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

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Keywords: (1,3)(1,2)- β -D-glucan, lactic acid bacteria, Real-time PCR, spoilage, cider

ABSTRACT

Ropiness in natural cider is a relatively frequent alteration, which is derived from the production of exopolysaccharides (EPS) by some lactic acid bacteria. Ropy ciders are mainly founded after bottling, being refused by the consumers. Most of the EPS+ isolates from ropy cider synthesize a 2-substituted (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 and targeting *gtf* gene was developed for detection and quantification of these bacteria in cider. Specific primers were designed and amplification conditions were optimized. All (1,3)(1,2)- β -D-glucan producing strains showed amplification with specific primers. Quantification was linear over a range of 5 logarithmic units, both with serial decimal dilutions of purified DNA and with *P. parvulus* 2.6 calibrated cell suspensions. Furthermore, the procedure has been applied to monitor the evolution of these spoiling bacterial populations in cider.

INTRODUCTION

Lactic acid bacteria (LAB) are able to produce a wide variety of exopolysaccharides (EPSs) which can be used as 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 the alcoholic beverages. In cider (Fernández et al., 1995) and wine (Llaubères et al., 2008), 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 o correction of acidity) are not applied (Garai-Ibabe 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 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-substituted (1,3)- β -D-glucan and belong to the genera *Pediococcus* (Llaubères et al., 1990; Dueñas et al., 1997), *Lactobacillus* (Dueñas et al., 1998) and *Oenococcus* (Ibarburu et al., 2007; Dols-Lafargue et al., 2008). (1,3)- β -D-glucans are considered as biological response modifiers (Sletmoen and Stokke, 2008) and for this reason, the (1,3)(1,2)- β -D-glucan producer *Pediococcus parvulus* 2.6 (Mårtensson et al., 2003) was tested for fermentation of oat-based products to increase the potentially healthy properties of the native (1,3)(1,4)- β -D-glucan. 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). Cloning of the *gtf* gene from *P. parvulus* 2.6 and functional expression of its encoded glycosyltransferase in *Streptococcus pneumoniae* (Werning et al., 2006) and *Lactococcus lactis* (Werning et al., 2008) revealed that this enzyme is indeed responsible of the synthesis of the β -D-glucan. This *gtf* gene 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 *L. diolivorans* G77, *P. damnosus* and *O. oeni* I4 revealed that it possesses a 100, 99.9 and 98.8% identity, respectively, with their counterparts in *P. 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 glycosyltransferase domain and the fifth trans-membrane 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.

MATERIAL AND METHODS

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. 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% CO₂. To isolate and select rosy 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).

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 x g for 10 minutes, pellets were washed twice with 1 ml of Ringer's solution (Oxoid, Hampshire, England) and centrifuged at 8000 x g for 5 minutes. 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 minutes at 37 °C, samples were homogenized with 200 µl of lysis buffer and proteinase K. The homogenate was incubated at 70 °C for 30 minutes. DNA was purified through the column using two cleaning buffers supplied in the kit. DNA was eluted in 100 µl of ultra pure water and 5 µl was used for PCR amplification.

Conventional PCR. Amplification of the *gtf* gene by conventional PCR was carried out by using primers GTFF and GTFR that delimit a 417 bp fragment (Werning et al. 2006). GTFF primer is located in the codifying sequence of the glycosyltransferase domain and GTFR is located in the fifth transmembrane segment. PCR amplifications were conducted in a final volume of 50 µl containing: 1x PCR buffer (Bioline), 1.25 U of BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany), 0.2 µM of each primer, 3.5 mM of MgCl₂, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 25 µM of each dNTP and 5 µl of DNA template. DNA isolated from *P. parvulus* 2.6 was used as the amplification positive control. Negative control of amplification was performed with 5 µl of water instead of DNA template. Reactions were carried out in a 7500 Real Time PCR System (Applied Biosystems, Foster City, Calif.) thermal cycler. PCR products (15 µl) were electrophoresed through 0.8 % agarose (Sigma-Aldrich, Steinheim, Germany) gel in TAE buffer (40 mM Tris-acetate, pH 8.0; 1 mM Na₂EDTA). Cycling conditions were 1 x (94 °C, 5 min); 30 x (95 °C, 1 min; 55 °C, 1 min; 72 °C, 30 s), and 1 x (72 °C, 10 min).

qPCR SYBR Green I-based assay

Primer design. Two primers were designed with the Beacon Designer software (Bio-Rad, Spain) targeting the *gtf* gene encoding for the GTF glycosyltransferase (Wernig 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, GeneBank accession number AY551933, Werning et al., 2006) for each primer were GTF3 (5'-ATCAAGTCAAAGACCATAAGTCTCTATC-3', 2365-2392 in the putative carboxyl glycosyltransferase 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).

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 μ l, 1x buffer (SYBR-Green I PCR Buffer), 200 μ M each dATP, dCTP, dGTP, and 400 μ M 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 μ l (10 ng) of template DNA. Different concentrations of $MgCl_2$ (1.5, 3 and 4.5 mmol/l) and primer (100, 200 and 300 nmol/l 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, for each amplification plot.

Melting-curve analysis. Melting-curves were programmed in order to check the expected amplification product. The thermal protocol for dissociation is defined as 15 s at 95 °C, 20 s at 60 °C and 20 min slow ramp between 60 and 95 °C, after the qPCR program. The data for the dissociation curve is captured during this slow ramp. The melting curve was visualized with the software in the “Dissociation Window” using the corresponding 7500 System SDS software procedure (Applied Biosystems).

Quantification assays. Standard curves were calculated for quantification purposes using: i) Ten-fold dilutions of genomic DNA extracted from one ml of a log phase culture of *P. parvulus* 2.6, covering the range from 1 to 10⁵ CFU/reaction (determined by plate count on MRS); ii) Calibrated cell suspensions prepared from ten-fold dilutions of an overnight 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 10⁵ CFU/ml (determined by plate count on MRS). iii) Calibrated cell suspensions in cider prepared from ten-fold dilutions of a 36 h culture of *P. parvulus* 2.6. One 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 double-distilled water and 5 µl of DNA solution was used as template for qPCR amplification. PCR amplification reactions in triplicate were done per dilution; 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.

Sensitivity of qPCR assays for (1,3)(1,2)-β-D-glucan producing bacteria in artificially contaminated cider. Sensitivity assays were carried out in cider contaminated with a ten-fold serially diluted overnight culture of strain *P. parvulus* 2.6, *O. oeni* I4 or *L. diolivorans* G77, in separate, covering the range from 0.1 to 10⁵ CFU/ml (determined by plate count on MRS). One 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 double-distilled water and 5 µl of DNA solution was used as template for qPCR amplification. Reactions were carried out in triplicate per dilution.

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 cellars from two cider factories with frequent incidence of ropiness. Samples were collected during three consecutive months along alcoholic and malolactic fermentations and subjected to pH and density (g/l) measurements, in accordance with the methodology described by Dueñas et al., 1995. DNA was extracted from one ml aliquots using the DNeasy Blood and Tissue Kit (Qiagen) as described above. Purified DNA was recovered in 100 µl of double-distilled water 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 as an internal amplification control.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, 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.

RESULTS

Optimization of the qPCR reaction and specificity

A SYBR Green based qPCR procedure was developed by designing specific primers targeted to the *gtf* gene present in all β-glucan producers (Wernig et al., 2006). When tested *in silico* they

specifically matched the glycosyltransferases encoding genes for (1,3)(1,2)- β -D-glucan synthesis showing 100% homology in *P. damnosus* IOEB8801, *O. oeni* IOEB 0205, *L. diolivorans* G77 and *O. oeni* I4 sequences. In addition, primers effectiveness and specificity were tested *in vitro* by conventional PCR using purified DNA from LAB strains belonging to different species and genera (Table 1). They included β -glucan and heteropolysaccharide (HePS) producing strains and non-EPS producers. The expected 151 bp amplicon was only obtained in the (1,3)(1,2)- β -D-glucan producers.

Improved specificity and qPCR reaction efficiency was obtained using 200 nM of each oligonucleotide and 4.5 mM of MgCl₂. 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. Amplification was obtained for the β -glucan producing strains, the melting-curve analysis showed T_m values between 74.0 and 74.7 °C and C_T values obtained were in the range of 10.05-14.21 (average 11.78 $\pm$ 1.54), with the exception of strain *L. suebicus* CUPV 221 that showed a C_T of 25. Amplification signal was also observed in some EPS⁻ strains, both reference and cider isolates, showing C_T values ranging from 28.04 to 37.75. 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 statistical analysis of C_T data of these EPS⁻ strains, a critical value of 28.54 with the significant level set of 0.05 was established, in order to assess detection of (1,3)(1,2)- β -D-glucan producing strains.

Standard curves and detection limits of the qPCR

Table 2 shows the standard curves, amplification efficiency and C_T values derived from purified DNA of *P. parvulus* 2.6 and calibrated cell suspensions of *P. parvulus* 2.6 and *L. diolivorans* G77 in MRS and *O. oeni* I4 in MLO. Representative amplification plots corresponding to standard quantification curves of *P. parvulus* 2.6, DNA and cell suspensions are shown in Figure

1 1. They showed a linear correlation between Log_{10} input DNA and C_T with slope and R^2 (square
2 correlation coefficient after the linear regression) values very similar in three independent assays.
3 The slopes of the linear regression curves, calculated using genomic DNA or calibrated cell
4 suspensions as template were -3.58 and -3.31, respectively and close to the theoretical optimum
5 of -3.32 (Higuchi et al., 1993). R^2 values were above 0.98, indicating that the SYBR-Green PCR
6 system was highly linear. Quantification limit, defined as the lowest concentration in which the
7 linearity is maintained, was established in 0.43 and 26 CFU/reaction, using purified DNA and
8 cell suspensions, respectively. However, detection was possible in both cases at the lowest level
9 assayed, reaching 0.43 and 0.26 CFU/reaction, respectively. A high correspondence was found
10 between C_T values obtained at the same concentrations (CFU/reaction) from purified DNA or
11 calibrated cell suspensions of *P. parvulus* 2.6, which indicates that the DNA extraction
12 procedure used was efficient. Thus, both standard curves could be applied for quantification of
13 β -glucan producing LAB in natural cider, at levels above 26 CFU/reaction.

14 Regarding standard curves constructed from calibrated cell suspensions of *O. oeni* I4 and *L.*
15 *diolivorans* G77, both had slope values similar to the one corresponding to cell suspensions of *P.*
16 *parvulus* 2.6 (Table 2), with a correlation coefficient slightly lower for *L. diolivorans* G77. The
17 quantification limit was set at 58.3 and 39.3 CFU/reaction for *O. oeni* I4 and *L. diolivorans* G77,
18 respectively. Therefore, the quantification range was similar to that of *P. parvulus* 2.6, ranging
19 from 10 to 10^5 CFU/reaction. Due to the biodiversity of β -glucan producing strains and the lack
20 of information about the copy number of *gtf* gene in each species, a third approach was tested to
21 construct a "universal" standard curve to approach quantification of *gtf* gene in cider, using a ten-
22 fold serially diluted PCR product from *P. parvulus* 2.6 (Figure 2). Linear regression analysis of
23 the C_T values showed good linearity (R^2 0.998) between 3.19 and 3.19×10^7 molecules/reaction.
24 In summary, C_T values obtained from calibrated cell suspensions of *P. parvulus* 2.6, *O. oeni* I4,
25 and *L. diolivorans* G77 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 total genomic DNA or the 417 bp amplicon) linearity was extended over 7 log orders.

Sensitivity of the RTi-PCR 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).

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

The evolution of *gtf*⁺ LAB population during cidermaking was analyzed in 13 cellars from two cider factories with frequent incidence of ropiness, by qPCR and plate count on MRS. Samples were collected during three consecutive months along alcoholic and malolactic fermentations. Table 3 shows the physico-chemical parameters, pH and density, and the microbiological variations of β -glucan-producing LAB populations, measured by qPCR, over the cidermaking period. In some cellars a tendency to decrease of the C_T values was observed indicating an increase in LAB *gtf*⁺ populations.

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 showed in Table 4. C_T values of six ciders (S1 to S6) ranged between 15.14 and 19.33 and were significantly lower than those found in fermenting musts (Table 3), indicating

1 that these ropy ciders had higher spoiling *gtf*⁺ populations. Sample S7 showed a C_T value of
2 25.11 and sample S8 did not show amplification until it was diluted one-half (external
3 amplification control indicated absence of PCR inhibitors). Quantification of *gtf*⁺ populations
4 was performed by interpolating C_T values in the different standard curves. Levels obtained from
5 the different approaches were within a log range, which corroborates the adequate performance
6 of the developed qPCR method as a rapid procedure to estimate β -glucan producing LAB in
7 cider. Quantification of *gtf*⁺ populations derived from the different standard curves showed no
8 significant differences. Therefore, the amplicon-derived standard curve is proposed for the
9 routine estimation of *gtf*⁺ populations in cider.

11 DISCUSSION

12 (1,3)(1,2)- β -D-glucan producing lactic acid bacteria are responsible of the alteration named
13 ropiness or oiliness in natural cider which lead to high economic losses. On the other hand, the
14 synthesis of the β -glucan by these strains provides an interesting feature, as (1,3)- β -D-glucans
15 are a group of biologically active biopolymers and some of the β -glucans producers are being
16 tested as potential probiotics (Fernández de Palencia et al., 2009). In this work we have
17 developed a SYBR-Green I dye based real-time PCR method for the rapid detection and
18 estimation of (1,3)(1,2)- β -D-glucan producing lactic acid bacteria.

19 The *gtf* gene has been detected in all (1,3)(1,2)- β -D-glucan producing LAB (Werning et al.,
20 2006) including different bacterial species usually found in cider. Therefore detection of these
21 cider spoiling bacteria was based on the use of specific primers GTF3/GTF4 targeting the *gtf*
22 gene. The qPCR assay, followed by the melting curve analysis, confirmed the generation of a
23 single PCR product from the β -glucan producers with a T_m of 74 °C. Despite amplification was
24 only obtained in EPS producers by conventional PCR, the qPCR approach showed a slight
25 amplification signal in some of the EPS⁻ LAB strains tested which might indicate the presence of

1 these targets in low numbers or to the occurrence of similar but not identical targets. They
2 rendered C_T values significantly higher ($P(C_T < 28.54) = 0.05$) than those of the β -glucan
3 producers. This C_T (28.54) value was considered as the threshold to discriminate (1,3)(1,2)- β -D-
4 glucan producing strains from non producers.

5 The standard curves constructed using DNA or calibrated cell suspensions from *P. parvulus* 2.6
6 (ranging from 10^{-1} to 10^5 CFU/reaction) showed good linearity which confirmed the suitability
7 of the assay for the quantification of the β -glucan producing *P. parvulus* 2.6. Due to the
8 biodiversity of β -glucan producing strains and the lack of information about the copy number of
9 *gtf* gene in each species, a "universal" standard curve was constructed to approach quantification
10 of *gtf* gene in cider, based on a PCR amplification product from *P. parvulus* 2.6 that includes the
11 qPCR primer sequences. The linearity was maintained over 7 log magnitude orders.

12 The sensitivity of qPCR evaluated on calibrated cell suspensions of *P. parvulus* 2.6, incubated in
13 MRS broth or cider showed a detection limit of 2.6 and 16 CFU/reaction, respectively. Similarly
14 to other studies, these values were around one log lower than the values obtained from purified
15 DNA. This result has been explained by the DNA extraction step from cells (Alarcón et al.,
16 2006; Fernández et al., 2006). The standard curves obtained in both, MRS or cider, showed a
17 similar linear relationship over 5 magnitude orders, allowing quantification in a range from 10 to
18 10^5 CFU/reaction, but the amplification efficiency was lower in the samples from inoculated
19 cider. This decrease could be explained by the presence of inhibitory compounds in the
20 beverage, such as polyphenols. Similar inhibitory effect on PCR reaction has been reported in
21 wines by some authors (Delaherche et al., 2004; Martorell et al., 2005). With respect to the
22 quantification limits/detection limits, they are in the same range than the ones reported for other
23 LAB i.e. *Lactobacillus sakei* (Martin et al., 2006) and *Leuconostoc mesenteroides*.

24 In a previous work, we showed that the *gtf* gene is largely spread in LAB microbiota of
25 natural cider. It has been found in most of the species presented in cider, such as *O. oeni*, *L.*

1 *suebicus*, *L. collinoides*, *P. parvulus*, and *L. diolivorans* (Werning et al., 2006), being the two
2 last species the majority ones. On the other hand, as *O. oeni* is the most abundant microbiota
3 during malolactic fermentation in cider, we examined the sensitivity of the qPCR procedure
4 using calibrated cell suspensions of the (1,3)(1,2)- β -D-glucan producing *O. oeni* I4 and *L.*
5 *diolivorans* G77 strains. The standard curves constructed from cells of both strains in MRS
6 showed good quantification efficiency, since concentrations as low as 58.3 and 39.3
7 CFU/reaction could be amounted. The quantification range was also established between 10 and
8 10^5 CFU/reaction, as in *P. parvulus* 2.6.

9 As natural ciders are bottled without any stabilization treatment, quantification of *gtf*⁺
10 LAB populations can be useful to evaluate the risk of β -glucan spoilage before bottling. In this
11 context, the developed qPCR method was used for quantification of the (1,3)(1,2)- β -D-glucan
12 producers along cidermaking process in factories with frequent incidence of ropiness. Significant
13 increase of the *gtf*⁺ population was detected in some of the vats after malolactic fermentation.
14 The qPCR procedure was also utilised to quantify *gtf* populations in ropy bottled ciders,
15 demonstrating clearly an enrichment of *gtf*⁺ lactic acid bacteria population, in comparison to the
16 levels found in fermenting musts. Although cell numbers were probably overestimated because
17 qPCR inability to differentiate between living and dead cells (Neeley et al., 2005), our results
18 indicate that, in most of spoiled ciders, the *gtf*⁺ population is the predominant microbiota,
19 ranging from 10^5 to 10^7 CFU/ml.

20 On the basis of these results, we conclude that the SYBR-Green I assay developed in this
21 study is suitable for a sensitive and rapid detection of the (1,3)(1,2)- β -D-glucan LAB producers
22 and provides a good tool for its early detection during cider making process. This knowledge
23 will allow cidermakers to make decisions to control and avoid spoilage of ciders by sulfiting or
24 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.

ACKNOWLEDGMENTS

This work was supported by the Ministerio de Educación y Ciencia (projects AGL2006-11932 and CSD2007-00063), Universidad del País Vasco (UPV/EHU) (EHU08/37) and the Diputación Foral de Gipuzkoa and the Generalitat Valenciana (GVACOMP2009-257). Programa Red Gipuzkoana de Ciencia, Tecnología e Innovación (co-financed by the European Union). Idoia Ibarburu acknowledges the Universidad del País Vasco for the predoctoral fellowship.

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Fig.1. Representative amplification plots corresponding to standard curves obtained from serial dilutions of *P. parvulus* 2.6: (a) genomic DNA in the range from 4.3 to 4.3×10^5 CFU/reaction and (b) DNA extracted from calibrated cell suspensions in the range from 26 to 2.6×10^5 CFU/reaction. PCR amplification reactions in triplicate were carried out per dilution. Insets show representative standard curves generated from the amplification data: C_T values are plotted against the concentration (CO) as $\log_{10}$ of (a) genomic DNA per reaction; (b) cell concentration in CFU per reaction (determined by plate count). (c) Amplification plots corresponding to the standard curve obtained from serial ten-fold dilutions of the *gtf* amplification product (417 bp) from *P. parvulus* 2.6.

(a)

(b)

(c)

Fig. 1.

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	
<i>Lactobacillus brevis</i>	CECT 216		> 40	60.7	–
<i>L. buchneri</i>	CECT 4111		35.06±0.32	74.7	–
<i>L. collinoides</i>	CECT 922 ^T		> 40	60.7	–
	CUPV ^d 231		8.46±0.12	74.4	+β-D-glucan (Ibarburu, 2009)
	CUPV ^e 234, 235	2	30.49±0.09	74.4	–
<i>L. delbrueckii</i> subsp <i>bulgaricus</i>	NCIMB 702772		> 40	60.9	+HePS (Grobbs et al., 1997)
<i>L. diolivorans</i>	LMG 19667 ^T		37.76±0.24	72.3	–
	CUPV ^d G77		10.96±0.16	74.1	+α– and β-D-glucan (Dueñas et al., 1998)
<i>L. helveticus</i>	NCIMB 700766		39.59±0.48	60.6	+HePS (De Vuyst et al., 2001)
<i>L. hilgardii</i>	CECT 4786 ^T ,	2	> 40	60.7	–
	CECT 4681		29.23±0.49	74.7	
<i>L. mali</i>	CECT 4149		31.15±0.09	74.4	–
<i>L. plantarum</i>	CUPV ^e 241, 242, 243	3	30.56±0.31	74.4	–
<i>L. suebicus</i>	CECT 5917		28.04±0.07	74.7	–
	CUPV 221 ^d		25.75±0.38	74.5	+β-D-glucan (Ibarburu, 2009)
	CUPV ^d 225, 226	2	38.64±0.14	60.6	+HePS (Ibarburu, 2009)
<i>Leuconostoc mesenteroides</i>	NRRL B742		38.25±0.32	74.2	+α-D-glucan (Monsan et al., 2001)
<i>Leu. mesenteroides</i> subsp <i>mesenteroides</i>	CECT 394		36.98±0.28	74.2	+α-D-glucan (Monsan et al., 2001)
<i>Pediococcus parvulus</i>	CECT 7350		35.49±0.15	74.2	–
	CUPV 2.6 ^d		16.80±0.037	74.1	+β-D-glucan (Dueñas et al., 1997)
	CUPV ^d 1, 2, 22, 23, 24, 26	6	10.31±0.36	74.4	+β-D-glucan
<i>P. damnosus</i>	CECT 793,	2	36.90±0.20	60.7	–
	CECT 4694		37.38±0.08	74.4	
<i>P. pentosaceus</i>	CECT 4695 ^T		35.19±0.18	74.7	–
<i>Oenococcus oeni</i>	CECT 217 ^T		28.34±0.26	74.7	–
	CECT 218		34.40±0.16	74.7	–
	CUPV I4 ^d		12.71±0.44	74.2	+β-D-glucan and HePS (Ibarburu et al., 2005)
	CUPV ^e 302-309, 3010, 3011, 3013-3015, 3017-3020	17	31.18±1.65	74.4	–
<i>Streptococcus thermophilus</i>	NCIMB 700859		37.93±0.25	74.2	+HePS

^aInstitutional 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). ^bqPCR parameters: C_T±SD and T_M. ^cEPS phenotype (+ or –) assigned by visual examination. EPS type was previously established by determination of the structures in the corresponding references. ^dIsolated from Basque Country ropy cider. ^eIsolated from Basque Country non-ropy cider. ^TType strain. Shadowed boxes correspond to (1,3)(1,2)-β-D-glucan producers.

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

	DNA		Cell suspensions					
	<i>P. parvulus</i> 2.6		<i>P. parvulus</i> 2.6		<i>O. oeni</i> I4		<i>L. diolivorans</i> G77	
R ²	0.998		0.984		0.977		0.942	
sc	y=−3.58x+35.68		y=−3.31x+31.28		y=−3.41x+36.20		y=−3.49x+35.78	
AE	0.9		1		0.96		0.93	
n	7		5		5		5	
	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	$C_T \pm SD^b$
	4.3x10 ⁵	14.70 ± 0.138	2.6x10 ⁵	14.26 ± 0.58	5.83x10 ⁵	14.76 ± 0.24	3.93x10 ⁵	17.33 ± 0.84
	4.3x10 ⁴	17.82 ± 0.16	2.6x10 ⁴	18.74 ± 0.16	5.83x10 ⁴	18.22 ± 0.12	3.93x10 ⁴	22.53 ± 0.36
	4.3x10 ³	21.33 ± 0.11	2.6x10 ³	22.01 ± 0.04	5.83x10 ³	23.09 ± 0.33	3.93x10 ³	26.68 ± 0.49
	4.3x10 ²	25.06 ± 0.05	2.6x10 ²	24.20 ± 0.69	5.83x10 ²	27.27 ± 0.42	3.93x10 ²	27.93 ± 0.32
	43	28.40 ± 0.15	26	28.19 ± 0.15	58.3	30.33 ± 0.08	39.3	31.60 ± 0.50
	4.3	32.24 ± 0.16	2.6	27.47 ± 0.45 [§]	5.83	31.51 ± 0.24 [§]	3.93	32.08 ± 1.06 [§]
	0.43	35.69 ± 0.32	0.26	26.17 ± 1.23 [§]	-	-	-	-

R², 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.

^aCFU/reaction calculated from DNA concentrations (4.2 ng corresponds to 4.3x10⁵ CFU); ^bMean C_T values ± standard deviation (SD) from three independent cultures, each performed in triplicate, ^cMean values of three separate cell cultures expressed as CFU/reaction; [§]Values that are not linear and they were not considered for the linear regression analysis.

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

nd, not determined. ^aSampling event: 1, October; 2, November; 3, December. ^bMean C_T value $\pm$ standard deviation (SD). Reactions were performed in triplicate. *, These samples were considered positive for the presence of β -glucan producers with $P < 0.05$.

Table 4. Quantification of β -glucan producer 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	<i>P. parvulus</i> 2.6 cell suspensions in MRS	<i>P. parvulus</i> 2.6 cell suspensions in cider	<i>O. oeni</i> I4 cell suspensions in MRS	<i>L. diolivorans</i> G77 cell suspensions in MRS
S1	18.31 $\pm$ 0.61	5.38	6.19	6.75	5.82	6.14	7.15	6.91
S2	18.46 $\pm$ 0.46	5.41	6.15	6.71	5.77	6.10	7.10	6.86
S3	19.33 $\pm$ 0.47	6.16	5.91	6.47	5.51	5.88	6.85	6.61
S4	17.67 $\pm$ 0.13	7.16	6.36	6.93	6.01	6.31	7.33	7.09
S5	15.14 $\pm$ 0.46	7.09	7.5	7.64	6.78	6.98	8.08	7.81
S6	15.34 $\pm$ 0.13	5.00	7	7.58	6.72	6.92	8.02	7.76
S7	25.11 $\pm$ 0.62	7.41	3.72	4.25	3.16	3.76	4.55	4.36
S8	21.55 ^c $\pm$ 0.14	6.23	4.70	5.25	4.24	4.69	5.59	5.38

^aMean C_T value $\pm$ standard deviation (SD); ^bTotal counts on MRS agar.); ^cValue corresponding to the dilution 1/2 of the sample.