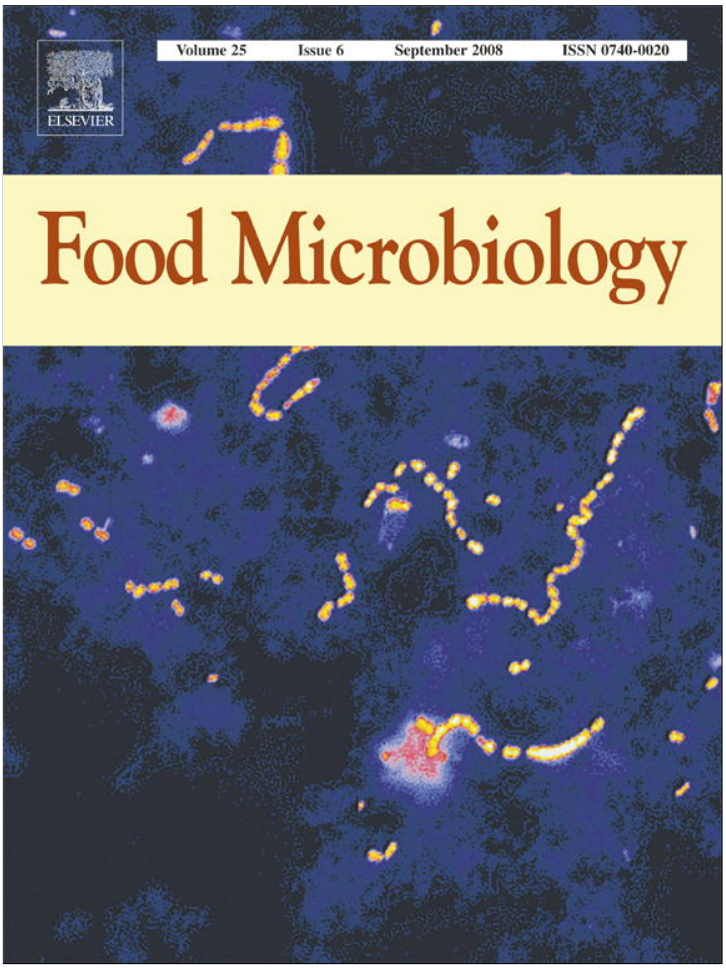

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Short communication

Present knowledge of the bacterial microflora in the extreme environment of sugar thick juice

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ABSTRACT

The diversity of the bacterial population in sugar thick juice, an intermediate product in the production of beet sugar, which exhibits an extreme, osmophilic environment with a water activity value (a_w) less than 0.86, was assessed with both culture-dependent and -independent 16S ribosomal RNA (rRNA) gene-based analyses. In comparison with previous studies, the number of different thick juice bacterial species increased from 29 to 72. Remarkably, a limited, gram-positive, culturable flora, encompassing species of *Bacillus, Staphylococcus* and mainly *Tetragenococcus* dominated thick juice during storage, while a more heterogeneous and unculturable fraction of *Acinetobacter*, *Sporolactobacillus* and *Thermus* species could be detected in freshly produced thick juice. Notably, almost all bacteria detected in the thick juice were also detected in the air, emphasising the importance of further investigation and assessment of strategies to reduce (air) contamination during processing and storage. The discovery of the contamination source may be used for the development of management strategies for thick juice degradation resulting from microbial activity.

1. Introduction

Sugar beet (*Beta vulgaris* L.) is an important crop cultivated widely in Europe. The beets are annual vegetables penetrating 25–40 cm into the ground and they are processed to crystal sugar in a sequence of operations, including washing and juice extraction, clarification, evaporation and crystallisation. Sugar thick juice is one of the intermediate products in this production of beet sugar, more precisely the concentrated juice after evaporation with a total soluble solids content of about 69 °Bx and a slightly alkaline pH of 9.0. Storing thick juice beyond extraction and refining has been commonly practiced by many sugar companies worldwide, since it was first introduced in 1960 in USA. However, even under good storage practices, thick juice degradation resulting from microbial contamination still occurs. The most pronounced symptoms of degradation are a reduction in pH from 9 to 5–6 and typically, an increase in reducing sugar content due to microbial growth (Sargent et al., 1997; Willems et al., 2003), resulting in financial loss.

Although numerous studies have reported on the microflora of sugar beets and the extraction juice (Hollaus and Klaushofer, 1973; Bugbee et al., 1975; Belamri et al., 1991), only few authors report the presence and growth of bacteria in the highly concentrated thick juice (ICMSF, 2005; Van der Poel et al., 1998; Willems et al., 2003). As thick juice has a sucrose content of 66–72 °Bx, corresponding with an a_w value of 0.85–0.80, only a limited bacterial microflora is expected to grow in this extreme environment (Grant, 2004). In the past, the knowledge about microbial growth in thick juice was limited to a few osmophilic yeasts and moulds (ICMSF, 2005). Nevertheless, the presence of bacteria in thick juice has recently been better documented. While in some studies, bacteria were only vaguely described as mesophiles (Sargent et al., 1997) or gram-positive cocci (Hein et al., 2002), other studies report good identifications to the species level (Van der Poel et al., 1998, Willems et al., 2003; Justé et al., 2008a, b). Until now, 29 different species have been described to occur in thick juice (Table 2).

In this article, an up to date overview of all bacteria isolated from sugar thick juice is presented, enlarging the knowledge of

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osmophilic thick juice microflora and revealing the unexpected tolerant character of some well-known bacteria.

2. Material and methods

2.1. Thick juice samples

Thick juice samples were collected during the sugar campaigns from 2004 till 2007. During these 4 years, representative thick juice samples from both industrial and pilot scale laboratory storage tanks (Justé et al., 2008a, b) were analysed including as well freshly produced thick juice, long-term-stored thick juice in good condition as degraded thick juice. Industrial samples originated from 14 different factories located in three different European countries, i.e. Belgium, France and Germany.

2.2. Microbiological plating

Thick juice was sampled weekly during storage and analysed using traditional plating methods. Table 1 presents the microbiological parameters analysed as well as the incubation conditions used, as described in detail by Willems et al. (2003). The term 'Fastidious Bacteria' (FB) is defined as a collective term for those bacteria that form colonies on Columbia agar with sheep blood (CAwSB, Oxoid Limited) after 3–6 days of incubation at 30 °C. In addition, 13 samples of both degraded and non-degraded thick juice were plated on CAwSB plates and incubated under aerobic, micro-aerophilic and anaerobic circumstances at 30 °C.

Colonies that appeared macro-morphologically different from others were purified by streak plating on tryptone soy agar (TSA).

2.3. DNA extraction

Genomic DNA was extracted from both pure colonies and 20-ml-thick juice samples using the phenol–chloroform extraction protocol described by Lievens et al. (2003). In case of colony extraction, only those colonies that appeared macro-morphologically different from others were used. Thick juice samples were selected from both freshly produced thick juice, having low classical plate counts, and from long-stored thick juice with high plate counts. DNA yield and purity was determined spectrophotometrically.

2.4. Sequencing of bacterial 16S rRNA genes and internal transcribed spacer (ITS) regions of yeasts

In order to determine the identity of the bacterial colonies, PCR amplification and sequencing of the 16S rRNA gene was performed. Amplification was performed as previously described (Justé et al., 2008a) using the universal primers 27f (AGAGTTTGATCCTGGCTCAG) or 516f (TGCCAGCAGCCGCGGTA) and 926r (CCGTCAATTCCTTTRAGTTT), 1492r (GGTTACCTTGTTACGACTT) or 1541r (AAGGAGGTGATCCAGCCGCA). For yeasts, target ITS regions between the small and the large subunit of the rRNA gene were amplified using the primers ITS1-F and ITS4 (Gardes and Bruns, 1993). Sequencing was done on purified PCR products using an Applied Biosystems 373A automated sequencer using the primers 27f and/or 926r or 1492r for the bacteria and the reverse primers ITS4 for the yeasts. Resulting sequences with $Q > Q_{20}$ were deposited in the EMBL/Genbank/DDBJ nucleotide sequence database under accession numbers EU637594–EU637646 and EU660421–EU660432. The presumptive identity was determined as the most homologous sequences obtained (>97% homology; Drancourt et al., 2000) by comparison to the GenBank database by using the BLAST program located at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997; Benson et al., 2004).

2.5. 16S rRNA gene clone libraries

Bacterial 16S rRNA gene clone libraries were created from DNA extracts from both freshly produced and long-stored thick juice samples, as described by Justé et al. (2008b) with primers 27f and 1492r. The PCR product was ligated into vector pCR2.1-TOPO with the TOPO T-A cloning kit (Invitrogen, Merelbeke, Belgium) following the manufacturer's protocol. At least 10 transformed colonies were randomly picked from each library and sequenced using primer M13f. Fragments detected in the clone library were identified as described above.

3. Results and discussion

All bacterial species detected are presented in Table 2, differentiating between the isolates detected with culture-based (C) and non-culture-based techniques (NC). The selection of the colonies was made macro-morphologically. This criterion was preferred since at the start of the experiments, a significant amount of macro-morphologically identical colonies were identified based on 16S rDNA sequencing and appeared to have (presumptive) identical identities. Consequently, only those colonies that appeared macro-morphologically different from others were analysed in further experiments. In total, 53 different species were identified in this study, of which 39 were isolated using plating and 21 were detected straight from thick juice samples. Seven species were detected with both approaches, including *Tetragenococcus halophilus*, *T. muriaticus*, *Aerococcus viridans*, *S. equorum*, *S. haemolyticus, Bacillus* spp., *B. licheniformis and Leuconostoc mesenteroides*. Not surprisingly, these bacteria represent the most abundant fraction of the thick juice microflora.

In comparison with previous work (Table 2), out of the 53 identified species in this study, 43 species (83%) were never detected before in thick juice, encompassing 18 species identified

Table 1
Microbiological parameters, growth media and aerobic incubation conditions for monitoring different groups of microorganisms during long-term thick juice storage

Microbiological parameter	Growth media	Incubation temperature (°C)	Incubation time (days)
Aerobic colony count	Plate count agar (PCA)	30	3
Lactic acid bacteria	De Man Rogosa Sharpe agar (MRSA)	30	3
Yeasts and moulds	Oxytetracycline glucose yeast extract agar (OGYEA)	25	5
Osmophilic flora	De Whalley agar (DWA)[a]	25	5
Fastidious colony count	Columbia agar with 5-7% sheep blood (CAwSB)[b]	30	6

[a] DWA was prepared as follows: yeast extract, 5.0 g; caseinepeptone, 2.0 g; starch, 2.0 g; glycerine, 1.0 g; NH4Cl, 2.0 g; glucose, 20.0 g; sucrose and agar, 16.0 g per l of deionised water.

[b] Beside the usual aerobic incubation, 26 strategic samples from degraded and non-degraded thick juice had as well been incubated anaerobic and microaerophilic.

Table 2
Overview of all microflora known to occur in sugar thick juice, determined with both culture-based (C) and non-culture-based (NC) techniques as acquired in literature and in this research

Class/Family	Presumptive ID	Code	Day of storage	Detection	Similarity (%)	Lenght (bp)	Genbank	Reference
Bacteria								
Actinobacteria								
Corynebacteriaceae	*Corynebacterium xerosus*	C-TJ1	0	C	100	618	EU637608	E
Dermacoccaceae	*Dermacoccus* sp.	C-TJ2	168	C	99	762	EU637609	E
Dietziaceae	*Dietzia* sp.	C-TJ3	168	C	99	607	EU637610	E
Gordoniaceae	*Gordonia* sp.	C-TJ4	168	C	98	868	EU637611	E
	G. polyisoprenivorans	C-TJ5	168	C	99	798	EEU637612	
Microbacteriaceae	*Microbacterium* sp.	C-TJ6	168	C	100	800	EU637613	E
	M. arborescens	C-TJ7	119	C	99	792	EU637614	E
	Curtobacterium sp./*C. flaccumfaciens*	C-TJ8	0	C	99	710	EU637615	E
Micrococacceae	*Arthrobacter* sp./*A. bergeri*	C-TJ9	0	C	100	783	EU637616	E
	Kocuria sp.	C-TJ10	0	C	99	776	EU637617	E
	K. rhizophila	ID4123		C	99	1472	EU660422	B
	Micrococcus luteus	C-TJ11	All	C	99	698	EU637618	E
	M. lylae [b]	ID4401		C	–	–	–	B
Streptomycetaceae	*Streptomyces* sp.	C-TJ12	0	C	99	788	EU637619	E
Bacilli								
Aerococcaceae	*Aerococcus* sp./*A. viridans*	ID4403; C-TJ13	All	C, NC	99	850	EU637620	B; E
Bacilliaceae	*Anoxybacillus flavithermus*	NC-TJ14		NC	99	412	EU637607	E
	Bacillus sp.	C-TJ14	All	C, NC	99	761	EU637621	E
	B. cereus	C-TJ15		C	100	840	EU637622	B; E
	B. circulans	C-TJ16	14	C	99	833	EU637623	E
	B. clausii	C-TJ17	152	C	99	737	EU637624	E
	B. coagulans	NC-TJ1	270	NC	98	771	EU637594	E
	Bacillus sp./*cryopeg/globisporus*	C-TJ18	152	C	99	850	EU637625	E
	B. flexus	C-TJ19	All	C	99	811	EU637626	E
Bacilliaceae	*B. halodurans*	C-TJ20	0	C	100	724	EU637627	E
	B. licheniformis	C-TJ21	All	C, NC	99	738	EU637628	E
	B. psychrodurans	ID4391		C	99	1312	EU660425	B
	B. pumilus	C-TJ22		C	99	806	EU637629	B; E
	B. silvestris	C-TJ23		C	100	808	EU637630	E
	B. simplex	ID4402		C	100	1503	EU660430	B
	B. sphaericus	-		C		–	–	B
	B. subtilis	C-TJ24	152	C	98	799	EU637631	B; E
	Geobacillus sp.	NC-TJ2	0	NC	99	779	EU637595	E
	Sporosarcina globispora	ID4389		C	99	1504	EU660424	B
Carnobacteriaceae	*Desemzia incerta*	ID4395		C	99	1503	EU660427	B
Enterococcaceae	*Enterococcus* sp.	C-TJ25	73	C	100	803	EU637632	E
	Tetragenococcus halophilus	ID 4406; C-TJ26	All	C	99; 99	1511; 613	EU660432; EU637633	B; C; D; E
	T. muriaticus	C-TJ27	All	C, NC	97	615	EU637634	D; E
Lactobacillaceae	*Lactobacillus brevis* [a]	-		C	–	–	–	B
	L. buchneri/genomosp./parakefiri	NC-TJ3	0	CL	100	412	EU637596	E
	L. lactis lactis [a]	-		C	–	–	–	B
	L. delbrueckii [a]	-		C	–	–	–	B
Paenibacillaceae	*Paenibacillus* sp.	NC-TJ4	0	NC	99	758	EU637597	E
	P. polymyxa	C-TJ28	43	C	98	898	EU637635	E
	Aneurinibacillus migulans	C-TJ29		C	99	838	EU637636	E
Staphylococcaceae	*Staphylococcus* sp.	ID4386; C-TJ30	All	C	98	999	EU637637	B; D; E
	S. aureus	ID4388		C	100	1504	EU660423	B
	S. epidermis	ID4393		C	99	1503	EU660426	B
C-TJ33	*S. equorum*	ID4122; C-TJ31	All	C, NC	100; 99	1408; 837	EU660421; EU637638	B; C; D; E
	S. haemolyticus	C-TJ32	All	C, NC	99	667	EU637639	E
	S. pasteuri/Staphylococcus sp.	C-TJ33	All	C	98	965	EU637640	E
	S. saprophyticus	ID4404; C-TJ34	–; 168	C	99; 99	703; 1505	EU660431; EU637641	B; C; D; E
	S. xylosus	C-TJ35		C	99	835	EU637642	E
	Uncultered *Staphylococcus/S. epidermis*	C-TJ36		C	99	703	EU637643	E
	Uncultered *Staphylococcus* / *S. lugdunensis*	C-TJ37		C	99	786	EU637644	E
Bacteria								
Bacilli								
Sporolactobacillaceae	*Sporolactobacillus inulinus*	NC-TJ5	0	NC	98	760	EU637598	E
Streptococcaceae	*Streptococcus macedonicus* [c]	ID4387	C		–	–	–	B
	Lactococcus raffinolactis [c]	ID4396		C	–	–	–	B
Clostridia								
Syntrophomonadaceae	*Caldicellulosiruptor acetigenus/C. lactoaceticus*	NC-TJ6	0	NC	98	474	EU637599	E
	Caldicellulosiruptor kristjansonii	NC-TJ7	0	NC	99	621	EU637600	E
Proteobacteria								
Enterobacteriaceae	*Enterobacter* sp.	ID4399		C	–	–	–	B
Leuconostocaceae	*Leuconostoc* sp.	-		C	–	–	–	A

Table 2 (*continued*)

Class/Family	Presumptive ID	Code	Day of storage	Detection	Similarity (%)	Lenght (bp)	Genbank	Reference
Bacteria	*Leuconostoc mesenteroides*	C-TJ38	0	C, NC	99	719	EU637645	E
Proteobacteria								
Moraxellaceae	*(uncultered) Acinetobacter/bacterium clone*	NC-TJ8	0	NC	99	774	EU637601	E
	Acinetobacter sp.	NC-TJ9	0	NC	99	623	EU637602	E
Pseudomonadaceae	*Pseudomonas* sp.	ID4398; C-TJ39	168	C	99; 99	1489; 776	EU660428; EU637646	B; E
	Pseudomonas sp. (*P. fluorescens complex*)[b]	ID4405		C	–	–	–	B
	P. fragi [a]	ID4399B		C	–	–	–	B
Xanthomonadaceae	*Stenotrophomonas maltophilia* [b]	ID4392	C	–	–	–		B
Archea								
Thermococci								
Thermococcaceae	*Thermus* sp.	NC-TJ10	0	NC	99	732	EU637603	E
	T. scotoductus	NC-TJ11	0	NC	99	406	EU637604	E
	T. Thermophilus/sp.	NC-TJ12	0	NC	98	674	EU637605	E
	Thermus sp./*T. fiji*/uncultured bacterium	NC-TJ13	0	NC	99	733	EU637606	E

–: No data available. Detection techniques used: C = culture-based technique, NC = non-culture-based technique.References A = Van der Poel et al., 1998; B = Willems et al., 2003; C = Justé et al., 2008a; D = Justé et al., 2008b; E = This research.

[a] Determined with API.

[b] Determined with Biolog.

[c] Determined with SDS-PAGE.

with non-culturable methods and 29 species classified with culture-based sequencing. As a result, four species were detected with both methods. Nevertheless, five taxa that had been identified in the past (Willems et al., 2003) could not be detected in this study, including strains of *Desemzia*, *Enterobacter, Lactococcus raffinolactis, Stenotrophomonas maltophila* and *Streptococcus macedonicus*. As a result, taking all studies together, 72 different species have been reported in sugar thick juice so far, including 18 species that could not be cultured with the plating methods used.

It should be noted that several thick juice bacteria are also known to occur in other steps of the sugar refinery process, including taxa like *Bacillus*, *Lactobacillus*, *Leuconostoc*, *Pseudomonas*, *Streptomyces* and *Thermus* (Belamri et al., 1991; Bugbee et al., 1975; Hollaus et al., 1997; Klaushofer et al., 1971; Pollach et al., 2002; Samaraweera et al., 1995; Van der Poel et al., 1998).

From most matrices, the majority of microorganisms cannot be cultivated using the widely used laboratory methods (Ampe et al., 1999; Yang et al., 2001; Rapp and Giovannoni, 2003; Tyson and Banfield, 2005). Nevertheless, only a small fraction of the thick juice bacteria (25%) could not be detected by culture-based methods. More in particular, these bacteria were only found in freshly produced thick juice and encompassed thermophiles like *Thermus* and/or anaerobes like *Caldicellulosiruptor* and *Sporolactobacillus,* two groups that were not expected to be detected under the culture conditions used. Nevertheless, under appropriate conditions, one should be able to cultivate these bacteria as well. However, although *Acinetobacter* spp. were only detected using the culture-independent method, these aerobic proteobacteria should have been detected with the plating methods used. This discrepancy could possibly be explained by the lower detection limit of plating methods. Alternatively, the bacterium may have been overlooked on the plates by the selection criteria used or the DNA could have been derived from non-viable cells as well. In case of the bacteria that were identified during thick juice storage, no bacteria were found by the culture-independent method that were not picked up by the plating methods used. Bacteria detected only with the culture-based techniques are not as dominant as the *Bacillus*, *Staphylococcus* and *Tetragenococcus* spp. and are therefore not uncovered with universal primers. The high bacterial background during storage can thus result in more sensitive culture-based methods, emphasising the importance of these techniques (Joseph et al., 2003; Burns et al., 2004). All together, one can conclude that the culturable flora covers almost the total thick juice microflora during storage.

The bacterial microflora in freshly produced thick juice is generally present at low densities, varying from 1 to 10^3 cfu/ml (Justé et al., 2008a, b). In addition, this bacterial community is relatively diverse. However, during storage, this relatively heterogeneous flora evolves to dominance of *Tetragenococcus* species (Justé et al., 2008b), typically reaching concentrations till 10^7 cfu/ml. Nevertheless, *Bacillus* and *Staphylococcus* species might be present during storage as well (Table 2), though in lower densities never exceeding 10^3 cfu/ml (Justé et al., 2008a, b).

The effects of thick juice microflora on thick juice degradation are difficult to assess as the concentration of sucrose in thick juice is very high. Nevertheless, the dominance of *Tetragenococcus* species results mostly in an increase of lactic acid and reducing sugars concentration, clearly indicating their importance on sugar loss (Justé et al., 2008b). However, the importance of bacilli and staphylococci cannot be measured and is therefore unclear.

Anaerobic counts on CAwSB were very low (<20 cfu/ml), while the micro-aerophilic and aerobic counts were almost identical at 10^5 cfu/ml (data not shown) and revealed identical identifications. As a consequence, almost no flora was overlooked by incubating solely under aerobic circumstances.

To our knowledge, so far only few reports are available describing the (osmophilic) bacterial flora of an environment with a_w values as low as in sugar thick juice ($a_w<0.86$), making comparisons with other matrices rather difficult. Maple syrup ($a_w = 0.75$), which is obtained in an analogous manner to beet sugar, is produced from naturally growing maple trees after collection and evaporation of the maple sap. Although the microflora of the unconcentrated maple sap has been well described (Lagacé et al., 2004, 2006; ICMSF, 2005), maple syrup itself has never been studied as is the case for palm syrup (ICMSF, 2005). In contrast, for honey ($a_w = 0.5$–0.6) the composition of the microbial community was studied by Iurlina and Fritz (2005) reporting the presence of several bacteria including *Bacillus cereus*, *B. pumilus*, *B. laterosporus* and *Pseudomonas larvae.*

An interesting question related to the diversity of bacteria detected in thick juice is how these bacteria end up in thick juice. After all, the severe heat treatment during evaporation (approximately 15 min at 130 °C and 15 min at 85 °C) kills

in principle all vegetative bacteria, which was confirmed in laboratory experiments (data not shown). All detected thick juice bacteria except for *Tetragenococcus* spp. have been isolated from the air, suggesting that air contamination could be an important contributing factor to the thick juice microbial composition. Therefore, installing an air filter at the top of the thick juice tank might reduce this contamination. At present, the source of contamination for *Tetragenococcus* remains still unclear.

Beside the dominant thick juice bacteria, a few yeasts and moulds were detected during thick juice storage as well, though in rather low concentrations ($<10^3$ cfu/ml). BLAST analysis of ITS sequences obtained from industrially stored thick juice samples revealed the presence of multiple yeast and mould species, including *Candida bombi*, *C. zemplinina*, *Cordyceps sinensis*, *Cystofilobasidium infirmo-miniatum*, *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Penicillium camemberti/commune*, *Pichia* sp., *Pichia anomala*, *Rhodotorula minuta*, *Torulaspora* sp. and *Torulaspora delbruecki* (data not shown), which are all known to occur in food matrices like cheese, yoghurt, wine, maple sap and sugar melasse (Sheneman and Costilow, 1959; Caggia et al., 2001; Petersen et al., 2002; Samaraweera et al., 2003; Passoth et al., 2006). In addition, these species can also be found in the air (Sugita and Nakase, 1998; Sampaio et al., 2001; Yamamoto et al., 2002). In contrast to stored thick juice, no eukaryotes were found in freshly produced thick juice.

Further studies are needed for examination and for assessment of strategies to reduce microbial contamination during processing and storage. Nevertheless, a major challenge therefore is to rapidly detect and identify all microorganisms of interest. In this regard, the data gathered in this study could be used to develop a new strategy to detect and identify the species that can occur in sugar thick juice and may have a potential role in its degradation. In fact, a DNA macroarray (Lievens et al., 2003) has already been developed for simultaneous detection and identification of the most prominent thick juice bacteria, encompassing detector oligonucleotides for the genera *Bacillus, Kocuria, Staphylococcus* and *Tetragenococcus*, as well as for the species *A. viridans, L. mesenteroides* and *T. halophilus* (Justé et al., unpublished results). Ultimately, such detection system could contribute to the development of management strategies for thick juice degradation resulting from microbial activity.

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