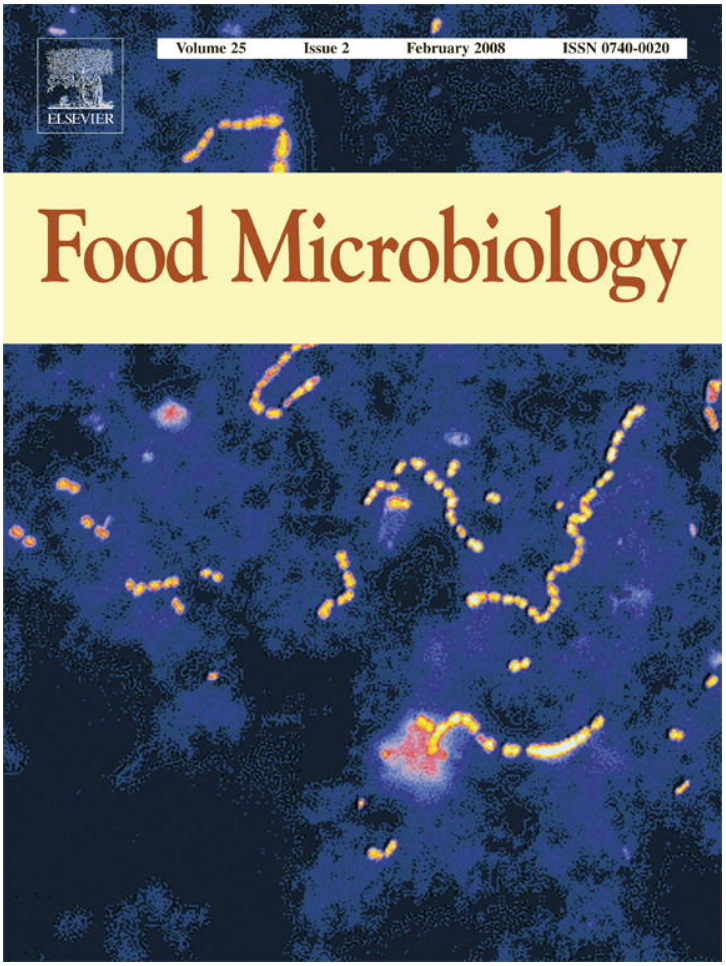

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Short communication

Predominance of *Tetragenococcus halophilus* as the cause of sugar thick juice degradation

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Abstract

The industrial storage of sugar thick juice was simulated on a laboratory scale. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis and the application of Clone Libraries in parallel with classical microbiology were used to study the bacterial diversity and all revealed a dominance (>99%) of *Tetragenococcus halophilus* during storage. The degradation of thick juice correlated with the appearance of L-lactic acid and high concentrations of *T. halophilus*. In addition, pure cultures of *T. halophilus* induced degradation of sterile thick juice. A specific PCR was developed to detect *T. halophilus* and industrial thick juice samples from Belgium, Germany and France all contained *T. halophilus*, suggesting a consistent association of this organism with thick juice. *T. halophilus* has been known only as a halophile thus far, and this report is the first to show an association of this organism with a sugar-rich environment.

Keywords: *Tetragenococcus halophilus*; Terminal restriction fragment length polymorphism; Clone library; Sugar thick juice; Lactic acid bacteria; Specific PCR

1. Introduction

Sugar thick juice is an intermediate product in the production of beet sugar. It is the concentrated product of evaporation of beet juice with a total soluble solids content corresponding to about 69 °Bx and a slightly alkaline pH of 9.0. Previous research (Willems et al., 2003; Justé et al., 2007) and industrial practice (Asadi, 2006) have shown that thick juice stability depended on the adequate control of process parameters including solids content, pH and temperature, since these directly influence microbial growth. 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 pH 9 to pH 5–6 and typically, an increase in reducing sugar content (Sargent et al., 1997; Willems et al., 2003), resulting in economical losses.

However, studies of the causes of thick juice degradation have yielded conflicting results. Sargent et al. (1997) and Hein et al. (2002) suggested that the pH drop during thick juice degradation is caused by an increase in lactic acid. In contrast, results from Willems et al. (2003) showed no correlation between thick juice degradation and any acid monitored during storage, including lactic, acetic, propionic, butyric, valeric, isovaleric, hexanoic and formic acid.

The causal microflora held responsible for thick juice degradation also differed in previous reports. Sargent et al.

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(1997) proposed that mesophilic flora detected on plate count agar (PCA) caused the pH drop, while Willems et al. suggested a correlation of thick juice degradation with previously undetected 'fastidious bacteria' (FB), a component of the microbial flora that does not grow on the conventional non-selective bacterial plating media. The hypothesis was formulated that thick juice degradation comes down to a process of succession, resulting from changing competitive ability under autogenically changing environmental conditions. The ratio of fastidious and mesophilic counts was proposed as the perfect monitoring tool, since both bacterial populations were reported to co-evolve during storage with a characteristic shift to dominance of the FB at degradation. Justé et al. (2007) however were able to show a strong correlation ($r = 0.99$) between the FB counts as such and the start of degradation in thick juice not containing any biocides. Treatment with a commercially available formulation of hop β-acids significantly delayed both juice degradation (pH fall) and the development of FB, implicating the FB as the cause of degradation. The FB were characterized as catalase-negative, Gram-positive cocci by Hein et al. (2002) and Willems et al. (2003). More recently, we tentatively identified these bacteria as *Tetragenococcus halophilus* based on 16S rDNA sequencing (Justé et al., 2007).

In this paper, the predominance of *T. halophilus* during thick juice storage and degradation is confirmed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis and rDNA clone libraries. These methods are culture independent and therefore provide a more comprehensive representation of the thick juice microflora than previous studies (Hugenholtz et al., 1999). In addition, we show that the acidification observed during thick juice degradation is caused by lactic acid production, and that pure cultures of *T. halophilus* can cause degradation of sterile thick juice. Finally, a species-specific PCR for *T. halophilus* was developed and used to screen industrial thick juice samples from different countries for the presence of this dominant flora.

2. Materials and methods

2.1. Thick juice storage experiment and sampling

Long-term thick juice storage experiments were conducted to monitor degradation and microbial dynamics in pilot-scale storage tanks. Each of these tanks consisted of an upright closed cylinder (dimensions: $Ø_i$: 20 cm, H_i: 200 cm, V_i: 62.8 l) surrounded by a temperature-controlled water jacket of 27.7 l. Thick juice with 69 °Bx was collected in sterile 25 l polyethylene buckets after the evaporation and cooling stage in an industrial sugar refinery (Tiense Suiker, Tienen, Belgium). The effect of solids content and pH on thick juice stability was analysed (Justé et al., 2007) at 25 °C, a challenging but industrially relevant temperature, using a factorial design for 13 cylinders (Systat Software Inc., San Jose, CA, USA) (Table 1A). All thick juice cylinders were stored for 168 days. Weekly, 20 ml thick juice samples were aseptically obtained from each cylinder and analysed for pH, volatile acids, lactic acid, microflora and reducing sugars.

2.2. Viable counts

Table 1B presents the microbiological parameters analysed during thick juice storage as well as the incubation conditions used, as described in detail in 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,

Table 1
Experimental characteristics from the thick juice storage experiment

A. Experimental design of the pilot scale thick juice storage experiment[a]

pH	Brix (°)		
	65	67	69
9.2	C7/C8	C10	C3/C4
9.0	C9	C13	C11
8.8	C5/C6	C12	C1/C2

B. Microbiological parameters, growth media and aerobic incubation conditions for monitoring diverse microbial groups during long-term thick juice storage

Microbiological parameter	Growth media	Incubation temperature (°C)	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	25	5
Fastidious colony count	Columbia agar with 5–7% sheep blood (CawSB)	30	6

[a]C1–C13 represent the 13 cylinders stored at 25 °C with their corresponding Brix (°) and pH.

Oxoid Limited) after 3–6 days of incubation at 30 °C (Justé et al., 2007).

2.3. Acid analysis

Volatile fatty acids and lactic acid were determined on a Varian 3800 gas chromatograph equipped with a stand alone 8200 auto-sampler and a Genesis headspace sampler for the volatile acids and lactic acid, respectively; and a 1079 Varian injector and a standard Varian flame ionization detector. The analysis of volatile acids included the detection of acetic, propionic, butyric, isobutyric, valeric, isovaleric and hexanoic acid. Sample treatment and analyses settings were as described in Willems et al. (2003).

2.4. DNA extraction

Genomic DNA was extracted from 20 ml thick juice samples using the phenol–chloroform extraction protocol described by Lievens et al. (2003).

2.5. Sequencing of bacterial 16S rRNA genes

Sequencing was performed on purified PCR products from pure colonies obtained with the universal bacterial primers 27f and 1492r (Lane, 1991). Samples were analysed on an Applied Biosystems 373A Automated Sequencer and resulting sequences were compared to online databases 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). Multiple sequence alignment were performed using CLUSTAL X. Phylogenetic analyses were performed using the neighbour-joining algorithm of the CLUSTAL X software package and visualized using Treeview.

2.6. Analysis of 16S rDNA T-RFLP

Both a degraded (C9) and a non-degraded (C3) thick juice sample were analysed with T-RFLP in order to determine the diversity of the bacterial community. T-RFLP analysis targeting 16S rDNA was performed using the universal bacterial primers 27f (5′-AGAGTTT-GATCCTGGCTCAG-3′) (5′FAM-labelled) (Liu et al., 1997) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) (unlabelled) (Marchesi et al., 1998). Amplification reactions were done by PCR in a 20 µl reaction volume, containing 1 µl template DNA and using 0.15 mM digoxigenin-11-d-UTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM of each primer, and 1 unit Titanium Taq DNA polymerase (Clontech Laboratories Inc., Palo Alto, CA, USA). Samples were denatured at 94 °C for 2 min and then subjected to 30 cycles 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. Amplicons from four replicate PCR reactions were pooled for each sample and purified with a Qiaquick® kit (Qiagen Inc.) according to the manufacturer's protocol. About 200 ng of PCR product were digested for 4 h at 37 °C in a total volume of 20.0 µl with either *Hha*I, *Msp*I or *Rsa*I (New England Biolabs, Frankfurt am Main, Germany). Restriction fragment analyses were conducted on an Applied Biosystems 373A Automated Sequencer. The similarity of communities could be estimated by visual comparison of the electropherograms, and by identification with T-RFLP Analysis Program (TAP) (Marsh et al., 2000).

2.7. Application of clone libraries

Bacterial clone libraries were created from the same degraded and non-degraded thick juice samples as analysed with T-RFLP, using the same PCR protocol except that non-labelled primers 63f (CAG GCC TAA YAC ATG CAA GTC) and 1387r were used. 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. Ninety-five white colonies were randomly picked from each library and sequenced using the M13 primer. Multiple sequence alignment were performed using CLUSTAL X. Phylogenetic analyses were performed using the neighbour-joining algorithm of the CLUSTAL X software package. Phylogenetic trees were constructed using CLUSTAL X and visualized using Treeview.

2.8. Specific PCR assay development and verification

In order to design species-specific primers that target the 16S rDNA sequence of *T. halophilus*, we used the ARB program and database (ARB Project, Technische Universität München, http://www.arb-home.de/) (Ludwig et al., 2004) in combination with our own sequences of thick juice Tetragenococci (Justé et al., 2007 and this study). Several PCR primers were designed, but only one set of primers was selected based on its specificity and sensitivity. The selected primers 193f (5′-AGC TCA AAG GCG CTT TAC-3′) and 480r (5′-TTC TGG TCA GCT ACC GTC-3′) were tested for their specificity with phylogenetically related species and well-known thick juice contaminants (Table 4A). The optimized PCR temperature profile consisted of a preliminary denaturation of 2 min at 94 °C, followed by 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 62 °C and 45 s elongation at 72 °C, with a final extension at 72 °C for 10 min. Finally, the species-specific PCR protocol was tested for its ability to detect target species in degraded and non-degraded thick juice samples obtained from 12 industrial refinery plants in Belgium, France and Germany.

3. Results and discussion

3.1. Culturable microflora: viable counts and identification

Lactic acid bacteria, aerobic colony counts, yeasts and moulds were either absent or present only in low numbers

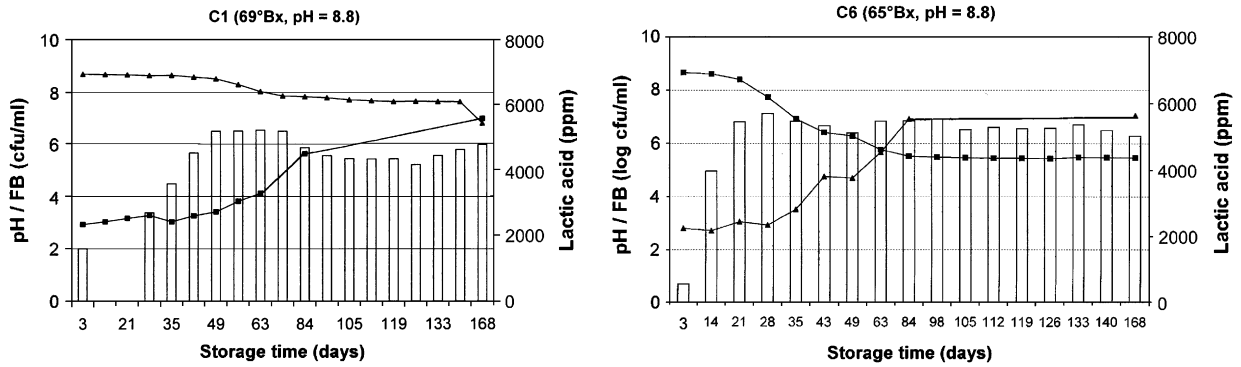

Fig. 1. Representative dynamics of pH (▲), fastidious bacteria (bars) and lactic acid (■) concentration during thick juice storage.

($<10^3$ cfu/ml) in all cylinders (data not shown). In addition, these populations remained relatively stable during thick juice storage, regardless of whether thick juice was degraded or not. Remarkably, the FB became dominant in all cylinders, reaching concentrations of up to 10^6–10^7 cfu/ml (Fig. 1). The dominance of these FB ($>10^6$ cfu/ml), tentatively identified as *T. halophilus* (Justé et al., 2007), was strongly correlated ($r = 0.96$) with the start of degradation, defined as the day at which the pH drops more than one unit below the initial value. All cylinders showed the dominance of these phenotypically identical FB although for example C1 (69 °Bx and pH = 8.8) only degraded after 112 days of storage as shown in Fig. 1. This discrepancy between the dominance of the FB and the decrease in pH was not observed in our first pilot-scale experiment (Justé et al., 2007). No other culturable flora could be detected and therefore be linked to degradation.

Ten FB isolates from both degraded and non-degraded thick juice were sequenced on their 16S rRNA genes in order to achieve a first, quick identification of the dominant culturable microflora. The 10 sequences thus obtained, were deposited in the EMBL/Genbank/DDBJ Nucleotide Sequence Database under accession numbers EU054441–EU054450. Comparison to this online database by using the BLAST program revealed the same presumptive identity for all 10 isolates, more specifically, 99% homology with *T. halophilus* (Fig. 3). These results show the same dominant culturable microflora as an earlier thick juice storage experiment (Justé et al., 2007). As a consequence, thick juices originating from different thick juice campaigns show comparable dominant culturable microflora after pilot-scale storage.

3.2. Acid analysis

Volatile acids and lactic acid (LA) were monitored during the entire storage period in order to explain the observed drop in pH during thick juice storage (Sargent et al., 1997; Hein et al., 2002; Willems et al., 2003). All volatile acids remained relatively stable during thick juice storage regardless of whether thick juice was degraded or not (data not shown), confirming earlier results of Willems et al. (2003). The concentration LA, however, was clearly correlated with the pH drop ($r = 0.87$). All degraded thick juices contained increased levels of LA as presented in

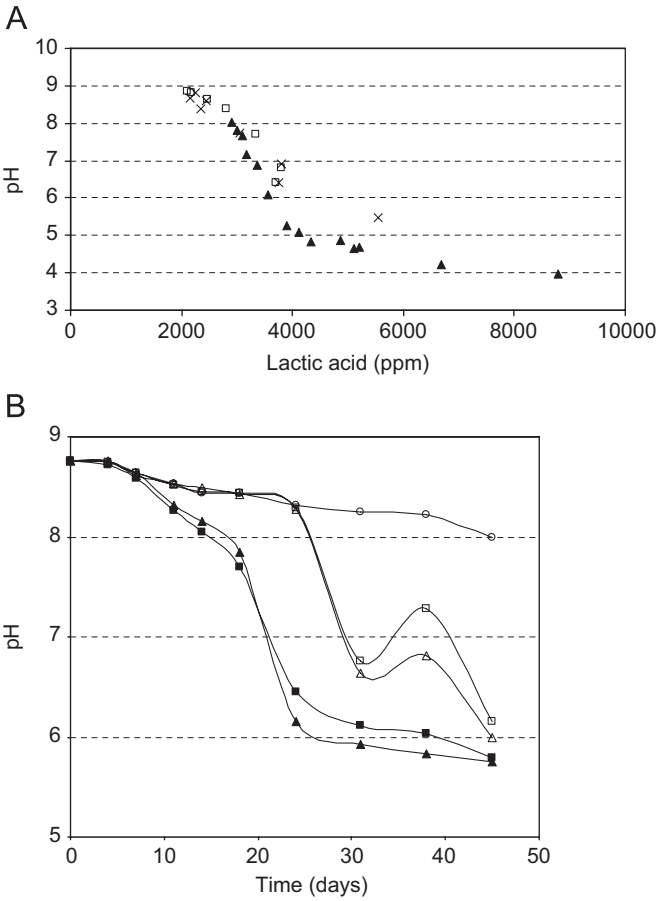

Fig. 2. Dynamics of the pH during thick juice storage in parallel with the lactic acid concentration. (A) Comparison of the pH-decrease in thick juice observed in artificially acidified thick juice and in the experimental pilot-scale storage (C5 and C6). (▲) Fresh thick juice gradually acidified with L-lactic acid. (□, ×) Experimental drop in pH during 272 days of thick juice storage in respectively C5 and C6. (B) Dynamics of the pH during thick juice storage after inoculation of sterilized thick juice with *Tetragenococcus halophilus*. Two different concentrations of two different isolates were used as inoculum. (▲) 10^5 cfu/ml *T. halophilus* isolated from industry, (△) 10^2 cfu/ml *T. halophilus* isolated from industry, (■) 10^5 cfu/ml *T. halophilus* isolated from the pilot scale, (□) 10^2 cfu/ml *T. halophilus* isolated from the pilot scale, (○) Control, non-inoculated thick juice.

Table 2A. Thick juice cylinders without degradation or drop in pH, revealed a constant or only slightly increased concentration of LA. An analysis of frozen thick juice samples of an earlier storage experiment (Justé et al., 2007) confirmed the positive correlation between the increase in LA and the drop in pH ($r = 0.94$) (Table 2B). These data fit with the observations of Sargent et al. (1997) and Hein et al. (2002).

The hypothesis that the increased concentration of LA caused the drop in pH was further tested by adding L-LA to fresh thick juice. Fig. 2A shows that addition of L-LA produces a similar pH reduction as the concentrations of LA found in the thick juice cylinders in the storage experiment. Since *T. halophilus* is a homo-fermentative lactic acid bacterium (Uchida, 1982), we propose that it is the LA produced by this organism that causes the observed thick juice degradation.

Notably, and in contrast to all other volatile acids, the concentration of acetic acid occasionally increased slightly at the top of the thick juice cylinders (data not shown). The different behaviour of thick juice at the surface has been reported several times (Pollach et al., 1999; Hein et al., 2002) and may be explained by the different conditions at the surface including the availability of oxygen and the presence of condensed water on the tank walls that can dilute the upper layer, creating favourable conditions for microorganisms. Interestingly, Gürtler et al. (1997) demonstrated that *T. halophilus* grown under aerobic conditions produced mainly acetate, in contrast to the dominant production of lactic acid in anaerobically grown cultures.

3.3. T-RFLP analyses and application of clone libraries

Both a degraded and a non-degraded thick juice sample were analysed with T-RFLP in order to assess the complexity of the bacterial community and identify the causative agent. Only one restriction fragment was detected for all three enzymes for both degraded and non-degraded thick juice samples. The obtained terminal restriction fragments for *Hha*I, *Msp*I and *Rsa*I were respectively 212(±1), 561(±1) and 901(±1) bp long, being consistent with the presence of *T. halophilus* as the dominant species (>99%) (Table 3). With the current sequences available, no other single representative could be found matching all

Table 2
Overview of the concentration of lactic acid (LA) and pH after a short period of 49 days and at the end of thick juice storage (168 days)

A. Thirteen thick juice cylinders incubated at 25 °C in the Brix experiment[a]

Cylinder	Brix (°)	pH initial	After 49 days of storage		After 168 days of storage	
			ΔLA (ppm)	ΔpH	ΔLA (ppm)	ΔpH
C1	69	8.68	1313	0.18	3239	1.90
C2	69	8.66	0	0.15	2275	1.95
C3	69	9.80	929	0.00	3710	1.51
C4	69	9.04	1084	0.19	3179	1.97
C5	65	8.85	1517	2.64		
C6	65	8.67	1498	2.39	3388	3.21
C7	65	9.10	4434	3.91		
C8	65	9.13	3170	2.99	4472	3.73
C9	65	8.84	3609	3.13	4572	3.56
C10	67	9.07	2136	2.38		
C11	69	8.83	0	0.00	3304	2.10
C12	67	8.72	1202	1.63		
C13	67	8.76	1914	1.64		

B. Nine thick juice cylinders from an earlier hop experiment during which thick juice was stored for 272 days (Justé et al. 2007)[b]

Cylinder	Hop	*T* (°C)	pH initial	After 49 days of storage		After 272 days of storage	
				ΔLA (ppm)	ΔpH	ΔLA (ppm)	ΔpH
K1	40	20	8.74	<0	0.09	330	1.22
K2	40	20	8.7	<0	0.10	133	1.48
K3	0	20	8.77	<0	0.71	2217	3.38
K4	0	20	8.64	1193	3.13	1932	3.54
K5	0	30	8.66	2663	3.63	3253	3.60
K6	0	30	8.65	1660	3.33	3054	3.34
K7	40	30	8.66	<0	0.40	989	2.07
K8	40	30	8.69	<0	0.39	306	2.02
K9	20	25	8.67	<0	0.59	823	1.93

[a]Thick juice that already degraded after 49 days of storage was not sampled for the entire storage period of 168 days.
[b]Samples were stored at −20 °C before analysis.

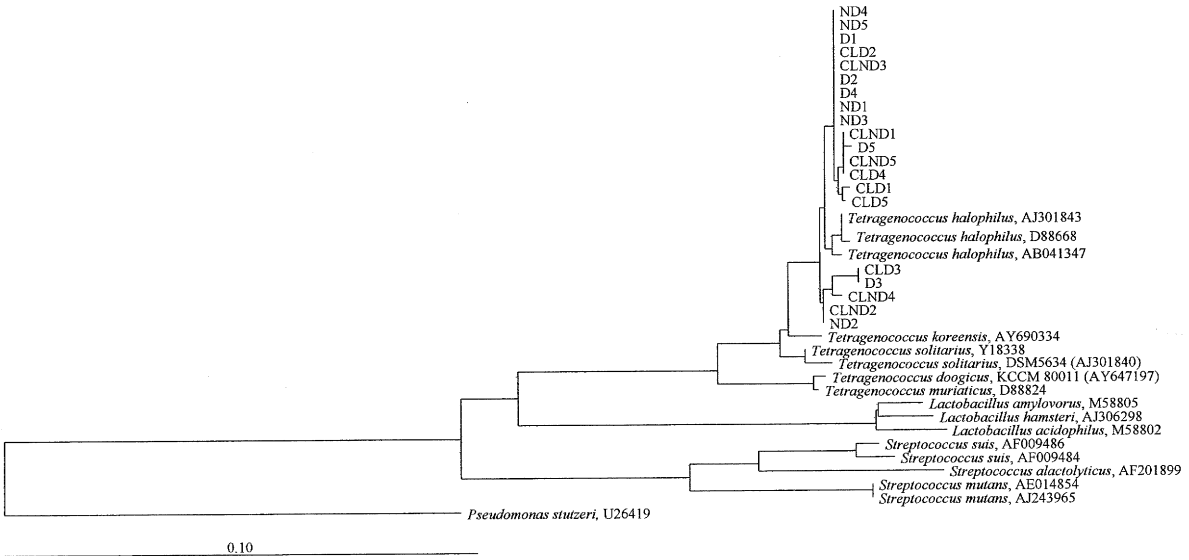

Fig. 3. Phylogenetic tree of 10 fastidious bacteria (D1–10 and ND1–10) and 10 randomly chosen clones (CLD1–10 and CLND1–10) from both degraded (D) and non-degraded (ND) thick juice based on 16S rDNA sequence analysis. All isolates and clones had a 99% sequence similarity and clustered with *Tetragenococcus halophilus*. *Pseudomonas stutzeri* is used as out group. GenBank/EMBL/DDBJ accession numbers for all 16S rDNA sequences are given. Bar: 10% sequence divergence.

Table 3
Predicted and observed lengths of Terminal Restriction Fragments (TRF)

Restriction enzyme	Bacteria	Expected TRF (bp)	Observed TRF (bp)
*Hha*I	*T. halophilus*[a]	212–213	
	Thick juice sequences	211–212	211–212
*Msp*I	*T. halophilus*[a]	560–562	
	Thick juice sequences	560–561	560–561
*Rsa*I	*T. halophilus*[a]	897–899	
	Thick juice sequences	n.a.	901–902

n.a.: not available.
[a]Based on all sequences available in Genbank.

three restriction digests. It should be reminded that accurate quantification becomes more difficult as the fragments become longer (Marsh, 2005).

The absence of other detectable fragments with this 'universal' primer set suggests that if other populations were present they were at levels <1% of the community (Forney et al., 2004). The conclusion is that no unculturable microflora could be detected and thus linked to thick juice degradation.

In parallel, bacterial rDNA clone libraries from the same representative thick juice samples were characterized by 16S rDNA sequencing and compared to sequences available in Genbank. As observed in the T-RFLP analyses, *T. halophilus* appeared to be the dominant bacterium, representing all 95 clones for both degraded and non-degraded thick juice. Phylogenetic analyses showed a mixed cluster for the 190 *T. halophilus* sequences from both degraded (C9) and non-degraded (C3) thick juice (data not shown). The phylogram in Fig. 3 demonstrates this mixed clustering for 10 randomly chosen clone sequences (Genbank accession numbers EU054431–EU054440). There are two possible explanations for this apparent strain-level sequence variation in *T. halophilus* populations. The sequence differences could reflect strain differences consistent with observations by Uchida (1982) and Röling and van Verseveld (1996), who demonstrated varying substrate utilization patterns, including the carbon source sucrose, for different *T. halophila* isolates. Alternatively, the physiology of these sequence variants may be identical but the thick juices may differ in their physiochemical parameters, creating more favourable or unfavourable conditions for thick juice degradation. It is known for example that a higher Brix index prolongs stable thick juice storage (Sargent et al., 1997; Willems et al., 2003), which is confirmed in our experiment (Table 2A). Moreover, *T. halophilus* is known for its different behaviour under different growth conditions (Gürtler et al., 1997).

3.4. *Tetragenococcus halophilus* can cause thick juice degradation

The hypothesis that *T. halophilus* as such is able to cause thick juice degradation by lactic acid production

was tested by inoculating sterile thick juice with pure cultures of *T. halophilus* isolated from thick juice. The dynamics of the pH during 40 days of storage is presented in Fig. 2B. A pH decrease was observed similar to the decrease occurring in the pilot-scale experiment. Moreover, the pH decrease occurred more rapidly and was stronger when more concentrated inocula of *T. halophilus* were added. These results indicate that *T. halophilus* can cause thick juice degradation similar to what is detected in commercial storage vats. It should be noted that uninoculated sterile thick juice had a small decrease in pH during storage without any bacteria becoming detectable by classical plating. A slightly increased concentration of LA was measured (data not shown), possibly indicating the transformation of sucrose into lactic acid by a purely chemical reaction as suggested previously (Justé et al., 2007; Manley-Harris et al., 1980).

Originally isolated from anchovy pickles (Orla-Jensen, 1919), *T. halophilus* has been found in several other food products like Japanese soy sauce (Nagakawa and Kitahara, 1959), Indonesian soy sauce (Röling and van Verseveld, 1996), Japanese fermented puffer fish ovaries (Kobayashi et al., 2000), Thai fish sauce (Thongsant et al., 2002) and Indonesian shrimp paste (Kobayashi et al., 2003). These are all salted foods and *T. halophilus* has therefore been considered as a halophile thus far. This report is the first to show an association of this organism with a sugar-rich environment. Generally, the microflora of high-sugar foods is dominated by xerophilic filamentous fungi and osmophilic yeasts (Grant, 2004), and the dominance of a halophilic bacterium in sugar thick juice is therefore unexpected. In principle, the stresses imposed by salt (cations) and by sugar (uncharged organic solutes) are not necessarily the same (Grant, 2004). Many osmotolerant microorganisms (e.g. *Staphylococcus aureus* and *Zygosaccharomyces rouxii*) also tolerate high salt concentrations (Eriksen and McKenna, 1999; Scott, 1957), while the opposite is not necessarily true: microorganisms isolated from saturated salt lakes ($a_w = 0.75$), for example, were not able to tolerate similar a_w values imposed by inorganic solutes (Kushner, 1978). The salt tolerance of *T. halophila* was shown to be enhanced in the presence of well established compatible solutes like ectoine, dimethylsulfoniopropionate, proline, choline, glycine betaine and L-carnithine, and the latter three compounds were shown to be actively taken up and contribute to intracellular sodium ion homeostasis (Robert et al., 2000). In *S. aureus*, compatible solutes in the medium contribute to both NaCl and sucrose tolerance (Vilhelmsonn and Miller, 2002), and it seems likely therefore that also in *T. halophilus*, uptake of the above mentioned compatible solutes will confer both halo- and osmotolerance. Nevertheless, this remains to be shown, and an extensive genetic and physiological characterization of both halophile and osmophile *T. halophili* is ongoing in our laboratory.

3.5. Specific PCR for Tetragenococcus halophilus

Based on its high density and the ability of pure *T. halophilus* cultures to decrease the pH, we believe that this organism plays an essential role in thick juice degradation. Therefore, a species-specific PCR was developed to detect *T. halophilus* using the primers 193f and 480r. The non-target species listed in Table 4A were not amplified with the specific primers, whereas *T. halophilus* could be detected with a sensitivity of 40 fg of genomic DNA in pure water which represents 20 cells of *T. halophilus* or 1 cfu/ml since 20 ml was routinely extracted.

In order to test the applicability of these results, industrial thick juice samples were analysed for *T. halophilus* with the specific PCR assay. All analysed samples, collected immediately after thick juice production

Table 4
Development and industrial thick juice results for the specific PCR for *Tetragenococcus halophilus*

A. Bacterial strains used to check the specificity of the species-specific PCR for *Tetragenococcus halophilus*

Strain	Source[a]
Kocuria rhizophila	LMG 8816
Staphylococcus equorum	LMG19116
Bacillus cereus	LMG 2098
Aerococcus viridans	LMG 17931
Lactobacillus plantarum	LMG 9205
Leuconostoc mesenteroides (*sub. dextranicum*)	LMG 6908
Tetragenococcus muriaticus	LMG 18498
Tetragenococcus solitarius	LMG 12890
Tetragenococcus halophilus	LMG 11490
Tetragenococcus halophilus[b]	Thick juice

B. Presence of *Tetragenococcus halophilus* in industrial thick juice samples from different countries as analysed with the species-specific PCR and classical plating on Tryptone Soy Agar (TSA)

Sample code	Country	Specific PCR for *T. halophilus*	Fastidious bacteria on TSA (cfu/ml)
I1–I2	BE	+	1×10^2
I3–I4	BE	+	<100
I8–I19	GE	+	<100
I20	FR	++	4.2×10^5
I21	FR	++	4.0×10^5
I22	FR	++	3.7×10^5
I23	FR	++	3.8×10^5
I24	FR	++	1.2×10^5
I25	FR	++	1.3×10^5
I26	FR	++	1.3×10^5
I27	FR	++	1.2×10^5
I28	FR	++	2.1×10^5
I29	FR	++	1.9×10^5

+ =  ; ++ =  .

[a]LMG; BCCM/LMG = Belgian Co-ordinated Collection of Micro-organisms, Laboratory of Microbiology, University of Ghent, Belgium.

[b]As determined by sequencing of 16S gene and comparison to online databases by using the BLAST program.

from sugar companies in Belgium, Germany and France, contained *T. halophilus* (Table 4B). French samples contained remarkably more *T. halophilus* DNA in comparison to the low levels found in the Belgian and German thick juice samples (Table 4B). Classical plating revealed a concentration of 10^5 cfu/ml in the French samples, whereas no *T. halophilus* colonies could be detected in the German samples (<100 cfu/ml or 4000 fg DNA) compared to one single colony in two Belgian samples (detection limit = 100 cfu/ml). Sequencing confirmed the identity of the PCR product from the French and German samples as *T. halophilus* 16S rDNA, indicating the specificity of the assay in industrial samples with a natural mixed microflora. Probably the concentration of *T. halophilus* in the German thick juice samples was below the detection limit of the classical plating (100 cfu/ml or 4000 fg DNA), although the detection of free DNA or dead cells cannot be excluded. The developed specific PCR assay can be useful for source tracking of *T. halophilus*. Starting from the beets, every refinery intermediate can be analysed to track this fastidious bacterium. As a non-sporeformer, *T. halophilus* is very unlikely to survive the severe heat treatment during the evaporation of beet juice in the sugar industry, which corresponds to at least 12 min above 100 °C. Nevertheless, thick juice from French refineries contained already 10^5 cfu/ml *T. halophilus* right after production, which suggests that *T. halophilus* may be able to establish as a house microflora in sugar refineries. In addition, the specific PCR enables monitoring during thick juice storage. The sensitive and semi-quantitative PCR assay allows early detection of the target organism, facilitating appropriate handling like biocide treatment (e.g. hop extract, Justé et al., 2007) or further processing of the thick juice. Additionally, more strain specific information about the *T. halophilus* isolated from degraded and non-degraded thick juice might provide options for control of thick juice degradation.

However, an industrially degraded thick juice sample that contained 10^7 cfu/ml FB in 1 ml, was negative for the detection of *T. halophilus* with specific PCR. Sequencing of the 16S rRNA gene from 14 FB isolates (Genbank accession numbers EU054451–EU054464), revealed a 97% similarity with the 16S rDNA from *Tetragenococcus muriaticus* available in Genbank.

Future research will focus on the typing of several *Tetragenococcus* spp. colonies isolated from characteristically degraded and non-degraded thick juice, both from industry and from the pilot-scale experiments, in order to elucidate its precise role in thick juice degradation.

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