*	LMG 13346^T
Kocuria rosea	LMG 14226	*Staphylococcus saprophyticus*	LMG 13350^T
Kocuria varians	LMG 14232	*Staphylococcus saprophyticus*	B328
Lactobacillus casei	LMG 6904^T	*Staphylococcus xylosus*	LMG 20217^T
Lactobacillus delbrueckii	LMG 6412^T	*Staphylococcus xylosus*	A30
Lactobacillus delbrueckii subsp. *lactis*	LMG 7942^T	*Streptococcus gallolyticus* subsp. *macedonicus*	LMG 15061
Lactobacillus iners	LMG 18914^T	*Tetragenococcus halophilus*	LMG 11490^T
Lactobacillus johnsonii	LMG 9437	*Tetragenococcus halophilus*	T2
Lactobacillus plantarum	LMG 9205	*Tetragenococcus halophilus*	T6
Lactococcus lactis	LMG 6890^T	*Tetragenococcus halophilus*	T9
Lactococcus raffinolactis	LMG 13095^T	*Tetragenococcus halophilus*	T14/ IAM 1674
Leuconostoc lactis	LMG 8894^T	*Tetragenococcus halophilus*	T19/ JCM 2015
Leuconostoc mesenteroides	B208	*Tetragenococcus muriaticus*	LMG 18498^T
Leuconostoc mesenteroides subsp. *cremoris*	LMG 6909^T	*Tetragenococcus solitarius*	LMG 12890^T
		Trichococcus flocculiformis	DSMZ 2094^T

LMG Laboratory of Microbiology Ghent, Belgian Coordinated Collection of Microorganisms, Ghent, Belgium; *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; *IAM* Institute of Applied Microbiology, The university of Tokyo, Tokyo, Japan; *JCM* Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan; *A-, B-, T-and Z-numbers* bacteria from own experiments [Justé 2008; Justé et al. 2008a]

universal bacterial primers 341F and 516R (Muyzer et al. 1996) used to detect the presence of any prokaryotic DNA were designed as a control for the amplification and hybridization. All oligonucleotides, with the exception of Dig1, were synthesized with a 5′-C6-amino linker for covalent binding to a nylon membrane. As 3′-digoxigenin-labeled oligonucleotides cannot be synthesized with this linker, Dig1 was labeled with a 5′-C6-thiol linker.

DNA Array Production

A comprehensive DNA array was produced as described previously (Lievens et al. 2003; Fessehaie et al. 2003). Briefly, the selected oligonucleotides were diluted in sodium bicarbonate buffer containing 0.004% bromophenol blue as a tracking dye and drawn into wells of a 384-well microtiter plate (two wells for each oligonucleotide) according to a predesigned array template (Fig. 1a). Oligonucleotides were then spotted on Immunodyne ABC membrane strips (PALL Europe Limited, Portsmouth, UK) using a 384-pin replicator (V & P Scientific, San Diego, CA, USA) at an amount of 8.0 fmol per spot. For the reference oligonucleotide Dig1, 2.0 fmol was printed. Membranes were air dried, blocked for 30 min at room temperature, again air dried, vacuum-packaged and stored at room temperature until use.

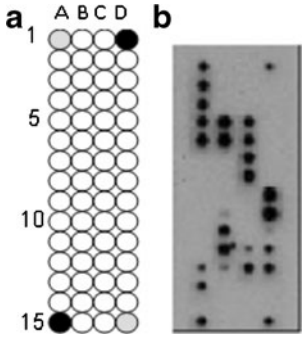

Fig. 1 **a** Representation of the developed DNA array. PCR-labeled amplicons hybridize to genus-specific oligonucleotides for *Staphylococcus* spp. (A2−5, C4−7), *Tetragenococcus* spp. (B3−4, D8−9), *Bacillus* spp. (A6−8, C8−10) and *Kocuria* spp. (B13−14, C2−3) and species-specific oligonucleotides for *Tetragenococcus halophilus* (B9−11, D10−12), *Aerococcus viridans* (B6−8, D2−4) and *Leuconostoc mesenteroides* (B1−3, D5−7). In addition to the immobilized target-specific oligonucleotides, the DNA array contains control oligonucleotides for the hybridization (*grey spots*) and a reference for detection (*black spots*). Based on the location of the signals, identification is performed. An example of an analyzed sample is presented in **b**, in which *Staphylococcus* spp., *Tetragenococcus* spp. and *T. halophilus* are detected

PCR Amplification, Labeling and Hybridization

The target 16S rDNA was amplified and simultaneously labeled with alkaline-labile digoxigenin using the eubacterial universal primers 8F and 1492R (Lane 1991). Samples were amplified in a reaction volume of 20 µL, containing a 0.15 mM digoxigenin-11-d-UTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM of each primer, 1 unit Titanium *Taq* DNA polymerase, 1× Titanium *Taq* PCR buffer (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA (5 ng genomic DNA from a pure culture or 1-15 ng from thick juice samples). Before amplification, DNA samples were denatured at 94 °C for 2 min. Next, 30 cycles were run consisting of 45 s at 94 °C, 45 s at 59 °C, and 45 s at 72 °C, with a final extension at 72 °C for 10 min. Hybridization, washing and detection were performed as described previously (Lievens et al. 2003). Hybridization signals were quantified and analyzed using Labworks 4.0 Image Acquisition and Analysis Software. Hybridization signal strength was reported as integrated optical density. All hybridizations were carried out at least twice.

Validation of the DNA Array

Specificity of the array was evaluated using labeled amplicons from all strains listed in Table 1. In addition, the array was validated using two industrial (non-degraded) thick juice samples taken at different stages during storage, including one sample taken right after production (I195) and one sample taken after seven months of storage (I76). Samples were homogenized and subsamples were used for DNA extraction (20 mL) and classical microbiological plating as described previously (Justé et al. 2008e). DNA samples were subjected to DNA array analysis and used for specific PCR detection of the selected target bacteria (Table 2). Recovered colonies on plate were identified by phenotypic characterization and specific PCR assays or 16S rDNA sequencing as described in Justé et al. (2008e)

Monitoring Thick Juice Bacterial Contaminants During Storage and Degradation

In an attempt to monitor the dynamics of the different microorganisms that occur during thick juice storage and degradation, long-term thick juice storage experiments were conducted using laboratory scale reactors (500 mL). Experiments were performed using thick juice samples from five different sugar refineries from three countries, which were collected either right after production or after 7 months of industrial storage (Table 3). Thick juice samples were stored aerobically at 30 °C for 185 days. At regular time intervals, 20 mL thick juice samples were analyzed for pH and the concentrations of lactic acid and reducing sugars. Genomic DNA was extracted simultaneously for DNA array analysis. Specific PCR assays (Table 2) and classical plating was used as reference methods.

Stability of DNA in Thick Juice

In order to check the stability of microbial DNA in sugar thick juice, the stability of DNA from heat-killed bacteria was monitored in the presence of different, living bacteria. First, the time needed to obtain dead bacterial cells with intact DNA was determined. Saturated Tryptone Soya Broth cultures of *Staphylococcus lugdunensis* were heated in a 60 °C warm water bath for a period of 0-120 min. Every 10-30 min, viability was checked with classical plating on TSA (incubation at 30 °C). In the case no colonies could be detected, the cultures were incubated for an additional 5 days at 30 °C to allow resuscitation, and again classical plating was performed. DNA stability was determined using the PCR assay for *Staphylococcus* species (Table 2). Heat-killed cells of *S. lugdunensis* with intact (16S r)DNA were obtained after 60-min heating at 60 °C.

10^7 cfu/mL killed *S. lugdunensis* cells were then added to 500 mL of autoclaved thick juice which was subsequently inoculated with 10^6 cfu/mL *T. halophilus* to ascertain bacterial activity in the sample (three replicates). A fourth thick juice sample only contained dead cells from *S. lugdunensis*. The samples were aerobically stored for 42 days at 30 °C. The juices were regularly sampled and analyzed with both classical microbiological counts (TSA, incubation at 30 °C) and a specific PCR assay for *Staphylococcus* species (Table 2).

Table 2 16S rDNA primers, with their corresponding sensitivity, developed for specific identification of the studied thick juice contaminants

Code	Target	Sequence (5′-3′)	Product length (bp)	Sensitivity (pg DNA)[a]	T_a (°C)
Av 5F Av 9R	*Aerococcus viridans*	CTTGAGTACAGAAGAGGAAT CCGTGGCGTGCTGATCCA	700	1	59
Ba 1F Ba 5R	*Bacillus* spp.	GGATAACTCCGGGAAACC TTTCACATCAGACTTAAG	452	10[b]	59
Koc 1F Koc 4R	*Kocuria* spp.	TGGTTKTGGATGGGCTCA GTCCGGAATTATTGGGCG	331	<1–40[c]	59
Lm 2F Lm 6R	*Leuconostoc mesenteroides*	AATACCGAATAAAACTTAGTG TTGTAGTCTGCAACTCGA	1176	1	59
St 9F St 8R	*Staphylococcus* spp.	TYAGGGAAGAACAWAYGT AATCCGAACTGAGAACAA	840	<1–40[d]	62
198F 480R	*Tetragenococcus halophilus*	AGCTCAAAGGCGCTTTAC TTCTGGTCAGCTACCGTC	282	0.01	62

F forward primer, *R* reverse primer, T_a annealing temperature used in the PCR program described in the "Materials and Methods" section.

[a] The sensitivity of a genus PCR can fluctuate between different species

[b] The presented sensitivity was determined with *B. cereus* and *B. cohnii* (10 pg)

[c] *K. kristina* (<1 pg), *K. rhizophila* (10 pg), and *K. palustris* (40 pg)

[d] *S. epidermis* (<1 pg), *S. cohnii* (<1 pg) and *S. equorum* (40 pg)

Results and Discussion

Design and Evaluation of the DNA Array

Based on the 16S rRNA gene, 68 oligonucleotides were designed and tested in a first hybridization experiment using labeled amplicons from a limited set of target and non-target species. Out of these 68 detector oligonucleotides, 20 oligonucleotides performed well under the hybridization conditions used, resulting in high specificity and sensitivity (Tables 4 and 5). In order to limit the number of oligonucleotides targeting nearly the same site of the 16S rRNA gene, 19 detectors (omitting OSta4) were selected for further experiments. There are several possible explanations why only 31% of the manually designed oligonucleotides performed well under real hybridization conditions. Firstly, the detection limit of an oligonucleotide is dependent on several factors and is, so far, not predictable. Single nucleotide shifts in the target site of the oligonucleotides may, for example, result in an altered sensitivity (Lievens et al. 2003). This may be explained by the formation of amplicon secondary structures preventing target hybridization (Lane et al. 2004). Secondly, although oligonucleotides can theoretically be designed to discriminate single-nucleotide polymorphisms when accounting for specific criteria such as the position of the mismatch and the sequence and length of the oligonucleotide (Lievens et al. 2006), so far no rules are available that exclude potential cross-reactivity with the designed oligonucleotides. Finally, sequences in GenBank do not necessarily represent all strains of a given species, certainly not when species in extreme environments are studied, and may lead to negative results when the oligonucleotides were derived from GenBank sequences only. Therefore, the suitability of developed oligonucleotides can only be evaluated by practical testing in the laboratory.

The 19 selected oligonucleotides included nine detectors for species identification and 10 oligonucleotides for genus identification (two to three oligonucleotides per target). *In silico* analysis using all available target sequences from

Table 3 Industrial thick juice samples used to evaluate the monitoring capacity of the DNA array for thick juice contaminants

Sampling date	Factory	Country	Sample	Description
14/07/2006	Factory 1	Belgium	I65	Degraded thick juice after 7 months of storage
14/07/2006	Factory 1	Belgium	I66	Non-degraded thick juice after 7 months of storage
14/07/2006	Factory 1	Belgium	I67	Non-degraded thick juice after 7 months of storage
19/10/2005	Factory 6	Germany	I68	Freshly produced thick juice
20/10/2005	Factory 10	France	I69	Freshly produced thick juice
20/10/2005	Factory 12	France	I70	Freshly produced thick juice
19/10/2005	Factory 3	Germany	I71	Freshly produced thick juice

Table 4 Sequences and sensitivity of the selected 16S rDNA detector oligonucleotides

Code	Specificity	Sensitivity (pg DNA)	Sequence (5′–3′)	Targeting site in the 16S rRNA gene[a]	Length	Tm (°C)[b]	GC (%)	Location on the DNA array
OAv 3	*Aerococcus viridans/urinaequi*	10–100[c]	AGGAATGTGGAACTCCATGT	665-684	20	58	45	B7/D3
OAv 4	*A.viridans/urinaequi*	10–100[c]	TAAGCGGGGGATAACATTCG	131-150	20	60	50	B8/D4
OAv7	*A. viridans/urinaequi*	1[c]	CGTGGATCAGCACGCCACG	1352-1370	19	64	68	B9/D5
OBa7	*Bacillus* spp.	1–10[d]	CTAAGGTGACTGCCGGTG	1110-1137	18	58	61	A6/C8
OBa9	*Bacillus* spp.	1–10[d]	GCGCAACCCTTGATCTTAGT	1162-1181	20	60	50	A7/C9
OBa11	*Bacillus* spp.	1–10[d]	ACCCTTGATCTTAGTTGCCA	1167-1186	20	58	45	A9/D13
OKoc5	*Kocuria* spp.	10–100[e]	TGGATGGGCTCACGGCCTAT	220-239	20	64	60	B13/C2
OKoc6	*Kocuria* spp.	10–100[e]	GATGGATGGGCTCACGGCCT	218-237	20	64	60	B14/C3
OLeu1	*Leuconsotoc mesenteroides*	10[f]	CTGGACTGCAACTGACGTTG	709-728	20	62	55	B1/D5
OLeu2	*L. mesenteroides*	10[f]	TTACTG GACTGCAACTGACG	706-725	20	60	50	B2/D6
OLeu8	*L. mesenteroides*	10[f]	TGGGAAGAACAG CTAGAATA	444-463	20	56	40	B3/D7
OSta4	*Staphylococcus* spp.	1–10[g]	GCACTCTARGTTGACTGCCG	1146-1165	20	62	55	A2/C4
OSta5	*Staphylococcus* spp.	1–10[g]	CTCTARGTTGACTGCCGGTG	1149-1168	20	62	55	A3/C5
OSta6	*Staphylococcus* spp.	1[g]	TGGGCACTCTARGTTGACTG	1143-1162	20	60	50	A4/C6
OSta7	*Staphylococcus* spp.	1[g]	CATAAAGTTGTTCTCAGTTCG	1286-1306	21	58	38	A5/C7
OTH1	*Tetragenococcus halophilus*	10[h]	AGCTCAAAGGCGCTTTACA	202-220	19	56	47	B10/D11
OTH2	*T. halophilus*	10[h]	AAGCTCAAAGGCGCTTTACA	201-220	20	58	45	B11/D12
OTH23	*T. halophilus*	1[h]	ACAGGAGAAAGAGGAAATGC	457-476	20	58	45	B9/D10
OTH13	*Tetragenococcus* spp.	0.1-1[i]	GCTTTCTGGTCAGCTACCGT	487-496	20	62	55	B4/D8
OTH14	*Tetragenococcus* spp.	0.1–1[i]	CTTTCTGGTCAGCTACCGTC	486-495	20	62	55	B5/D9
Dig1[k]	None		GTCCAGACAGGATCAGGATTG		21	64	52	A1/D14
O341F	All bacteria	0.1[j]	CCTACGGGAGGCAGCAG	346-362	17	58	71	A12/C11
O516R	All bacteria	0.1[j]	TGCCAGCAGCCGCGGTA	521-537	17	58	71	A13/C12

Sensitivity was determined using 10 μL PCR product

[a] The targeting site was determined using a type strain sequence, starting from primer 8F (AGAGTTTGATCCTGGCTCAG)

[b] T_m calculated using Wallace's rule

[c] *Aerococcus viridans* LMG 17931 and *A. viridans* A82

[d] *Bacillus* sp. Z78, *B. cereus* LMG2098, and *B. flexus* LMG 11155

[e] *Kocuria kristina* LMG 14216 and *K. rhizophila* LMG 8816

[f] *Leuconostoc mesenteroides* subsp. *dextranicum* LMG 6908

[g] *Staphylococcus epidermis* LMG 10273, *S. equorum* LMG 19116, and *S. lugdunensis* LMG 13346

[h] *Tetragenococcus halophilus* LMG 11490 and *T. halophilus* JCM 2015

[i] *Tetragenococcus halophilus* LMG 11490, *T. muriaticus* LMG 18498, and *T. solitarius* LMG 12890

[j] All bacteria listed in Table 1

[k] Lievens et al. [2003]

GenBank (2010-04) revealed that the oligonucleotides for *A. viridans*, *L. mesenteroides* and *T. halophilus* matched perfectly with all retrieved GenBank sequences. Using the *A. viridans* oligonucleotides, *A. viridans* could not be distinguished from *A. urinaeequi* because of the lack of sequence variation in the 16S rRNA gene (99.9% homology). For the genera *Tetragenococcus* and *Kocuria*, harboring five and 14 species, respectively, all species should be covered by the developed oligonucleotides as all GenBank sequences representing the different species of these genera perfectly matched to the oligonucleotides. However, since the genera *Bacillus* and *Staphylococcus* include about 230 and 49 different species respectively (GenBank Taxonomy Browser), design of oligonucleotides covering the whole genus may be more challenging. Therefore, for both genera, a set of 20 16S rDNA sequences from species belonging to phylogenetic highly divergent groups (Justé et al. 2008d; Takahashi et al. 1999) was retrieved from GenBank and *in silico* tested for hybridization with the developed oligonucleotides. For *Bacillus*, the selected oligonucleotides generally showed a perfect match with the tested GenBank sequences. However, one sequence, representing the species *B. subtilis* (GenBank Accession N° EF423592), only perfectly matched with oligonucleotide OBa7. Another sequence, representing *B. cohnii* (GenBank Accession N° X76437), did not match with any of the three *Bacillus* oligonucleotides. Nevertheless, as these sequences display only one mismatch to the oligonucleotides, hybridization to these oligonucleotides may still occur (Lievens et al. 2006). With regard to the oligonucleotides for *Staphylococcus* species, all tested sequences except

Table 5 Hybridization results[a] of digoxigenin-labeled PCR amplicons from selected bacterial cultures to the DNA array

		Oligonucleotides to detect																						
		Aerococcus viridans			*Bacillus* spp.			*Kocuria* spp.		*Leuconostoc*	*mesenteroides*	*Staphylococcus* spp.			*Tetragenococcus*	*halophilus*		*Tetragenococcus* spp.		Universal		Control
Bacterial species	Isolate	OAv3	OAv4	OAv7	OBa7	OBa9	OBa11	OKoc5	OKoc6	OLeu1	OLeu2	OLeu8	OSta5	OSta6	OSta7	OTH1	OTH2	OTH23	OTH13	OTH14	0341F	0516R	Dig1
Aerococcus christensenii	LMG 19525																				○	■	■
Aerococcus urinae	LMG 19526																				■	○	■
Aerococcus urinaequi	LMG 13989	■	■	■																	○	■	■
Aerococcus viridans	LMG 17931	■	■	■																	■	■	■
Aerococcus viridans	A82	■	■	■																	■	■	■
Arthrobacter globiformis	LMG 3813																○				○	○	■
Bacillus cereus	LMG 2098				■	■	■														■	■	■
Bacillus flexus	LMG 11155				■	■	■							○							■	■	■
Bacillus flexus	B236				■	■	■							○							■	■	■
Bacillus sp.	Z78				■	■	■														○	○	■
Carnobacterium divergens	LMG 9199																				○	■	■
Enterococcus faecalis	LMG 19433																				○	■	■
Kocuria kristinae	LMG 14216							○	○												○	○	■
Kocuria palustris	LMG 19167							■	■												○	○	■
Kocuria rhizophila	LMG 8816							■	○												○	○	■
Kocuria rosea	LMG 14226							■	■												○	○	■
Kocuria varians	LMG 14232							■	■												○	○	■
Lactococcus lactis	LMG 6890																				■	■	■
Lactobacillus plantarum	LMG 9205																				■	■	■
Lactobacillus raffinolactis	LMG 13095																				○	■	■
Leuconostoc lactis	LMG 8894																○					■	■
Leuconostoc mesenteroides subsp. *dextranicum*	LMG 6908									■	■	○									○	■	■
Leuconostoc mesenteroides subsp. *cremoris*	LMG 6909									■	■	○				○	○				○	○	■
Leuconostoc pseudomesenteroides	LMG 11482											■										○	■
Listeria ivanovii	LMG 19119																				■	■	■
Macrococcus hajeki	LMG 21711																				■	■	■
Micrococcus luteus	LMG 4050													○							■	■	■
Micrococcus lylae	LMG 14192																				○	○	■
Pediococcus parvulus	LMG 11486																				○	○	■
Pediococcus pentosaceus	LMG 11488																				○	○	■
Staphylococcus epidermis	LMG 10273												■	■	■						■	■	■
Staphylococcus equorum	LMG 19116												■	■	■						○	○	■
Staphylococcus lugdunensis	LMG 13346												■	■	■						■	■	■
Shewanella baltica	LMG 2250																				○	■	■
Streptococcus gallolyticus subsp. *macedonicus*	LMG 15061																				■	■	■
Tetragenococcus halophilus	LMG 11490															○	○	■	■	■	○	■	■
Tetragenococcus halophilus	T2															■	■	■	■	■	■	■	■
Tetragenococcus solitarius	LMG 12890																		■	■	■	■	■
Tetragenococcus muriaticus	LMG 18498																		■	■	○	■	■

Specificity was determined with 10 µL PCR product

[a] Hybridization strength is measured using the integrated optical density (IOD) and classified into three categories: blank = no signal (IOD < 1000); ○ = weak signal (IOD > 1000); ■ = strong signal (IOD > 5000).

one (*S. caseolyticus*, GenBank Accession N° D83359 having up to four mismatches) perfectly matched with the designed *Staphylococcus* oligonucleotides. Specificity of the selected detector oligonucleotides was tested using amplicons from all strains listed in Table 1. While most of the designed oligonucleotides were highly specific, some amplicons from closely related species showed a cross reaction (Table 5), e.g. with oligonucleotides OAv3, OAv4, OAv7 which were designed for the identification *of A. viridans* and with OSta6 and OTH1 and OTH2 which were designed for identification of *Staphylococcus* and *T. halophilus* strains, respectively. These experiments used a high amount of about 85 ng amplicons/ml hybridization buffer, which is unlikely to be PCR-generated from an environmental DNA extract. As

Table 6 Validation of the DNA array using two industrial samples, including one sample obtained from thick juice after seven months of storage (I76) and one sample from freshly produced thick juice (I195)

A. Hybridization results[a]

	Oligonucleotides to detect																						
	Aerococcus viridans			*Bacillus* spp.			*Kocuria* spp.		*Leuconsotoc mesenteroides*			*Staphylococcus* spp.			*Tetragenococcus halophilus*			*Tetragenococcus* spp.			Universal	Control
Sample	OAv3	OAv4	OAv7	OBa7	OBa9	OBa11	OKoc5	OKoc6	OLeu1	OLeu2	OLeu8	OSta5	OSta6	OSta7	OTH1	OTH2	OTH23	OTH13	OTH14	0341F	0516R	Dig1
I76												■	■	■	■	■	○	■	■	■	■	■
I195	○	○	■						○	■	■	○	■	■						■	■	■

Results were obtained with 20 µL PCR product

[a] Hybridization strength is measured using the integrated optical density (IOD) and classified into three categories: blank = no signal (IOD < 1000); ○ = weak signal (IOD > 1000); ■ = strong signal (IOD > 5000).

B. Results of specific PCR analyses[b]

	Specific PCR to identify					
	Aerococcus viridans	*Bacillus* spp.	*Kocuria* spp.	*Leuconostoc mesenteroides*	*Staphylococcus* spp.	*Tetragenococcus halophilus*
I76		+			++	++
I195	++			++	++	+

[b] PCR yield following gel electrophoresis is classified into three categories: blank = no signal; + = weak signal  ; ++ = strong signal 

cross-hybridization significantly diminishes at lower amplicon amounts (Lievens et al. 2006), these weak non-specific hybridizations are probably not relevant in practice. Furthermore, accuracy of the assay should be guaranteed by the use of multiple oligonucleotides per target species or genus.

In order to determine the detection limit of the DNA array, a DNA dilution series ranging from 1 ng to 0.1 pg was made for several target strains prior to PCR amplification. While the detection limit of the species-specific oligonucleotides was established for one strain, the detection limit of the genus-specific oligonucleotides was determined using two or three strains representing different species of the genus. In general, the detection limit of the array depended on the detector sequences used as different oligonucleotides showed different hybridization signals for a single test strain (Table 4). Oligonucleotides targeting nearly the same 16S rDNA site appeared to have comparable sensitivity. In addition, the data obtained in this study indicate that the detection limit of a genus-specific oligonucleotide is determined by the species that is detected, as demonstrated for example by the different detection limit of *Bacillus cereus* (10 pg DNA) and *B. flexus* (1 pg DNA) (Table 4). For the *Bacillus* and *Staphylococcus* species, generally the detection limit was 1 pg DNA, which is comparable with other molecular detection techniques (Lievens et al. 2003; Weller et al. 2000; Ercolini 2004; Ercolini et al. 2004; Szemes et al. 2005). *A. viridans* was detected up to 1 pg DNA. For *Tetragenococcus* species, a signal could still be observed in the last dilution, representing as little as 0.1 pg DNA (Table 4). In contrast to these relatively sensitive oligonucleotides, the oligonucleotides for *Kocuria* species appeared to be less sensitive (detection limit in the range of 10 to 100 pg DNA) (Table 4). In addition to the oligonucleotides,

the detection limit of the assay may be affected by the primers used for PCR amplification. Indeed, a comparison between the sensitivity of the assay when amplicons were generated with the primers 8F and 1387R (Marchesi et al. 1998) and 8F and 1492R revealed that the assay based on the latter combination appeared to be (10 to 100 times) more sensitive for almost all bacteria tested (data not shown). Since the sensitivity of a universal primer-based analysis may also be decreased by competition for PCR reagents between target and non-target DNA amplified by the same primer pair (Lievens et al. 2005b), the target DNA dilutions were also amplified in the presence of an excess non-target DNA and subsequently hybridized to the array. Similar to Lievens et al. (Lievens et al. 2005b), a 100-1,000-fold excess of non-target DNA influenced the sensitivity of the assay and diminished the detection limit with a factor of 10. In all other cases, sensitivity was not affected (data not shown).

Validation of the DNA Array Using Industrial Samples

For broad application in practice, identification from pure cultures is not very relevant as the array should preferably be used to assess the microorganisms directly from industrial samples. Therefore, the array was validated using two industrial samples, including sample I76 (seven months stored thick juice) and I195 (freshly produced thick juice). In both samples *Staphylococcus* species were found using the array (Table 6). Furthermore, *L. mesenteroides* and *A. viridans* were detected in the freshly produced thick juice I195, while these species were not detected in the stored thick juice (I76; Table 6). These results corroborate with earlier findings that freshly produced thick juice harbors a more diverse microflora than stored thick juice (Justé et al. 2008c). Generally, the results obtained with the DNA array were confirmed by standard plating methods and phenotypic characterization. In addition, the bacterial identity of the obtained cultures was confirmed by sequencing the 16S rRNA gene or by specific PCR, demonstrating the reliability of the array. When comparing the DNA array results with the results obtained by a direct PCR on DNA extracted from the thick juice samples, similar results were obtained (Table 6). However, in contrast to the DNA array analysis, *Bacillus* spp. and *T. halophilus* were detected by the specific PCR assays in sample I76 and I195, respectively. For both assays, a weak band was generated following gel electrophoresis. This discrepancy between both assays could be explained by a difference in specificity since the primers and oligonucleotides target different regions and thus different sequences. A more plausible explanation is the higher sensitivity that can be obtained with specific PCR assays as these only amplifies the target DNA, whereas the universal primers used for DNA array analysis also amplify DNA from non-target bacteria having annealing sites for these primers. However, the major advantage of the DNA array is that multiple species can be efficiently detected in a single assay. In addition, compared with a conventional PCR-based detection, interpretation of DNA array results is more straightforward since the formation of non-specific products may complicate detection using agarose gels. Furthermore, DNA arrays can relatively easily be extended to include more and other targets of interest (Lievens et al. 2005a). In this context, some thick juice samples from the last sugar beet campaign (2009) appeared systematically negative for *T. halophilus* with the specific PCR, although classical plating revealed the presence of micro-colonies, phenotypically identical to *T. halophilus*. These data suggest that another, or adapted microflora, might be important for thick juice degradation and therefore interesting to put on the membrane for fast detection.

Monitoring Thick Juice Bacterial Contaminants

Finally, the array was used to monitor thick juice contaminants during thick juice storage and degradation. Different thick juice samples were incubated on a laboratory scale and regularly screened for the presence of the selected bacteria during 185 days of storage (Table 7). An example of an outcome is presented in Fig. 1b. Screening was performed using the DNA array as well as the different PCR assays listed in Table 2 and classical plating. Classical plate counts for *T. halophilus* are presented in Table 7 as this bacterium was identified as the main cause for thick juice degradation (Justé et al. 2008a).

Samples which had been industrially stored (I65-I67), revealed a more diverse microflora than freshly produced thick juice (I68-I71; Table 7). Nevertheless, the samples I65-I67 all originate from one factory as well. Most diversity was observed for the degraded thick juice sample I65 (Table 7). At the start of the experiment (day 0), all target taxa including *Bacillus*, *Staphylococcus*, *Tetragenococcus*, *A. viridans* and *L. mesenteroides* were detected by the array in this sample. Compared with the other samples, the latter two species were only found in this sample. In the freshly produced thick juice samples from factories 10 and 12 (I69 and I70), hybridization signals were obtained for the *Tetragenococcus* and *T. halophilus* oligonucleotides. In the two other freshly produced samples originating from factory 3 and 6 (respectively, I71 and I68), none of the target bacteria were detected. Nevertheless, signals were obtained for the universal bacterial oligonucleotides (Table 7), indicating another bacterial flora might be present in these samples. Indeed, analysis of a 16S rDNA clone library of sample I71 revealed the presence of *Thermus*, *Acinetobacter*, *Anoxybacillus* and *Thermoanaerobacterium* strains (Justé

Table 7 Thick juice bacterial contaminants detected in different thick juice samples during 185 days of storage under laboratory conditions at 30 °C using the DNA array, specific PCR assays and classical microbiological plating

		DNA array[a]							Specific PCR[b]					Classical plating on TSA
Sample	Day of storage	*Aerococcus viridans*	*Bacillus* spp.	*Leucomostoc mesenteroides*	*Staphylococcus* spp.	*Tetragenococcus* spp.	*T. halophilus*	Universal	*Aerococcus viridans*	*Bacillus* spp.	*Staphylococcus* spp.	*T. halophilus*	Universal	
I65	0	■	○	○	■	■	○	○	++	++	++	+	+	Overgrown by *Bacillus* sp.
	11	■			■	■		○	++	++	++	+	+	2.7×10^5
	20	○			■	■		○	++	++	++	+	++	3.3×10^6
	29				○	■		○	++	++	++	+	++	4.4×10^6
	42	○			■	■		○	++	++	+	+	++	7.8×10^5
	60	○			■	■		○		+	++	+	++	2.0×10^7
	81	○			■	■		○		+	++	+	++	9.7×10^5
	185	○			■	■		○		++	++	+	++	9.4×10^4
I66	0				■	■	■	○		+	+	++	+	$3{,}5 \times 10^4$
	11					■	○	○		+	+	++	++	1.5×10^6
	20					■	■	○		+	+	++	++	1.8×10^6
	29					■	○	○		+	+	++	++	7.0×10^5
	42					■	■	○		+	+	++	++	1.4×10^6
	60				○	■	■	○		+	+	++	++	8.8×10^4
	81				○	■	■	■		+	+	++	++	1.4×10^5
	185				○	■	■	■		+	+	++	+	<100
I67	0				■	■	■	■		++	++	++	+	$4{,}8 \times 10^5$
	11				■	■	■	○		++	++	++	+	3.2×10^6
	20				■	■	■	■		++	++	++	++	3.6×10^6
	29				■	■	○	○		++	++	++	++	2.6×10^6
	42				■	■	■	■		++	++	++	++	3.8×10^6
	60			○	■	■	■	○		+	++	++	++	1.6×10^7
	81				■	■	■	■		+	++	++	++	1.3×10^6
	185				■	■	■	■		++	++	++	+	<100
I68	0							■					+	<100
	11					■	○	■				+	+	<100
	20					■		■					+	<100
	29							■					+	<100
	42							■					+	<100
	60							■					+	1.5×10^2
	81				○	○	○	■		+		+	+	<100
	185					○		■					+	<100
I69	0					■	○	■		+	+	++	++	1.2×10^6
	11					■	○	○		+	+	++	++	3.8×10^6
	20				■	■	○	■		+	+	++	++	3.5×10^5
	29				■	■	■	○		++	++	++	++	4.8×10^5
	42				■	■	■	○		++	++	++	++	9.8×10^5
	60				■	■	■	○		+	++	++	++	5.4×10^5
	81				■	■	■	■		+	++	++	++	5.6×10^5
	185				■	■	■	■		++	++	++	++	<100
I70	0					■	■	■		+	+	++	+	1.2×10^5
	11					■	○	○		+	+	++	++	8.8×10^5
	20					■	■	■		+	+	++	++	5.9×10^5
	29					■	○	○		+		++	++	1.2×10^6
	42					■	○	○		+		++	++	3.7×10^5
	60					■	■	○		+	+	++	++	4.1×10^5
	81				■	■	■	○		+	+	++	++	2.6×10^5
	185					■	■	○		+	+	++	++	9.5×10^2
I71	0							■		+			+	<100
	11						○	■		+		+	+	<100
	20					■		■		+		+	+	<100
	29							■		+			+	<100
	42							■		+			+	<100
	60					■	○	■		+		+	+	<100
	81					○	○	■		+		+	+	<100
	185							■					+	<100

Results were obtained with 20 µL PCR product

[a] Hybridization strength is measured using the integrated optical density (IOD) and classified into three categories: blank = no signal (<1000); ○ = weak signal (IOD > 1000); ■ = strong signal (IOD > 5000).

[b] PCR yield following gel electrophoresis is classified into three categories: blank = no signal; + = weak signal  ; ++ = strong signal 

et al. 2008b), for which no oligonucleotides were designed. However, *Bacillus* species were also found in the clone library, which were not detected by the array nor by the classical plating method. Remarkably, the sequences obtained did match in theory with all of our *Bacillus* oligonucleotides. On the contrary, using the specific PCR assay, *Bacillus* DNA was detected (Table 7). Also in the other samples, *Bacillus* spp. was detected using the PCR, again demonstrating a higher sensitivity for the specific PCR.

At the second sampling, at day 11, both I68 and I71 contained as well *T. halophilus*. Nevertheless, this bacterium could never be recovered on plate in these samples. In addition, the molecular data revealed a low concentration of *T. halophilus* during the whole period of storage, showing alternating positive and negative results during the period of storage, suggesting that the concentration is close to the detection limit. Remarkably, the bacterial community appeared relatively stable during thick juice storage. The species *Staphylococcus*, *Tetragenococcus* and *Bacillus* were present in all samples. *Aerococcus viridans* was only present in I65, but during the whole period of storage. *Leuconostoc mesenteroides* was only detected twice and in two different samples, more precisely I65 and I67. Surprisingly, high concentrations of *Tetragenococcus* spp. were detected on plate for I65, while the specific PCR for *T. halophilus* was only weakly positive. Moreover, the oligonucleotides for *Tetragenococcus* spp. were highly positive, while the species detectors were negative. A specific PCR on 20 colonies that were phenotypical identical to *T. halophilus* were all negative. The 16S rDNA sequence of a few isolates revealed the identity of a new species of *Tetragenococcus (Justé et al.* 2008c*)*, mostly relative to *T. muriaticus*, confirming the molecular results. The weak positive PCR for *T. halophilus* can be explained by a low target concentration, which could not be found on plate between the high concentrations of other *Tetragenococcus* spp. This was the first time that a *Tetragenococcus* species, different from *T. halophilus* was detected in thick juice. This industrial case of thick juice degradation by a new species of *Tetragenococcus* supports the relevance of the DNA array for thick juice analysis.

As shown in Table 7, this experiment resulted in a clear differentiation in microbial dynamics during storage of I68 and I71 and I69 and I70, all freshly produced thick juice (from campaign 2005-2006). Almost no bacteria could be counted in I68 and I71 during the whole period of 185 days storage and as expected, the pH was relatively stable and only few sucrose was lost (<0.4%). On the contrary, samples from tank 10 and 12 (I69 and I70) contained a high amount of *T. halophilus* during the experiment, even right after production. As expected, these samples degraded (decrease of pH) during storage and a considerable amount of sucrose up to 2.2% was lost. Remarkably, I68 and I71 originated from German sugar factories, while I69 and I70 were both samples from two French factories. Nevertheless, French freshly produced thick juice from the same refineries, from the next campaign (2006-2007), and produced according to the same process, showed the same low bacterial counts as the German samples from 2005-2006 and 2006-2007, excluding a difference in the production process as explanatory variable.

Notably, after 185 days of storage, a big discrepancy was observed between the molecular assays and classical plating for *T. halophilus*. While the microbial counts varied from 10^5 cfu/mL to below the detection limit (<100 cfu/mL), in all samples strong signals were obtained for *T. halophilus* using the molecular assays (Table 7), suggesting DNA from dead bacteria was detected.

Stability of DNA in Thick Juice

Since DNA based techniques cannot discriminate between dead and living microorganisms, the stability of DNA in the analyzed matrix is crucial. It is generally accepted that DNA from dead cells will be quickly metabolized by other microorganisms in microbiologically active environments, such as humid or aquatic environments (Lebuhn et al. 2004). However, the rate of DNA degradation in thick juice appeared to be extremely slow, as shown in Fig. 2. DNA from heat-killed cells of *S. lugdunensis* was found to be stable in thick juice for over 42 days, irrespective whether 10^6 cfu/mL living *T. halophilus* were present or not. Consequently, standard DNA applications in thick juice may overestimate certain species in the community limiting accurate monitoring. Indeed, matrices rich in easily degradable sugars like sugar thick juice could be expected to enhance stability of dead cells and their DNA. First of all, the activity of microorganisms in such extreme matrix is low (Rudi et al. 2005). Moreover, if sucrose can be used as carbon source, the proportion of the sucrose concentration compared to the concentration of ribose (as available in DNA) is enormous (about 10^8), favoring the use of sucrose. Despite this extreme high stability of DNA from dead cells in sugar thick juice, some samples from a previous thick juice storage experiment (Lane et al. 2004) (study about the

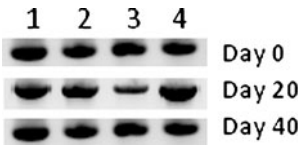

Fig. 2 Stability of DNA from 10^7 cfu/mL heat-killed *Staphylococcus lugdunensis* bacteria in autoclaved thick juice stored at 30 °C. In *1*, *2*, and *3*, living *T. halophilus* cells were additionally added to the sample at a concentration of 10^6 cfu/ mL; in *4* no additional cells were added

protective effect of hop extract during long term storage) were analyzed after about 300 days of storage and DNA from *T. halophilus* could not be detected, while it was present at day 150, suggesting the DNA will degrade eventually. In order to exclude detection of dead bacteria, addition of ethidium monoazide (Stark and Firestone 1995) or propidium monoazide (Nocker et al. 2006) should eliminate DNA from dead microorganisms. Alternatively, messenger RNA can be used as a target instead of DNA, in combination with reverse transcriptase PCR (Tan and Weis 1992).

In conclusion, our experiments illustrate the power of DNA arrays to simultaneously detect and identify thick juice contaminants, both from purified cultures and industrial samples. Efficient detection and identification of multiple contaminants such as *Bacillus*, *Staphylococcus* and *Tetragenococcus* species could be a major step forward in the quality control during thick juice storage. Knowledge of the bacterial load of stored thick juice is important to take fast and well-informed management decisions, like which tank should be processed first. In heavily degraded thick juice, production of bio-ethanol might be preferable to sugar crystallization. The developed technique is currently used to advise the sugar industry on the "microbial load" of their thick juice tanks right after production. Although it was found that different strains from the same species may have different thick juice-degrading capacity, the DNA array does not discriminate between such strains in its current format. Nevertheless, following characterization of the respective functional genes, the DNA array can likely be expanded with oligonucleotides to make this functional distinction. However, successful implementation of this technology, or any DNA-based method, in a monitoring program will require additional efforts to circumvent the high stability of DNA from dead bacteria in thick juice. Ultimately, correct interpretation of the hybridization patterns could fully aid food quality and processing management.

Acknowledgments We are grateful to the Südzucker company and the Lessius University College, Campus De Nayer for financing the PhD of A. Justé, and to the Tiense Suikerraffinaderij (Tienen, Belgium) and Südzucker (Germany) for supplying the thick juice and their committed cooperation. In addition, the authors thank Michael Waud for editing the manuscript.

References

Asadi M (2007) Beet-sugar handbook. John Wiley and Sons, Inc., Hoboken

Eom HS, Hwang BH, Kim DH, Lee IB, Kim YH, Cha HJ (2007) Multiple detection of food-borne pathogenic bacteria using 16S rDNA-based oligonucleotide signature chip. Biosens Bioelectron 22:845–853

Ercolini D (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Meth 56:297–314

Ercolini D, Mauriello G, Blaiotta G, Moschetti G, Coppola S (2004) PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. J Appl Microbiol 96:263–270

Fessehaie A, De Boer SH, Lévesque CA (2003) An oligonucleotide array for the identification and differentiation of bacteria pathogenic on potato. Phytopathology 93:262–269

Justé A (2008) Sugar thick juice degradation: study of the microbial community dynamics and control of the causative microflora during storage. Doctoral thesis, KULeuven. ISBN 978-8826-059-9

Justé A, Lievens B, Klingeberg M, Michiels CW, Marsh TL, Willems KA (2008a) Predominance of *Tetragenococcus halophilus* as the cause for sugar thick juice degradation. Food Microbiol 25:413–421

Justé A, Lievens B, Frans I, Klingeberg M, Michiels CW, Willems KA (2008b) Present knowledge of the bacterial microflora in the extreme environment of sugar thick juice. Food Microbiol 25:831–836

Justé A, Lievens B, Frans I, Klingeberg M, Marsh TL, Michiels CW, Willems KA (2008c) Genetic and physiological diversity of *Tetragenococcus halophilus* strains isolated from sugar- and salt-rich environments. Microbiology 154:2000–2010

Justé A, Lievens B, Frans I, Klingeberg M, Michiels CW, Marsh TL, Willems KA (2008d) Development of a DNA macroarray for monitoring microbial population dynamics during sugar thick juice storage. Commun Agric Appl Biol Sci 73:1–6

Justé A, Krause MS, Lievens B, Klingeberg M, Michiels CW, Willems KA (2008e) Protective effect of hop β-acids on microbial degradation of thick juice during storage. J Appl Microbiol 104:51–59

Lane DJ (1991) 16S and 23S rRNA sequencing, p 115-175. In: Stackebrandt E, Goodfellow M (ed) Nucleic acids techniques in bacterial systematics. Wiley, West Sussex, United Kingdom

Lane S, Evermann J, Loge F, Call DR (2004) Amplicon secondary structure prevents target hybridization to oligonucleotide microarrays. Biosens Bioelectron 20:728–735

Lebuhn M, Effenberger M, Garcés G, Gronauer A, Wilderer PA (2004) Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples. Water Sci Technol 50:263–270

Lievens B, Brouwer M, Vanachter ACRC, Lévesque CA, Cammue BPA, Thomma BPHJ (2003) Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiol Lett 223:113–122

Lievens B, Grauwet TJMA, Cammue BPA, Thomma BPHJ (2005a) Recent developments in diagnostics of plant pathogens: a review. Recent Res Devel Microbiol 9:57–79

Lievens B, Brouwer M, Vanachter ACRC, Lévesque CA, Cammue BPA, Thomma BPHJ (2005b) Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ Microbiol 7:1698–1710

Lievens B, Brouwer M, Vanachter ACRC, Lévesque CA, Cammue BPA, Thomma BPHJ (2006) Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis. FEMS Microbiol Lett 255:229–239

Lievens B, Claes L, Vanachter ACRC, Krause MS, Cammue BPA, Thomma BPHJ (2007) Assessing populations of a disease suppressive microorganism and plant pathogen using DNA arrays. Plant Sci 172:505–515

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes encoding for bacterial 16S rRNA. Appl Environ Microbiol 64:795–799

Muyzer G, Hottentrager S, Teske A, Wawer C (1996) Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA. A new molecular approach to analyze the genetic diversity of mixed microbial communities. In: Akkermans ADL, can Elsas JD, de

Bruijn FJ (eds), Molecular microbial ecology manual. Kluwer Academic Publishing, Dordrecht, 3.4.4.1-3.4.4.22

Nocker A, Cheung C-Y, Camper AK (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67:310–320

Rudi K, Flateland SL, Hanssen JF, Bengtsson G, Nissen H (2002) Development and evaluation of a 16S ribosomal DNA array-based approach for describing complex microbial communities in ready-to-eat vegetable salads packed in a modified atmosphere. Appl Environ Microbiol 68:1146–1156

Rudi K, Moen B, Drømtorp SM, Holck AL (2005) Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71:1018–1024

Samaraweera IS, Samaraweera U, Rheault DL, Mont RC (1995) Is L-lactic acid a true indicator of microbial infection? Int Sugar Journal 97:566–570

Sargent D, Briggs B, Spencer S (1997) Thick juice degradation during storage. Zuckerindustrie 122(8):615–621

Stark JM, Firestone MK (1995) Mechanisms for soil moisture effects on activity of nitrifying bacteria. Appl Environ Microbiol 61:218–221

Szemes M, Bonants P, de Weerdt M, Baner J, Landgren U, Schoen CD (2005) Diagnostic application of padlock probes—multiplex detection of plant pathogens using universal microarrays. Nucleic Acids Res 33:e70

Takahashi T, Satoh I, Kikuchi N (1999) Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int J Syst Bacteriol 49:725–728

Tan SS, Weis JH (1992) Development of a sensitive reverse transcriptase PCR assay, RT-PCR, utilizing rapid cycle times. PCR Meth Appl 2:137–143

Volokhov D, Rasooly A, Chumakov K, Chizhikov V (2002) Identification of Listeria species by microarray-based assay. Clin Microbiol 40:4720–4728

Weller SA, Elphinstone JG, Smith NC, Boonham L, Stead DE (2000) Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (Taqman) assay. Appl Environ Microbiol 66:2853–2858

Willems KA, Willems ML, Dardenne F, Klingeberg M, Michelberger T, Witte G (2003) Microbiological observations during storage of thick juice on a pilot and industrial scale. Proceedings CITS 2003, 22th General Assembly Madrid, Spain, May 18-21/2003

Xu D, Côté JC (2003) Phylogenetic relationships between Bacillus species and related genera inferred from comparison of 3′ end 16S rDNA and 5′ end 16S-23S ITS nucleotide sequences. Int J Syst Evol Microbiol 53:695–704