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Development of a Quantitative PCR for Detection of *Lactobacillus plantarum* Starters During Wine Malolactic Fermentation

Cho, Gyu-Sung[1], Sabrina Krauß[1], Melanie Huch[1], Maret Du Toit[2], and Charles M.A.P. Franz[1]*

[1]*Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institute, Federal Research Institute for Nutrition and Food, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, Germany*
[2]*Institute for Wine Biotechnology, Stellenbosch University, Private Bag X1, Matieland, ZA 7602, South Africa*

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A quantitative, real-time PCR method was developed to enumerate *Lactobacillus plantarum* IWBT B 188 during the malolactic fermentation (MLF) in Grauburgunder wine. The qRT-PCR was strain-specific, as it was based on primers targeting a plasmid DNA sequence, or it was *L. plantarum*-specific, as it targeted a chromosomally located plantaricin gene sequence. Two 50 l wine fermentations were prepared. One was inoculated with 15 g/hl *Saccharomyces cerevisiae*, followed by *L. plantarum* IWBT B 188 at 3.6 × 10^6 CFU/ml, whereas the other was not inoculated (control). Viable cell counts were performed for up to 25 days on MRS agar, and the same cells were enumerated by qRT-PCR with both the plasmid or chromosomally encoded gene primers. The *L. plantarum* strain survived under the harsh conditions in the wine fermentation at levels above 10^5/ml for approx. 10 days, after which cell numbers decreased to levels of 10^3 CFU/ml at day 25, and to below the detection limit after day 25. In the control, no lactic acid bacteria could be detected throughout the fermentation, with the exception of two sampling points where ca. 1×10^2 CFU/ml was detected. The minimum detection level for quantitative PCR in this study was 1×10^2 to 1×10^3 CFU/ml. The qRT-PCR results determined generally overestimated the plate count results by about 1 log unit, probably as a result of the presence of DNA from dead cells. Overall, qRT-PCR appeared to be well suited for specifically enumerating *Lactobacillus plantarum* starter cultures in the MLF in wine.

Keywords: LAB, *Lactobacillus plantarum*, malolactic fermentation, wine, starter cultures, qRT-PCR

*Corresponding author
Phone: +49 721 6625 225; Fax: +49 721 6625 453;
E-mail: Charles.Franz@mri.bund.de

The malolactic fermentation (MLF) is the secondary fermentation that takes place mostly after the alcoholic fermentation. It enhances wine sensory properties and increases its microbial stability [13]. This fermentation process is often carried out by one or more species of lactic acid bacteria (LAB) such as *Lactobacillus, Leuconostoc*, *Oenococcus*, and *Pediococcus* [15]. One of the LAB that may occur in the wine fermentation is *Lactobacillus* (*Lb.*) *plantarum*, and the current *Lb. plantarum* V22 starter culture is used as a starter for the MLF to overcome harsh wine conditions such as low pH and high alcohol levels, and to prevent the production of biogenic amines. This starter culture was released in 2010 by Lallemand [19]. *Lactobacillus plantarum* strains are also known to be closely associated with other fermented foods such as cheese, yoghurt, meat, and fermented plant products, and they often predominate in such fermentations [5, 10, 12, 22].

In order to enumerate viable bacteria in fermented foods, traditional culture-based plate counting methods are often used. This has several disadvantages, such as the long incubation times, unavailability of selective growth media for certain species, and possible inhibitory effects by bacterial cells occurring in the same sample that are able to outgrow the species of interest. The culture-independent, quantitative, real-time polymerase chain reaction (qRT-PCR) method has been shown to be a sensitive, highly specific, and rapid procedure for detection and quantification of pathogens, or of probiotic LAB in foods [6, 14, 16, 18, 20, 29]. Quantitative RT-PCR was tested as a rapid procedure for determining the viability and predominance of *Oenococcus* (*O.*) *oeni* starter cultures during wine fermentation [23].

In this study, *Lb. plantarum* strain IWBT B 188, previously isolated from wine in South Africa, was used as a starter culture in the MLF of "Grauburgunder" (Pinot

gris) white wine in Germany. The aim of this study was to develop a qRT-PCR method to quantitatively follow the starter strain in the MLF. The *Lb. plantarum* starter culture strains for MLF in wine need to establish themselves in a fermentation in which other LAB can be naturally present and predominate in the fermentation. The pH of wine produced in warm climates can be as high as pH 3.8 and this allows the growth of a variety of wine spoilage LAB [19]. Thus, qRT-PCR offers an opportunity for targeted detection of the MLF starter strain in a co-existing and competing autochthonous LAB micropopulation. The qRT-PCR in our study was therefore based on specific, custom-designed primers, which targeted a plasmid sequence and thus was strain-specific. Alternatively, bacteriocin-producing *Lb. plantarum*-specific primers for qRT-PCR were developed, which targeted the plantaricin EF bacteriocin genes and thus could help to distinguish the *Lb. plantarum* starter strain, and possibly also autochthonous bacteriocin-producing *Lb. plantarum* strains, from other LAB present in the MLF.

Materials and Methods

Bacterial Strains and Culturing Conditions

Lactobacillus plantarum strain IWBT B 188 isolated from South African wine at the Institute for Wine Biotechnology, Stellenbosch University, South Africa, was routinely grown in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 30°C and was kept as a stock culture at −80°C in MRS broth containing 20% (v/v) glycerol. The strain was subcultured at least twice before use in experiments. For preparation of the inoculum for wine fermentation, 5 ml of an overnight culture grown in MRS broth at 30°C for 18 h was transferred to 450 ml of adaptation medium [MRS medium containing (g/l) fructose, 40.0; D-(+)-glucose, 20.0; L-malic acid, 4.0; Tween 80, 1.0; and 6% ethanol (v/v); pH 4.6].

Wine Fermentation and Microbial Lactic Acid Bacterial Count Determinations

Lb. plantarum IWBT B 188 was used as a starter culture for the MLF of Grauburgunder white wine, which was produced in the 2010 harvest season by the winemaking cooperative of Zell-Weierbach in Baden-Wuerttemberg, Germany. Two 50 l volumes were fermented in glass fermentation jars. The wine yeast *Saccharomyces cerevisiae* subsp. *bayanus* (Lalvin EC-1118, Lallemand) was added (15 g/hl) to the grape juice, and three days after inoculation with the yeast (yeast count at this time was approx. 1×10^7 CFU/ml), the fermentation was also inoculated with 3.6×10^6 CFU/ml of the *Lb. plantarum* IWBT B 188 starter culture for MLF. The other 50 l fermentation was left uninoculated with the bacterial starter culture. For determining the viable counts in both the fermentations, the wine samples were diluted in a 10-fold dilution series with quarter-strength Ringer's solution (Merck), and spread-plated onto MRS agar at specified intervals for up to 32 days. The plates were incubated aerobically at 30°C and colonies were enumerated after 48 h.

Genomic and Plasmid DNA Isolation and Development of *Lb. plantarum*-Specific qPCR Primers that Targeted Plasmid DNA or Chromosomally Encoded *pln*EF Genes

The total genomic DNA of *Lb. plantarum* IWBT B 188 was isolated from 5 ml of an overnight culture grown at 30°C in MRS broth according to the method of Pitcher *et al.* [21], as modified by Björkroth and Korkeala [2] for Gram-positive bacteria. Plasmid DNA from Gram-positive bacteria was isolated by the method of Birnboim and Doly [1] as modified by van Belkum and Stiles [25]. In order to obtain a plasmid-specific DNA sequence that could be used for designing custom primers for quantitative real-time PCR, plasmid DNA from *Lb. plantarum* IWBT B 188 was used as template for RAPD-PCR with the LB2 (5'-GGT GAC GC-3') primer [28]. RAPD-PCR was done in a 50 µl volume containing 100 ng of template plasmid DNA, 10× *Taq* DNA polymerase buffer (GE Healthcare, Freiburg, Germany), 200 µM dNTP's (Peqlab, Erlangen, Germany), 50 pM LB2 primer, and 1.5 U *Taq* DNA polymerase (GE Healthcare). The PCR reaction was performed with an initial denaturation step at 94°C for 5 min, followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 2 min, ramping of 0.6°C per second to 72°C, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 7 min. An aliquot of the PCR products was separated using a 1.5% (w/v) agarose gel and visualized using a Fluorchem imager 5500 system (Alpha Innotech, USA). One of the PCR products showed only one band and was subsequently purified using PCR clean columns (Peqlab) and commercially sequenced at GATC Biotech (Constance, Germany). Based on this sequence, a strain-specific qRT-PCR primer pair, p188fw 5'-AGC AGG CCG AGT GAA ACG AGG T-3' and p188rev 5'-CGC CAT CGG CGA CTT CTG ATA C-3', which detected the *Lb. plantarum* IWBT B 188 strain containing this plasmid, was designed. In addition, the qRT-PCR primers plnEFfw 5'-CTA TTT CAG GTG GCG TTT TC-3' and plnEFrev 5'-GTG GAT GAA TCC TCG GAC AG-3', used to detect the plantaricin EF gene [3], were also used to follow the development of the starter culture in the wine fermentation. Although this latter primer pair that detects the *pln*EF genes is not strain-specific, the plantaricin genes are known to occur only in *Lb. plantarum* strains, and therefore the primers were thought to be at least specific for bacteriocin (plantaricin EF)-producing *Lb. plantarum* strains. The *pln*EF primers have been used successfully to specifically detect the *pln*EF gene in a number of studies [4, 19].

Enumeration of *Lb. plantarum* Starter Cultures in Wine During Malolactic Fermentation Using qRT-PCR

For determining the numbers of *Lb. plantarum* strain IWBT B 188 by quantitative PCR, DNA was isolated from 6 ml of wine, which was first centrifuged at low speed (200 ×*g*, 10 min) to remove non-dissolved particles, which was followed by a washing step in quarter-strength Ringer's solution at 7,500 rpm for 10 min to collect the bacterial pellet. Total DNA was isolated according to the method described above and used as template for qRT-PCR. Quantitative RT-PCR reactions were performed in 200 µl, 96-well plates (Bio-Rad, Munich, Germany), and each reaction contained the following components: 12.5 µl of IQ SYBR green PCR supermix (Bio-Rad), 4 µl of template DNA, and 100 pM of each primer in 25 µl volume. The qRT-PCR with both sets of primer (*i.e.*, primers for the plasmid sequence and primers for plantaricin EF) were separately done in an

iQ5 thermal cycler (Bio-Rad). Amplification conditions for qRT-PCR based on either plasmid or plantaricin EF primers were the same, and included one cycle at 95°C for 10 min for initial denaturation, 40 cycles of denaturing at 95°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. A melting curve analysis was done subsequently to determine the specificity of the PCR reaction, by denaturing from 55°C to 94°C immediately after the last cycle of each amplification and the fluorescence was recorded by the iQ5 thermocycler.

In order to generate a qRT-PCR standard curve that was able to relate cell counts to qRT-PCR signals, the *Lb. plantarum* IWBT B 188 strain was inoculated in MRS broth medium and grown until ca. 1×10^8 CFU/ml. Next, the culture was diluted in a 10-fold dilution series and DNA was isolated from 1 ml of each of the dilutions using the method described above. The DNA from each dilution was used as template for qRT-PCR with either the plasmid or plantaricin genes-specific primers. The standard curve was generated from quadruplicate cultures in MRS broth, plate count determinations, and DNA extractions. The PCR reaction efficiency ($E = 10^{1/-S}$-1, where S = slope) was calculated from the log-linear part of the standard curve [11].

RESULTS AND DISCUSSION

Plasmid DNA isolated from *Lb. plantarum* strain IWBT B 188 was shown to harbour more than one plasmid (Fig. 1), as multiple bands could be observed upon gel electrophoresis. A part of the plasmid nucleotide sequence was obtained by using the plasmid DNA as a template for RAPD-PCR product amplification, and a resulting fragment was sequenced and found to encode a partial sequence with homology to *orf9* encoded on the *Lactobacillus sakei* plasmid pRV500 (GenBank Acc. No. AF 438419). A GenBank database search showed that this nucleotide sequence also showed similarity to sequences of plasmids pRH 20690 (GenBank Acc. No. AB167898) and pSMB74 (GenBank Acc. No. U02482) from *Lactobacillus lindneri* and *Pediococus acidilactici*, respectively. The function of the gene product of *orf9* is unknown, but in plasmid pSMB74 from *Pediococcus acidilactici*, the homologous gene was involved in plasmid replication and maintenance according to the GenBank information. The strain-specific primers p188fw (5'-AGC AGG CCG AGT GAA ACG AGG T-3') and p188rev (5'-CGC CAT CGG CGA CTT CTG ATA C-3') were custom designed based on this sequence. The primers were tested on three different, plasmid-containing *Lb. plantarum* strains (*i.e.*, IWBT-B188, IWBT-B184, and BFE 5092), and a PCR product with the expected size was only obtained for strain IWBT- B188 (results not shown). A similar approach to obtain nucleotide sequence on which to design primers for qRT-PCR was previously described, also based on generation of a DNA fragment in genotyping experiments such as RAPD-PCR [8] or AFLP [27]. Xiang *et al.* [27], for example, used RT-PCR to follow the survival of 10 bacterial strains (*Bacillus subtilis*, *Escherichia hermanii*, and *Pseudomonas* spp.) from the Canadian Domestic Substance List (CDSL) in soil under laboratory conditions, and were able follow the persistence of these bacteria for up to 180 days of incubation with a detection limit of 1.3×10^2 to 3.25×10^5 CFU/g. Fujimoto *et al.* [9] used RT-PCR for the quantitative detection of the *Bifidobacterium breve* strain Yakult (BbrY) in human feces, in a volunteer study in which volunteers ingested 1×10^7 CFU of BbrY/day and that lasted 10 days. They detected only the viable cells, as they used a propidium monoazide treatment that combined a DNA intercalating dye for covalently linking DNA in dead cells after photoactivation. They were able to enumerate the BbrY strain at levels of 7.5 (±1.5) log CFU/g, which correlated well with the 10^6 to 10^9 cells/g in feces after 10 days determined by spread-plating on selective medium [9].

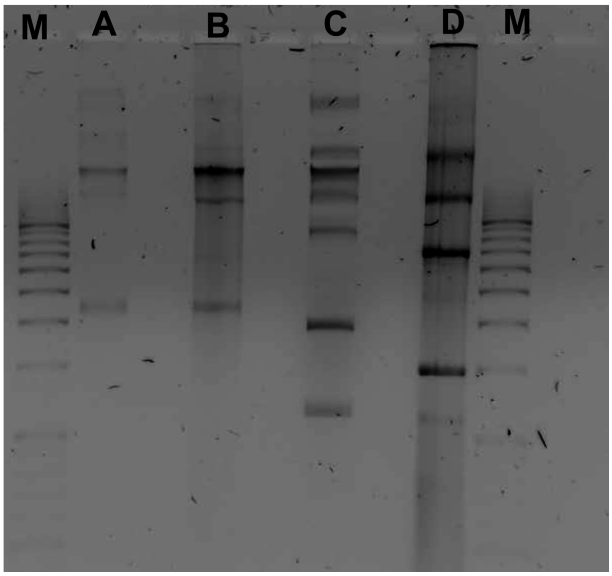

Fig. 1. Plasmid DNA profiles of *L. plantarum* strain IWBT B188 and selected *L. plantarum* reference strains.
M: Basepair marker; A: *L. plantarum* BFE 905 [7]; B: *L. plantarum* PCS20 [4]; C: *L. plantarum* IWBT B 188; D: *L. plantarum* BFE 5092 [5].

Strain-specific primer sets have also been developed on the basis of RAPD-PCR DNA fragments for enumeration of the probiotic *Lb. casei* strain Shirota [8] and *Lactococcus lactis* subsp. *cremoris* [17] in human feces, in studies on the ability of these probiotics to colonize the human gastrointestinal tract. In both studies, the primers were determined to be strain-specific by testing them against various intestinal bacteria.

These previous examples, however, were based on chromosomal target genes. Töwe *et al.* [24] used four functional genes located on different plasmids, as well as a chromosomal gene, to compare the amplification efficiencies for qRT-PCR. When used at a certain dilution range, the plasmid and chromosomal DNA samples had similar

amplification efficiencies, and thus both types of DNA may be used with equal successful in qRT-PCR [24].

Lactobacillus plantarum IWBT B 188 was grown in pure culture in MRS broth to produce standard curves that relate the cell count with qRT-PCR detection signals. Two different standard curves were constructed using the two different primer pairs described above, targeting either the plasmid DNA *orf9* gene sequence or the chromosomal *pln*EF gene sequence. At the same time, the qRT-PCR amplification signals were determined using 10-fold diluted total DNA as template from the same pure culture. When plasmid-targeted primers based on the *orf9* gene were used for qRT-PCR, the minimum detection number of cells was determined to be ca. 5×10^3 CFU/ml. The correlation between the Ct values and the CFUs was highly linear, and the slope of the qRT-PCR generated standard curve equation was −1.753 with a correlation coefficient of 0.99 (Fig. 2A). The PCR reaction efficiency calculated from the log-linear part of the standard curve (see Materials and Methods) in this experiment was 0.789. The other standard curve, which was generated using the primers targeting the chromosomally encoded *pln*EF gene, showed that the detection limit of bacterial cells based on the qRT-PCR standard curve was slightly lower than that determined from the standard curve of the plasmid-encoded *orf9* gene. Thus, as little as 5×10^2 CFU could be detected using qRT-PCR based on the chromosomally encoded *pln*EF gene. The slope of the liner equation was −1.5416, (R^2=0.99) and showed a somewhat better amplification efficiency of 0.913 (Fig. 2B). As a result, the quantification was accurate in the range corresponding to 5×10^2 to 5×10^8 CFU/ml in pure culture sample using qRT-PCR based on both primer pairs. This was slightly better than the detection limits previously determined for real-time PCR primers detecting probiotics in feces, for which the minimum detection levels were $10^{4.6}$ and 10^5, respectively [8, 9]. This higher detection limit may be a result of the more complex matrix or also because of the presence of high amounts of different genetic material, as it is known that the bacterial density in feces is approx. 10^{11} to 10^{12} cells /g in the colon [26].

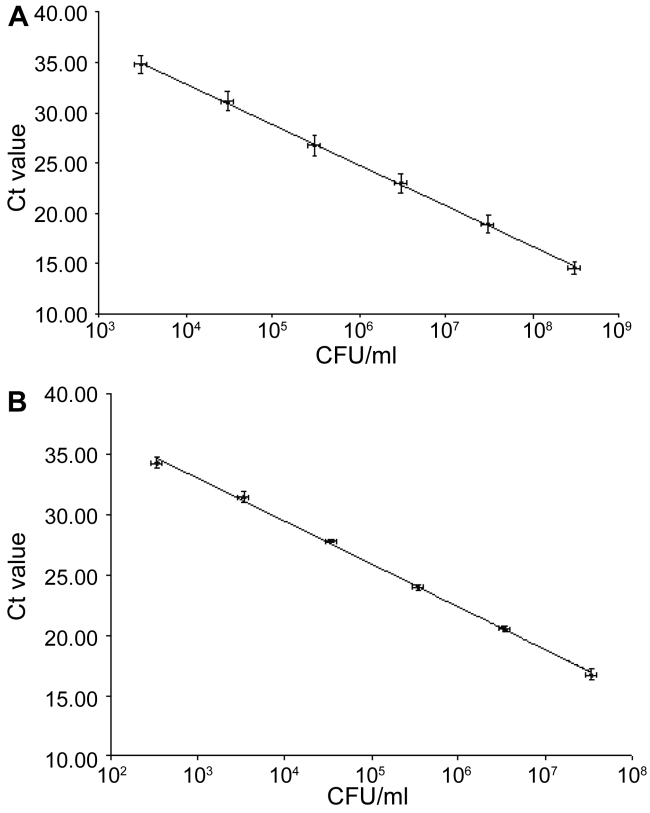

Fig. 2. Standard curves showing the linear relationship of the number of cycles and log CFU for serially 10-fold diluted culture and DNA.

The Ct values are the results from nine replicate measurements of each target gene, and the standard deviation is shown. (**A**) Plasmid DNA targeted standard curve showing the linear regression coefficient (R^2) of 0.99 (y= -1.753Ln(x) + 48.617). (**B**) Chromosomal DNA targeted standard curve showing the linear regression coefficient (R^2) of 0.99 (y= -1.5416Ln(x) + 43.647).

A previous study of qRT-PCR done on plasmid-encoded genes from a bacterial sample in a pure culture showed that the qRT-PCR efficiencies of the reactions with plasmid DNA as template ranged from 0.80 to 0.87 in different dilutions of the plasmid DNA [24]. These amplification efficiencies were considered suitable for deriving bacterial cell counts from qRT-PCR standard curve data [24]; therefore, the similar, or slightly higher, amplification efficiencies of this study also indicated the suitability of utilizing the standard curve based on the *orf9* and *pln*EF gene qRT-PCR reactions for determining bacterial counts.

The amplification specificity of the qRT-PCR reaction with the different primer pairs was also investigated by melting curve analysis of the qRT-PCR products after the final amplification step. Using the melting curve analyses, no nonspecific peaks could be detected in both of the reactions; that is, with both the *pln*EF or the *orf9* gene primer pairs (data not shown). In addition, no peak was obtained in the no-template control. This indicated that a specific amplification of the correct product was achieved in the PCR reaction and that neither primer pair dimers nor unspecific PCR products interfered with the qRT-PCR reaction.

In order to determine the *Lb. plantarum* IWBT B 188 cell count during the MLF in wine, the total DNA of microorganisms (yeast and bacteria) in the Grauburgunder white wine was purified and used as template in qRT-PCR with SYBRGreen. At the same time, the LAB population was determined at each interval of the wine fermentation by plating onto MRS agar. The bacterial counts, as determined by conventional plate counting method, were related to the Ct value obtained in qRT-PCR with either of

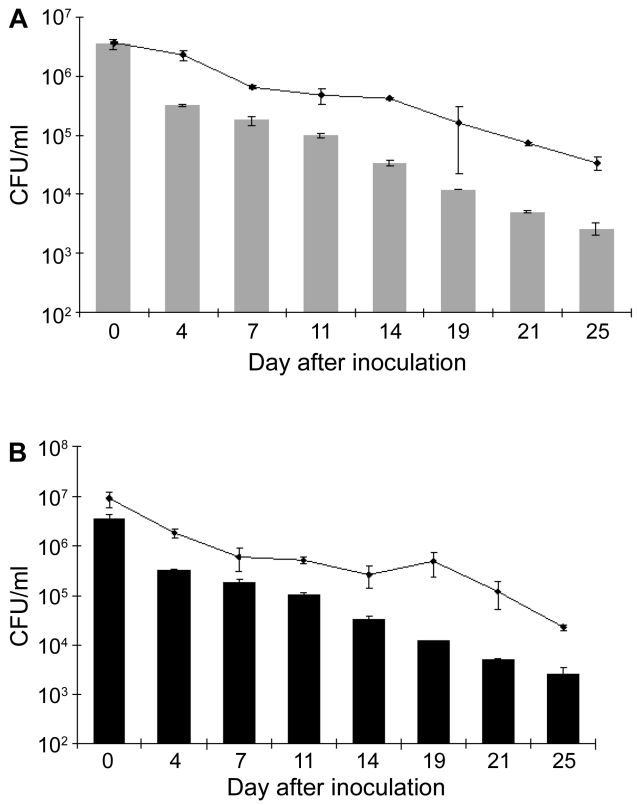

Fig. 3. Viable counts determined on MRS agar at specific time points correlated with the qRT-PCR count results (lines).
The plasmid-targeted qRT-PCR of the *orf9* gene (**A**) and chromosomal DNA-targeted qRT-PCR of the *pln*EF gene (**B**) results are means of triplicate samples. Error bars represents standard deviations from three independent tests.

the *pln*EF or *orf9* gene primer pairs (Fig. 3), and this value was correlated to the bacterial count using the standard curve.

Based on plate counts, the *Lb. plantarum* IWBT B 188 cells were shown to survive in wine despite the adverse condition resulting from high alcohol concentrations (*ca.* 12% vol. ethanol; result not shown) and low pH (*ca.* pH 3.5; result not shown). The cell counts decreased steadily from approx. 5×10^6 CFU/ml on day 0 to approx. 10^3 CFU/ml on day 25, but the cell counts stayed above 1×10^5 CFU/ml for about the first 10 days. The qRT-PCR, which was carried out with the primer pair targeting the *orf9* gene on the plasmid, showed a good agreement to the viable count determined by plate counting on day 0, but deviated by approx. 1 log unit in subsequent determinations up to day 25. In general, the qRT-PCR counts were 1 log higher than the viable counts determined by plate counting.

The result of qRT-PCR counts based on the chromosomally encoded *pln*EF gene showed an overall similar trend. Again, the counts determined by both methods were in good agreement on day 0, after which there was a less than 1 log difference up to day 14. After this, the difference in counts obtained by these methods were larger than 1 log unit on sampling days 19 and 21, but then the difference again decreased to less than 1 log difference on day 25 (Fig. 3B).

Taken together, the difference between plate counting results compared with qRT-PCR count determinations was approx. 1 log less on the plate counts at almost every sampling time point, except day 0 (Fig. 3B). As the fermentation progresses, the viable count determinations done by spread plating showed that bacteria died over the time course of this study. The DNA of dead cells can, however, still serve as a template if cells lysed, and this may have resulted in the approx. 1 log CFU higher bacterial counts assessed by qRT-PCR. Moreover, viable but damaged and thus non culturable cells, which may have developed during the long incubation in this hostile environment, may still have been quantifiable by qRT-PCR, but not by plate counts. Fujimoto *et al.* [8] also showed that qRT-PCR overestimated the cell counts of *Lb. casei* Shirota in feces in a human volunteer study, and attributed this difference also to the presence of dead cells or an underestimation due to the use of selective medium. The order of underestimation was similar as in our study, at *ca.* 1 log CFU/g.

The autochthonous LAB micropopulation in wine that was not inoculated with the starter culture occurred in very low numbers, and counts of approx. 10^2 CFU/ml could be detected only on days 0 and 14. On all other days, the LAB count on MRS agar was below the detection limit of 10^2 CFU/ml (results not shown). Moreover, qRT-PCR with samples from the control fermentation did not result in a signal for either the *pln*EF or the *orf9* primer pairs (results not shown). For the wine fermentation, therefore, both the primer pairs were considered specific for detection of *Lb. plantarum* IWBT B 188, and the counts deduced from the qRT-PCR experiments can be safely assumed to be based only on this strain's DNA.

As the chances that other autochthonous *Lactobacillus plantarum* strains that contain a similar plasmid or possess the *pln*EF gene also occur in high numbers in wine fermentations are rather slim, the qRT-PCR seems well-suited for following the development of this *Lb. plantarum* starter culture in wine fermentations. It should be remembered, however, that the counts obtained by this method may overestimate the actual count by up to 1 log unit. However, the method may nevertheless be suitable to give a fast indication of the development of the bacterial starter culture, and paired with enzymatic data, it may give valuable information on the stage of the MLF to the winemaker. This qRT-PCR method may also be adapted to other bacteriocinogenic *Lb. plantarum* strains that are used as starter cultures in other fermentations, based on other food matrices. If the raw materials in such other fermentations do not hamper *Lb. plantarum* growth, but rather allow a

rapid increase in viable cell numbers, then the qRT-PCR method may be even more successful, as during logarithmic growth it is unlikely that DNA from dead or injured cells can interfere with qRT-PCR and lead to such an overestimation as observed for these bacteria surviving in the harsh wine environment. This method may be an especially valuable tool to assess the success of the starter culture in wines made in warmer climates, which are known to have a higher pH and a more complex autochthonous micropopulation, and thus could be used to determine the success of the starter to establish itself in the MLF.

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