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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

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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

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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