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

Received: July 4, 2011 / Revised: August 2, 2011 / Accepted: August 9, 2011

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

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

---

*Corresponding author*

Phone: +49 721 6625 225; Fax: +49 721 6625 453; E-mail: Charles.Franz@mri.bund.de
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; (+)-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 (Latvin 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 × 10⁷ CFU/ml), the fermentation was also inoculated with 3.6 × 10⁶ 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 *plnEF* 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 Böhrkoth 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 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 CCT CTG ATA C-3′, which detected the *Lb. plantarum* strain containing this plasmid, was designed. In addition, the qRT-PCR primers plnEFfw 5′-CTA TTT CAG GTG GCC TTC TC-3′ and plnEFrev 5′-GTT GAT GAA TCC TCG AGC 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 primer pair that detects the plnEF 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 plnEF primers have been used successfully to specifically detect the plnEF 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