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A real-time PCR assay for detection and quantification of 2-branched (1,3)-β-D–glucan producing lactic acid bacteria in cider

Idoia Ibarburu [a], Rosa Aznar [b,c], Patricia Elizaquível [b], Nieves García-Quintáns [d], Paloma López [d], Arantza Munduate [a], Ana Irastorza [a], María Teresa Dueñas [a,*]

[a] Facultad de Ciencias Químicas, Universidad del País Vasco (UPV/EHU), Paseo Manuel de Lardizabal 3, 20018, San Sebastián, Spain
[b] Departamento de Microbiología y Ecología, Universitat de Valencia, Burjassot, Valencia, Spain
[c] Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Burjassot, Valencia, Spain
[d] Centro de Investigaciones Biológicas (CIB), Consejo Superior de Investigaciones Científicas (CSIC) Ramiro de Maeztu 9, 28040 Madrid, Spain

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ABSTRACT

Ropiness in natural cider is a relatively frequent alteration, mainly found after bottling, leading to consumer rejection. It is derived from the production of exopolysaccharides (EPS) by some lactic acid bacteria most of which synthesize a 2-branched (1,3)-β-D-glucan and belong to the genera *Pediococcus*, *Lactobacillus* and *Oenococcus*. This polysaccharide synthesis is controlled by a single transmembrane glycosyltransferase (GTF). In this work, a method based on quantitative PCR (qPCR) and targeting the *gtf* gene was developed for detection and quantification of these bacteria in cider. The newly designed primers GTF3/GTF4 delimit a 151 bp fragment within the 417 bp amplicon previously designed for conventional PCR. The inclusivity and exclusivity of the qPCR assay were assessed with 33 cider isolates belonging to genus *Lactobacillus, Oenoccocus* and *Pedioccocus,* together with reference strains of 16 species and five genera including β-glucan, α-glucan and heteropolysaccharide (HePS) producing strains and non-EPS producers. The qPCR assay, followed by the melting curve analysis, confirmed the generation of a single PCR product from the β-glucan producers with a T_m of 74.28 ± 0.08 and C_T values (10 ng DNA) ranging between 8.46 and 16.88 (average 12.67 ± 3.5). Some EPS^- LAB strains rendered C_T values ranging from 28.04 to 37.75 but they were significantly higher ($P(C_T<28.54)=0.05$) than those of the β-glucan producers. The assay showed a wide quantification range of 5 log units using calibrated cell suspensions of *Pediococcus parvulus* 2.6 and *Oenococcus oeni* I4. The linearity was extended over 7 log orders when calibration curves were obtained from DNA. The detection limit for β-glucan producing LAB in artificially contaminated cider was about 3×10^2 CFU per ml. The newly developed qPCR assay was successfully applied to monitor the cidermaking process, in 13 tanks from two cider factories, revealing a decrease in C_T values derived from an increase in β-glucan producing LAB populations. In addition, 8 naturally spoiled bottled cider were tested for the quantification of these organisms using the five standard curves constructed: *P. parvulus* 2.6 genomic DNA and *gtf* amplicon (417 bp), calibrated cell suspensions of *Pediococcus parvulus* 2.6, *Lactobacillus diolivorans* G77 and *Oenococcus oeni* I4 and results were compared to LAB total counts on MRS. Levels obtained from the different approaches were within a log range and showed no significant differences. Therefore, the amplicon-derived standard curve is proposed for the routine estimation of gtf^+ populations in cider.

1. Introduction

Lactic acid bacteria (LAB) are able to produce a wide variety of exopolysaccharides (EPSs) which can be used as a starter to improve the texture and stability of some dairy products (Ruas-Madiedo et al., 2008). However, these EPSs have deleterious effects on the organoleptic properties of alcoholic beverages. In cider (Fernández et al., 1995) and wine (Llaubères et al., 1990), EPS-producing LAB are responsible for an alteration, called "ropiness" or "oiliness," characterized by a viscous, thick texture and oily feel, which although not appreciably altering the taste, renders the products unpleasant to the palate.

In the Basque Country (North Spain), "natural" ciders are produced in small factories according to traditional methods and the usual oenological procedures (sulfur dioxide treatment, clarification or correction of acidity) are not applied (Garai et al., 2006). As natural ciders are not microbiologically stabilized before bottling, ropy bottled ciders can be encountered and refused by the consumers, resulting in considerable financial loss to cidermakers. Therefore, early detection of these spoiling bacteria, not only in bottled ciders but also during cider

* Corresponding author. Facultad de Químicas, Universidad del País Vasco (UPV/EHU), Paseo 18 Manuel de Lardizabal 3, 20018, San Sebastián, Spain. Tel.: +34 943018170; fax: +34 943015270.
E-mail address: mariateresa.duenas@ehu.es (M.T. Dueñas).

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making, would lead to processing decisions (i.e. sulfiting) to overcome this drawback.

Most of the EPS-producing bacteria isolated from ropy cider and wine synthesize an identical 2-branched (1,3)-β-D-glucan and belong to the genera *Pediococcus* (Llaubères et al., 1990; Dueñas-Chasco et al., 1997), *Lactobacillus* (Dueñas-Chasco et al., 1998) and *Oenococcus* (Ibarburu et al., 2007; Dols-Lafargue et al., 2008). This polysaccharide synthesis is controlled by a single transmembrane glycosyltransferase (GTF), which belongs to the COG1215 membrane-bound glycosyltransferase family, and polymerizes glycosyl residues from UDP-glucose (Walling et al., 2005; Werning et al., 2006). It is encoded by the *gtf* gene (Werning et al., 2008), which has a different genomic location in cider and wine-spoiling LAB strains, as it is present on plasmids in pediococci and lactobacilli and on the chromosome in *O. oeni* (Werning et al., 2006; Dols-Lafargue et al., 2008). Determination of its nucleotide sequence in *Lactobacillus diolivorans* G77, *Pediococcus damnosus* and *Oenococcus oeni* I4 revealed that it possesses a 100, 99.9 and 98.8% identity, respectively, with their counterparts in *Pediococcus parvulus* 2.6 (Werning et al., 2006; Dols-Lafargue et al., 2008).

In a previous study, a PCR assay was developed for the detection of (1,3)(1,2)-β-D-glucan producing LAB with primers targeted to the coding sequences of the putative glycosiltransferase domain and the fifth transmembrane segment of the GTF, respectively (Werning et al., 2006). It allowed the detection of (1,3)(1,2)-β-D-glucan producing *Pediococcus*, *L. diolivorans* and *O. oeni* to date reported as cider spoilers. PCR based methods have quickly been replacing more traditional assays in the microbiological analysis of food since they are rapid and specific detection systems. Nowadays, real-time or quantitative PCR (qPCR) is one of the most promising tools in food control. It is based on the detection of a fluorescent signal and allows the automated detection of amplicons without post-PCR manipulation, thus reducing the risk of cross-contamination (McKillip and Drake, 2004). qPCR has successfully been applied to detect and quantify the presence of *Aspergillus carbonarius* in wine grapes (Selma et al., 2008), and yeasts (Hierro et al., 2007; Tessonnière et al., 2009), acetic (González et al., 2006) and lactic acid bacteria (Neeley et al., 2005) in wine. In addition, Delaherche et al. (2004) developed a real-time PCR method for specific detection and quantification in spoiled wine of β-glucan producing *P. damnosus* strains. However, qPCR procedures have not yet been tested in cider. In this work a qPCR procedure has been developed for the detection and quantification of (1,3)(1,2)-β-D-glucan producing bacteria in cider. Further, it has been tested in naturally contaminated cider following a DNA purification step.

2. Material and methods

2.1. Bacterial strains, culture media and growth conditions

Bacterial strains used in this work include 33 cider isolates belonging to genus *Lactobacillus, Oenococcus* and *Pediococcus* together with reference strains of 16 species and five genera. Both strains and sources are listed in Table 1. Cider isolates were obtained from our culture collection at the Department of Applied Chemistry, University of Basque Country (CUPV). They had been isolated from spoiled cider along a large period (from 1994 to 2007) and some of the ropy strains had been biochemically identified and genetically characterized (Werning et al., 2006; Ibarburu et al., 2007; Garai-Ibabe et al., 2010). Reference cultures were supplied by the Spanish Type Culture Collection (CECT), the National Collection of Industrial, Marine and Food Bacteria (NCIMB), the ARS Culture Collection (NRRL) and the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG). LAB strains were stored at −80 °C in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain), containing 20% (v/v) glycerol. Before experimental use, bacteria were propagated in MRS broth at 28 °C in an atmosphere containing 5% CO_2. To isolate and select ropy strains from cider, aliquots of the different samples were spiked on modified MRS (Pronadisa, Madrid, Spain) with 10 g/l of fructose and tomato juice (10% v/v).

2.2. DNA isolation

DNA from pure cultures and cider samples was isolated using the DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). One millilitre aliquots were taken and following centrifugation at 8000×*g* for 10 min, pellets were washed twice with 1 ml of Ringer's solution (Oxoid, Hampshire, England) and centrifuged at 8000×*g* for 5 min. Pellets were resuspended in 180 µl of lysozyme (20 mg/ml) in TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8). After 30 min at 37 °C, samples were homogenized with 200 µl of lysis buffer and proteinase K (600 mAU/ml). The homogenate was incubated at 70 °C for 30 min. DNA was purified through the column using two cleaning buffers supplied in the kit. DNA was eluted in 100 µl of ultra pure water (Sigma) and 5 µl was used for PCR amplification.

2.3. Conventional PCR

Amplification of the *gtf* gene by conventional PCR (Gene Amp PCR System 2400, Perkin Elmer, USA) was carried out using primers GTFF and GTFR, which delimit a 417 bp fragment, and according to the method described by Werning et al. (2006).

2.4. qPCR SYBR Green I-based assay

2.4.1. Primer design

Two primers were designed with the Beacon Designer software (Bio-Rad, Spain) targeting the *gtf* gene encoding for the GTF glicosiltransferase (Werning et al., 2006). Primer sequences were checked against sequences available in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence and target position (*P. parvulus* 2.6 *gtf* sequence, GenBank accession number AY551933, Werning et al., 2006) for each primer were GTF3 (5′-ATCAAGTCAAAGACCATAAGTCTCTATC-3′, 2365–2392 in the putative carboxyl glycosytransferase domain of GTF protein) and GTF4R (5′TAAATAATTGTGTTACTAGTGGAATGTGC-3′, 2515–2486, in the fourth transmembrane segment of GTF protein). They delimit a 151 bp fragment. Oligonucleotides were synthesized by Eurofins MWG/operon (Ebersberg, Germany).

2.4.2. Set up of the qPCR reaction: amplification conditions

qPCR reactions were performed using SYBR Green I Core Reagents (Applied Biosystems, Madrid, Spain). Reactions were done in triplicate for each strain. Amplification mixtures for qPCR, contained in a final volume of 20 µl, 1x buffer (SYBR Green I PCR Buffer), 200 µM each dATP, dCTP, dGTP, and 400 µM dUTP; 1U of AmpErase uracil N-glycosidase; 1 U of AmpliTaq Gold DNA polymerase; 3.5 mM of $MgCl_2$; 200 nM of each primer and 5 µl (10 ng) of template DNA. Different concentrations of $MgCl_2$ (1.5, 3 and 4.5 mM) and primer (100, 200 and 300 nM of each) were assayed. qPCR assays were carried out in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif.) programmed to hold at 50 °C for 5 min, to hold at 95 °C for 10 min, and to complete 40 cycles of 95 °C for 15 s and 55 °C for 1 min. PCR results were given as the increase in the fluorescence signal of the reporter dye detected and visualized by the 7500 System SDS Software provided with the version 1.4 (Applied Biosystems). C_T values (threshold cycle) represent the PCR cycle in which fluorescence first increased, over a defined threshold (set to a fluorescence value of 0.09), for each amplification plot. Melting curve analysis was determined according to manufacturers' instructions (SDS software 1.4, Applied Biosystems).

2.4.3. Quantification assays

Standard curves were calculated for quantification purposes using: i) Ten-fold dilutions of genomic DNA extracted from 1 ml of a log

Table 1
Bacterial strains used in this study.

Species	Strain and source[a]	N°	qPCR[b]		EPS phenotype/type of EPS[c]
			C_T	T_M	
Lactobacillus brevis	CECT 216		>40	60.7 ± 0	–
Lactobacillus buchneri	CECT 4111		35.06 ± 0.32	74.7 ± 0.23	–
L. collinoides	CECT 922[T]		>40	60.7 ± 0	–
	CUPV[d] 231		8.46 ± 0.12	74.4 ± 0.17	+/β-D-glucan (Ibarburu, 2009)
	CUPV[e] 234, 235	2	30.49 ± 0.09	74.4 ± 0.15	–
Lactobacillus delbrueckii subsp *bulgaricus*	NCIMB 702772		> 40	60.9 ± 0	+/HePS (Grobben et al., 1997)
L. diolivorans	LMG 19667[T]		37.76 ± 0.24	72.3 ± 0.49	–
	CUPV[d] G77		10.96 ± 0.16	74.1 ± 0	+/α- and β-D-glucan (Dueñas-Chasco et al., 1998)
Lactobacillus helveticus	NCIMB 700766		39.59 ± 0.48	60.6 ± 0	+/HePS (De Vuyst et al., 2001)
Lactobacillus hilgardii	CECT 4786[T],	2	> 40	60.7 ± 0	–
	CECT 4681		29.23 ± 0.49	74.7 ± 0	
Lactobacillus mali	CECT 4149		31.15 ± 0.09	74.4 ± 0	–
Lactobacillus plantarum	CUPV[e] 241, 242, 243	3	30.56 ± 0.31	74.4 ± 0.15	–
L. suebicus	CECT 5917		28.04 ± 0.07	74.7 ± 0	–
	CUPV 221[d]		16.88 ± 0.66	74.5 ± 0	+/β-D-glucan (Ibarburu, 2009)
	CUPV[d] 225, 226	2	38.64 ± 0.14	60.6 ± 0	+/HePS (Ibarburu, 2009)
Leuconostoc mesenteroides	NRRL B742		38.25 ± 0.32	74.2 ± 0.28	+/α-D-glucan (Monsan et al., 2001)
Leu. mesenteroides subsp *mesenteroides*	CECT 394		36.98 ± 0.28	74.2 ± 0.28	+/α-D-glucan (Monsan et al., 2001)
P. parvulus	CECT 7350		35.49 ± 0.15	74.2 ± 0	–
	CUPV 2.6[d]		16.80 ± 0.037	74.1 ± 0	+/β-D-glucan (Dueñas-Chasco et al., 1997)
	CUPV[d] 1, 2, 22, 23, 24, 26	6	10.31 ± 0.36	74.4 ± 0.19	+/β-D-glucan
P. damnosus	CECT 793,	2	36.90 ± 0.20	60.7 ± 0	–
	CECT 4694		37.38 ± 0.08	74.4 ± 0	
Pediococcus pentosaceus	CECT 4695[T]		35.19 ± 0.18	74.7 ± 0	–
O. oeni	CECT 217[T]		28.34 ± 0.26	74.7 ± 0	–
	CECT 218		34.40 ± 0.16	74.7 ± 0	–
	CUPV I4[d]		12.71 ± 0.44	74.2 ± 0.17	+/β-D-glucan and HePS (Ibarburu et al., 2007)
	CUPV[e] 302–309, 3010, 3011, 3013–3015, 3017–3020	17	31.18 ± 1.65	74.4 ± 0.26	–
Streptococcus thermophilus	NCIMB 700859		37.93 ± 0.25	74.2 ± 0	+/HePS

[a] Institutional names: CUPV, Colección de la Universidad del País Vasco (Spain); CECT, Colección Española de Cultivos Tipo (Spanish Type Culture Collection, University of Valencia, Burjassot, Spain); NCIMB, National Collection of Industrial and Marine Bacteria (Aberdeen Scotland, UK); NRRL, Agricultural Research Service (NRRL) Culture Collection (Peoria, Illinois, USA).
[b] qPCR parameters: $C_T \pm SD$ and $T_M \pm SD$.
[c] EPS phenotype (+ or −) assigned by visual examination. EPS type was previously established by determination of the structures in the corresponding references.
[d] Isolated from Basque Country ropy cider.
[e] Isolated from Basque Country non-ropy cider.
[T] Type strain.

phase culture of *P. parvulus* 2.6, covering the range from 1 to 10^5 CFU/reaction (determined by plate count on MRS); ii) Calibrated cell suspensions prepared from ten-fold dilutions of a log phase culture of each strain *P. parvulus* 2.6, and *Lactobacillus diolivorans* G77 in MRS, and *Oenococcus oeni* I4 in MLO (Ibarburu et al., 2007), covering the range from 1 to 10^5 CFU/ml (determined by plate count on MRS). In both assays, 1 ml from each dilution was subjected to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen). Purified DNA was recovered in 100 µl of ultra pure water (Sigma) and 5 µl of DNA solution was used as template for qPCR amplification. PCR amplification reactions were done in triplicate; and iii) Ten-fold dilutions of a 417 bp PCR amplification product, obtained by conventional PCR from strain *P. parvulus* 2.6 as previously described (Werning et al., 2006). This amplicon includes the targets for qPCR primers.

2.4.4. Sensitivity of qPCR assays for (1,3)(1,2)-β-D-glucan producing bacteria in an artificially contaminated cider

Sensitivity assays were carried out from calibrated cell suspensions prepared from ten-fold dilutions in cider of a log phase culture (36 h) of *P. parvulus* 2.6, obtained also by incubation in cider, and covering the range from 0.1 to 10^5 CFU/ml (determined by plate count on MRS). 1 ml aliquots were taken for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen) as described above. Purified DNA was recovered in 100 µl of ultra pure water (Sigma) and 5 µl of DNA solution was used as template for qPCR amplification. Reactions were carried out in triplicate per dilution.

2.5. Analysis of ropy and non-ropy cider samples by qPCR

The qPCR procedure was applied for the quantitative detection of gtf^+ LAB population during cidermaking in 13 tanks from two cider factories with frequent incidence of ropiness. Samples were collected during three consecutive months: during the simultaneous alcoholic and malolactic fermentations (October sample) and during maduration period, before bottling (November and December samples). Density (g/l) and pH measurements were determined as described by Dueñas et al. (1995). DNA was extracted from 1 ml aliquots using the DNeasy Blood and Tissue Kit (Qiagen) as described above. Purified DNA was recovered in 100 µl of ultra pure water (Sigma) and 5 µl of DNA solution was used as template for qPCR amplification. Reactions were carried out in triplicate. The absence of PCR inhibitors in the samples matrix was tested using as template purified DNA from *P. parvulus* 2.6 together with 5 µl DNA from the non-inoculated negative control. This reaction performed in triplicate was considered an internal amplification control.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPPS Inc., Chicago, Illionois, USA). With C_T values obtained from EPS^- negative strains, a lower limit of the unilateral confidence interval for a significance level of 5% was determined.

3. Results and discussion

3.1. Optimization of the qPCR reaction and specificity

In this work, a SYBR Green based qPCR procedure was developed by designing specific primers targeting the *gtf* gene present in all β-glucan producers (Werning et al., 2006). When tested *in silico* they showed 100% homology with glycosyltransferases encoding genes for (1,3)(1,2)-β-D-glucan synthesis in *P. damnosus* IOEB8801, *O. oeni* IOEB 0205, *L. diolivorans* G77 and *O. oeni* I4 sequences. Specificity was further tested *in vitro* by conventional PCR and the expected 151 bp amplicon, was only obtained in the (1,3)(1,2)-β-D-glucan producers (Table 1).

Improved specificity and qPCR reaction efficiency was obtained using 200 nM of each oligonucleotide and 4.5 mM of $MgCl_2$. Primer specificity was analyzed by qPCR using the optimized reaction conditions and purified DNA (10 ng/reaction) from the 54 strains listed in Table 1. This assay, followed by the melting curve analysis, confirmed the generation of a single PCR product from the β-glucan producers, with Tm values between 74.1 and 74.5. C_T values obtained ranged between 8.46 and 16.88 (average 12.67 ± 3.5). Despite amplification was only obtained in EPS producers by conventional PCR, the qPCR approach showed a slight amplification signal in some of the EPS^- LAB strains, both in reference strains and in cider isolates, with C_T values ranging from 28.04 to 37.76. These results indicate a low efficiency in amplification that could be attributed to either the presence of these targets in low numbers or to the occurrence of similar but not identical targets. From the statistical analysis of the C_T data of these EPS^- strains, a critical value of 28.54 with the significant level set at 0.05 was established, in order to assess the detection of (1,3)(1,2)-β-D-glucan producing strains. They rendered C_T values significantly higher ($P(C_T<28.54) = 0.05$) than those of the β-glucan producers. This C_T (28.54) value was considered the threshold to discriminate (1,3)(1,2)-β-D-glucan producing strains from non producers.

3.2. Standard curves and detection limits of the qPCR

As shown in previous studies, the *gtf* gene is present in most of the naturally occurring LAB species in cider, such as *O. oeni*, *Lactobacillus suebicus, Lactobacillus collinoides, P. parvulus,* and *L. diolivorans* (Werning et al., 2006). Of them, *P. parvulus,* and *L. diolivorans* are the predominant species meanwhile *O. oeni* became the most abundant microbiota during malolactic fermentation. In order to approach quantification of β-glucan producers, standard curves were constructed using *P. parvulus* 2.6, *O. oeni* I4 and *L. diolivorans* G77 as representative producer strains. Standard curves derived from both, purified DNA and calibrated cell suspensions of *P. parvulus* 2.6, showed a linear correlation between Log_{10} input DNA and C_T in the range from 10^{-1} to 10^5 CFU/reaction. Slope values were −3.58 and −3.31, respectively, very close to the theoretical optimum of −3.32 (Higuchi et al., 1993) and R^2 (square correlation coefficient after the linear regression) values were above 0.98, indicating that the SYBR Green PCR assay was highly linear (Table 2). Quantification limit, defined as the lowest concentration in which the linearity is maintained, was established in 0.43 and 26 CFU/reaction (5.2×10^2 CFU/ml), using purified DNA and cell suspensions, respectively. Moreover, detection was possible in both cases at the lowest level assayed, reaching 0.43 and 0.26 CFU/reaction (5.2 CFU/ml), respectively. A high correspondence was found between C_T values obtained from purified DNA and calibrated cell suspensions of *P. parvulus* 2.6 for equivalent concentrations (CFU/reaction), which indicates that the DNA extraction procedure used was efficient. Standard curves obtained from calibrated cell suspensions of the (1,3)(1,2)-β-D-glucan producing *O. oeni* I4 and *L. diolivorans* G77 strains showed slope values similar to the one corresponding to the cell suspensions of *P. parvulus* 2.6 (Table 2). The quantification limit was set at 58 and 393 CFU/reaction for *O. oeni* I4 and *L. diolivorans* G77, respectively. Therefore, the quantification range is between 10 and 10^5 CFU/reaction, similar to that of *P. parvulus* 2.6, except for *L. diolivorans* G77 that ranged from 10^2 to 10^5 CFU/reaction.

Due to the biodiversity of β-glucan producing strains and the lack of information about the copy number of *gtf* gene in each species, a third approach was tested to construct a "universal" standard curve to approach quantification of *gtf* gene in cider, using a ten-fold serially diluted PCR product from *P. parvulus* 2.6, that included the qPCR primer sequences. Linear regression analysis of the C_T values showed good linearity (R^2 0.998) between 3.19 and 3.19×10^7 molecules/reaction (Table 2).

In summary, C_T values obtained from calibrated cell suspensions of *P. parvulus* 2.6, and *O. oeni* I4 showed a linear relationship over 5 orders of magnitude, and a quantification range between 10 and 10^5 CFU/reaction. However, when using *P. parvulus* 2.6 DNA (either

Table 2
Standard curves, amplification efficiency and C_T values obtained from *P. parvulus* 2.6 genomic, DNA, DNA extracted from calibrated cell suspensions of *P. parvulus* 2.6, *O. oeni* I4 or *L. diolivorans* G77, and PCR amplification product from *P. parvulus* 2.6.

	DNA		Cell suspensions						PCR product	
	P. parvulus 2.6		*P. parvulus* 2.6		*O. oeni* I4		*L. diolivorans* G77		*P. parvulus* 2.6	
R^2	0.998		0.984		0.977		0.987		0.998	
sc	$y = -3.58x + 35.68$		$y = -3.31x + 31.28$		$y = -3.41x + 36.20$		$y = -4.66x + 35.37$		$y = -3.64x + 33.99$	
AE	0.9		1		0.96		0.64		0.88	
n	7		5		5		4		7	
	CFU/reac[a]	$C_T \pm SD$[b]	CFU/reac[c]	$C_T \pm SD$[b]	CFU/reac[c]	$C_T \pm SD$[b]	CFU/reac[c]	$C_T \pm SD$[b]	molecules/reac[d]	$C_T \pm SD$[b]
	–	–	–	–	–	–	–	–	3.19×10^6	12.14 ± 0.13
	4.3×10^5	14.70 ± 0.138	2.6×10^5	14.26 ± 0.58	5.83×10^5	14.76 ± 0.24	3.93×10^5	16.38 ± 0.31	3.19×10^5	15.61 ± 0.05
	4.3×10^4	17.82 ± 0.16	2.6×10^4	18.74 ± 0.16	5.83×10^4	18.22 ± 0.12	3.93×10^4	21.44 ± 0.98	3.19×10^4	19.30 ± 0.21
	4.3×10^3	21.33 ± 0.11	2.6×10^3	22.01 ± 0.04	5.83×10^3	23.09 ± 0.33	3.93×10^3	26.99 ± 0.49	3.19×10^3	22.99 ± 0.27
	4.3×10^2	25.06 ± 0.05	2.6×10^2	24.20 ± 0.69	5.83×10^2	27.27 ± 0.42	3.93×10^2	30.06 ± 0.38	3.19×10^2	26.39 ± 0.23
	43	28.40 ± 0.15	26	28.19 ± 0.15	58.3	30.33 ± 0.08	39.3	31.95 ± 0.41[§]	31.9	30.00 ± 0.41
	4.3	32.24 ± 0.16	2.6	27.47 ± 0.45[§]	5.83	31.51 ± 0.24[§]	3.93	31.97 ± 0.61[§]	3.19	34.52 ± 0.40
	0. 43	35.69 ± 0.32	0.26	26.17 ± 1.23 [§]	–	–	–	–	–	–

R^2, square correlation coefficient, sc, standard curve created by plotting C_T value versus log concentration. Equation describing data obtained by linear regression, AE, Amplification efficiency, n, number of points on the standard curve corresponding to the number of dilutions of DNA.

[a] CFU/reaction calculated from DNA concentrations (4.2 ng corresponds to 4.3×10^5 CFU).

[b] Mean C_T values ± standard deviation (SD) from three independent cultures, each performed in triplicate.

[c] Mean values of three separate cell cultures expressed as CFU/reaction.

[d] Molecules/reaction calculated from PCR amplification product.

[§] Values that are not linear and they were not considered for the linear regression analysis.

Table 3
Quantification of β-glucan producing LAB populations in ropy ciders using SYBR Green qPCR.

Sample	$C_T \pm$ DS[a]	LAB plating (Log CFU/ml)[b]	Quantification from standard curves (Log CFU/ml)					
			Amplicon	DNA	*P. parvulus* 2.6 cell suspensions in MRS	*P. parvulus* 2.6 cell suspensions in cider	*O. oeni* I4 cell suspensions in MRS	*L. diolivorans* G77cell suspensions in MRS
S1	18.31 ± 0.61	5.38	6.19	6.75	5.82	6.14	7.15	6.91
S2	18.46 ± 0.46	5.41	6.15	6.71	5.77	6.10	7.10	6.86
S3	19.33 ± 0.47	6.16	5.91	6.47	5.51	5.88	6.85	6.61
S4	17.67 ± 0.13	7.16	6.36	6.93	6.01	6.31	7.33	7.09
S5	15.14 ± 0.46	7.09	7.5	7.64	6.78	6.98	8.08	7.81
S6	15.34 ± 0.13	5.00	7	7.58	6.72	6.92	8.02	7.76
S7	25.11 ± 0.62	7.41	3.72	4.25	3.16	3.76	4.55	4.36
S8	21.55[c] ± 0.14	6.23	4.70	5.25	4.24	4.69	5.59	5.38

[a] Mean C_T value ± standard deviation (SD).
[b] Total counts on MRS agar.
[c] Value corresponding to the dilution ½ of the sample.

total genomic DNA or the 417 bp amplicon) linearity was extended over 7 log orders.

3.3. Sensitivity of the qPCR assay on artificially contaminated cider samples

The qPCR procedure developed was tested for *gtf*$^+$ LAB detection using cider contaminated with serial ten-fold dilutions of *P. parvulus* 2.6. The regression analysis showed a good linearity for concentrations between 16 and 1.65×10^5 CFU/reaction ($R^2 = 0.97$) and the slope was of −3.81. These values indicate that linearity of the amplification reaction was also kept in the inoculated cider. However, amplification efficiency (AE = 0.83) was slightly lower than that obtained from cell suspensions in MRS (AE = 1). Therefore, the sensitivity of qPCR evaluated on calibrated cell suspensions of *P. parvulus* 2.6, in cider or in MRS broth, showed detection limits around one log lower than the values obtained from purified DNA. This result can be explained by the DNA loss produced during the extraction step (Alarcón et al., 2006; Fernández et al., 2006). Furthermore, the standard curves obtained in both, MRS or cider, showed a linear relationship over 5 magnitude orders, allowing quantification in a range from 10 to 10^5 CFU/reaction, but the amplification efficiency was lower in the samples from inoculated cider. This decrease could be explained by the presence of inhibitory compounds such as polyphenols in the beverage. Similar inhibitory effect on PCR reaction has been reported in wines by some authors (Delaherche et al., 2004; Martorell et al., 2005). With respect to quantification and detection limits, they are in the same range than the ones reported for other LAB i.e. *Lactobacillus sakei* (Martín et al., 2006) and *Leuconostoc mesenteroides* (Elizaquível et al., 2008).

3.4. Detection of (1,3)(1,2)-β-D-glucan producing bacteria along cidermaking process and ropy bottled ciders

As sulfiting is not a usual practice in Basque Country cidermaking, alcoholic and malolactic fermentations take place simultaneously and, thereafter, LAB population became always dominant during the maturation period (Dueñas et al., 1995). In addition, natural ciders are not microbiologically stabilized before bottling and, as a consequence, bottled ciders are prone to spoilage by β-glucan producers. In this context, the developed qPCR method was used for quantification of the (1,3)(1,2)-β-D-glucan producers along cidermaking in 13 vats from two cider factories, with frequent incidence of ropiness (Table 3). In some tanks, a statistically significant decrease of C_T values was found ($P<0.05$), with Tm values of the single amplification product specific for the β-glucan producing LAB, ranging from 74.1 to 74.5, as described above,. All these results indicated a clear increase in LAB *gtf* $^+$ populations during the maturation period, after alcoholic and malolactic fermentations.

The qPCR procedure was also used to estimate the *gtf*$^+$ populations in 8 ropy ciders and they were compared to the levels of total lactic acid bacteria estimated by total counts on MRS. Results are shown in Table 4. Amplification was detected in all samples and melting curves analysis showed that Tm values ranged from 74.1 to 74.4. The C_T values of six ciders (S1 to S6) ranged between 15.14 and 19.33 and were significantly lower than those found throughout the cidermaking process (Table 3), demonstrating a clear enrichment of *gtf*$^+$ LAB populations, in comparison to the

Table 4
Evolution of physico-chemical parameters and β-glucan producing bacteria along cidermaking process.

Sample	pH			Density (g/l)			qPCR SYBR Green ($C_T \pm$SD)[b]		
	1[a]	2	3	1	2	3	1[a]	2	3
I5	3.77	3.95	3.93	1.027	0.997	0.995	32.59 ± 0.64	24.54 ± 0.37*	24.19 ± 0.30*
I7	3.75	4.01	3.96	1.029	0.995	0.994	35.21 ± 0.68	31.81 ± 0.63	31.88 ± 0.16
I8	3.77	3.99	3.92	1.027	0.998	0.996	29.68 ± 0.40	26.07 ± 0.44*	28.48 ± 0.26*
I9	3.72	4.03	3.96	1.038	0.999	0.996	32.65 ± 0.49	28.35 ± 0.01*	29.93 ± 0.61
I10	3.71	4.03	3.95	1.001	0.997	0.995	35.44 ± 0.30	33.23 ± 0.32	26.17 ± 0.08*
I12	3.65	4.02	4.00	1.022	0.995	0.995	33.27 ± 0.26	33.96 ± 0.25	30.93 ± 0.67
I13	3.68	4.04	4.04	1.027	0.999	0.997	36.5 ± 0.31	33.18 ± 0.01	37.61 ± 0.67
I14	3.77	3.99	3.95	1.011	0.997	0.996	34.56 ± 0.75	30.47 ± 0.25	30.33 ± 0.37
A2	3.99	3.97	3.94	1.025	0.997	0.995	40	27.26 ± 0.41*	27.13 ± 0.22*
A4	4.18	4.07	4.02	1.027	0.998	0.995	32.79 ± 0.87	34.54 ± 0.46	30.11 ± 0.09
A5	3.92	4.12	4.03	1.035	1.000	0.998	35.58 ± 0.60	26.07 ± 0.24*	nd
A6	4.15	4.11	nd	1.023	0.998	0.996	40	31.53 ± 0.45	30.46 ± 0.25
A10	3.89	3.95	3.88	1.010	1.001	0.997	40	40	40

nd, not determined.
[a] Sampling event: 1, October; 2, November; 3, December.
[b] Mean C_T value ± standard deviation (SD). Reactions were performed in triplicate.
* These samples were considered positive for the presence of β-glucan producers with $P<0.05$.

levels found along this period. Sample S7 showed a C_T value of 25.11 and sample S8 did not show amplification until it was diluted one-half (external amplification control indicated absence of PCR inhibitors). Quantification of gtf^+ populations approached by interpolating C_T values in the different standard curves, showed values within a log range, which corroborates the adequate performance of the developed qPCR method as a rapid procedure to estimate β-glucan producing LAB in cider. Quantification of gtf^+ populations derived from the different standard curves showed no significant differences. Therefore, the amplicon-derived standard curve is proposed for the routine estimation of gtf^+ populations in cider. Although cell numbers were probably overestimated because of the qPCR inability to differentiate between living and dead cells (Neeley et al., 2005), our results indicate that in most of spoiled ciders the gtf^+ population is the predominant microbiota, ranging from 10^5 to 10^7 CFU/ml.

On the basis of these results, we conclude that the SYBR Green I assay developed in this study is suitable for a sensitive and rapid detection of the (1,3)(1,2)-β-D-glucan LAB producers and provides a good tool for its early detection during cider making process. This knowledge will allow cidermakers to make decisions to control and avoid spoilage of ciders by sulfiting or by other microbiological stabilization method. This constitutes a great advantage in comparison to current situation, in which cider contamination by β-glucan producing lactic acid bacteria is not detected until occurrence of ropiness and the beverage is refused by the consumer.

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References

Alarcón, B., Vicedo, B., Aznar, R., 2006. PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. Journal of Applied Microbiology 100, 352–364.

Delaherche, A., Claisse, O., Lonvaud-Funel, A., 2004. Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. Journal of Applied Microbiology 97, 910–915.

De Vuyst, L., De Vin, F., Vaningelmem, F., Degeest, B., 2001. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. International Dairy Journal 11, 687–707.

Dols-Lafargue, M., Lee, H.Y., Le Marrec, C., Heyraud, A., Chambat, G., Lonvaud-Funel, A., 2008. Characterization of *gtf*, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in wine. Applied and Environmental Microbiology 74, 4079–4090.

Dueñas, M.T., Irastorza, A., Fernández, K., Bilbao, A., 1995. Heterofermentative *Lactobacilli* causing ropiness in Basque country ciders. Journal of Food Protection 58, 76–80.

Dueñas-Chasco, M.T., Rodríguez-Carvajal, M.A., Tejero-Mateo, P., Franco-Rodríguez, G., Espartero, J.L., Irastorza-Iribas, A., Gil-Serrano, A.M., 1997. Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6. Carbohydrate Research 303, 453–458.

Dueñas-Chasco, M.T., Rodríguez-Carvajal, M.A., Tejero-Mateo, P., Espartero, J.L., Irastorza–Iribas, A., Gil-Serrano, A.M., 1998. Structural analysis of the exopolysaccharides produced by *Lactobacillus* spp. G-77. Carbohydrate Research 307, 125–133.

Elizaquível, P., Chenoll, E., Aznar, R., 2008. A TaqMan-based real-time PCR assay for the specific detection and quantification of *Leuconostoc mesenteroides* in meat products. FEMS Microbiology Letters 278, 62–71.

Fernández, K., Dueñas, M., Irastorza, A., Bilbao, A., del Campo, G., 1995. Characterization and DNA plasmid analysis of ropy *Pediococcus damnosus* spp. strains isolated from Basque Country ciders. Journal of Food Protection 59, 35–40.

Fernández, M., Del Río, B., Linares, M.D., Martín, M.C., Alvarez, M.A., 2006. Real-time polymerase chain reaction for quantitative detection of histamine-producing bacteria: use in cheese production. Journal of Dairy Science 89, 3763–3769.

Garai, G., Dueñas, M.T., Irastorza, A., Martín–Alvarez, P.J., Moreno-Arribas, M.V., 2006. Biogenic amines in natural ciders. Journal of Food Protection 69, 3006–3012.

Garai-Ibabe, G., Areizaga, J., Aznar, R., Elizaquivel, P., Prieto, A., Irastorza, A., Dueñas, M.T., 2010. Screening and selection of 2-branched (1, 3)-β-D-glucan producing lactic acid bacteria and exopolysaccharide characterization. Journal of Agricultural and Food Chemistry 58, 6149–6156.

González, A., Hierro, N., Poblet, M., Mas, A., Guillamón, J.M., 2006. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. FEMS Microbiological letters 254, 123–128.

Grobben, G.J., van Casteren, W.H.M., Schools, A., Oosterveld, A., Sala, G., Smith, M.R., Sikkema, J., de Bont, J.A.M., 1997. Analysis of exopolysaccharides produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Applied Microbiology and Biotechnology 48, 516–521.

Hierro, N., Esteve-Zarzoso, B., Mas, A., Guillamón, J.M., 2007. Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. FEMS Yeast Research 7, 1340–1349.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Ibarburu, I. 2009. Bacterias lácticas productoras de exopolisacáridos: análisis estructural de polisacáridos y detección molecular de estirpes que sintetizan β-(1,3)(1,2)-D-glucanos. PhD thesis, Universidad del País Vasco (UPV/EHU), Donostia-San Sebastián, Spain.

Ibarburu, I., Soria-Díaz, M.E., Rodríguez-Carvajal, M.A., Velasco, S.E., Tejero-Mateo, P., Gil-Serrano, A.M., Irastorza, A., Dueñas, M.T., 2007. Growth and exopolysaccharide (EPS) production by *Oenococcus oeni* I4 and structural characterization of their EPSs. Journal of Applied Microbiology 103, 477–486.

Llaubères, R.M., Richard, B., Lonvaud, A., Dubourdieu, D., Fournet, B., 1990. Structure of an exocellular β-D-glucan from *Pediococcus* sp., a wine lactic bacteria. Carbohydrate Research 203, 103–107.

McKillip, J.L., Drake, M., 2004. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. Journal of Food Protection 67, 823–832.

Martín, B., Jofré, A., Garriga, M., Pla, M., Aymerich, T., 2006. Rapid quantitative detection of *Lactobacillus sakei* in meat and fermented sausages by real-time PCR. Applied and Environmental Microbiology 72, 6040–6048.

Martorell, P., Querol, A., Fernández–Espinar, M.T., 2005. Rapid identification and enumeration of *Saccharomyces cerevisiae* cells in wine by Real-Time PCR. Applied and Environmental Microbiology 71, 6823–6830.

Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot, R.M., Remaud–Simeon, M., 2001. Homopolysaccharides from lactic acid bacteria. International Dairy Journal 11, 675–685.

Neeley, E.T., Phister, T.G., Mills, D.A., 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. Applied and Environmental Microbiology 71, 8954–8957.

Ruas-Madiedo, P., Abraham, A., Mozzi, F., de los Reyes-Gavilán, C.G., 2008. Functionality of exopolysaccharides produced by lactic acid bacteria. In: Mayo, B., López, P., Pérez-Martín, G. (Eds.), Molecular aspects of lactic acid bacteria for traditional and new applications. Research Signpost, Kerala, India, pp. 137–166.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. International Journal of Food Microbiology 122, 126–134.

Tessonnière, H., Vidal, S., Barnavon, L., Alexandre, H., Remize, E., 2009. Design and performance testing of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. International Journal of Food Microbiology 129, 237–243.

Walling, E., Gindreau, E., Lonvaud-Funel, A., 2005. A putative glucan synthase gene *dps* detected in exopolysaccharide-producing *Pediococcus damnosus* and *Oenococcus oeni* strains isolated from wine and cider. International Journal of Food Microbiology 98, 53–62.

Werning, M.L., Ibarburu, I., Dueñas, M.T., Irastorza, A., Navas, J., López, P., 2006. *Pediococcus parvulus gtf* gene encoding the GTF glycosyltransferase and its application for specific PCR detection of β-D-glucan-producing bacteria in foods and beverages. Journal of Food Protection 69, 161–169.

Werning, M.L., Corrales, M.A., Prieto, A., Fernández de Palencia, P., Navas, J., López, P., 2008. Heterologous expression of a 2-substituted-1, 3)-β-D-glucan in *Lactococcus lactis*. Applied and Environmental Microbiology 74, 5259–5262.