TÚLIO IGLÉSIAS MACHADO

POTENTIAL USE OF MULTIPLEX REAL-TIME PCR TO DEVELOP A KIT FOR THE IDENTIFICATION OF BEER-SPOILAGE MICROORGANISMS

Dissertation submitted to the Agriculture Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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ABSTRACT

MACHADO, Túlio Iglésias, M.Sc., Universidade Federal de Viçosa, September, 2022. **Potential Use of Multiplex Real-time PCR to Develop a Kit for Identification of Beer-spoilage Microorganisms**. Adviser: Marcos Rogério Tótola. Co-advisers: Alex Gazolla de Castro and Tiago Antônio de Oliveira Mendes.

Beer is the most consumed alcoholic beverage globally. Despite being considered a microbiologically stable beverage due to its intrinsic properties such as low pH, high CO_2, presence of antimicrobial compounds from hop and other factors, microbiological contamination in beer does happen, leading to off-flavor production with changes in flavor and aroma, viscosity, acidification, among other unwanted effects. This study explores the use of multiplex real-time PCR (qPCR) coupled to High Resolution Melting (HRM) analysis for the simultaneous detection and discrimination of beer-spoilage microorganisms genera. Orthologous sequences were identified using the OrthoMCL pipeline for primer design. The designed primers exhibited high specificity, generating distinct melting peaks for the target genera. Sensitivity was confirmed, with successful amplification at low DNA concentrations. The perfect alignment of primers with target regions significantly influenced sensitivity. The multiplex qPCR-HRM approach demonstrated efficacy in detecting beer-spoilage microorganisms in multiplex reactions. Nonetheless, sensitivity variations among primers underscore the importance of thoughtful design for multiplex reactions with primers within the same sensitivity range. Our pipeline is highly adaptable and can be applied not only to the detection of various beer-spoilage microorganisms but also to other segments within the food industry, pharmaceutical, oil & gas industry, among others, effectively enhancing cost-efficient quality control measures.

Keywords: Beer-spoilage. Quality control. Multiplex qPCR. HRM. Orthologous genes.

RESUMO

MACHADO, Túlio Iglésias, M.Sc., Universidade Federal de Viçosa, setembro, 2022. **Potential Use of Multiplex Real-time PCR to Develop a Kit for Identification of Beer-spoilage Microorganisms**. Orientador: Marcos Rogério Tótola. Coorientadores: Alex Gazolla de Castro and Tiago Antônio de Oliveira Mendes.

A cerveja é a bebida alcoólica mais consumida no mundo. Apesar de ser considerada uma bebida microbiologicamente estável devido às suas propriedades intrínsecas, como baixo pH, alto teor de CO_2, compostos antimicrobianos provenientes do lúpulo e outros fatores, a contaminação microbiológica na cerveja ocorre, levando à produção de sabores indesejados como mudanças no sabor e aroma, viscosidade, acidificação, entre outros efeitos indesejados. Este estudo explora o uso do PCR em tempo real (qPCR) multiplex combinada com análise de *High Resolution Melting* (HRM) para a detecção e discriminação simultânea de gêneros de microrganismos contaminantes da cerveja. Sequências ortólogas foram identificadas usando a pipeline OrthoMCL para o design de primers. Os primers desenhados apresentaram alta especificidade, gerando curvas de *melting* distintas para cada gênero. A sensibilidade foi confirmada, com amplificação baixas concentrações de DNA. O alinhamento perfeito dos primers com regiões-alvo influenciou significativamente a sensibilidade. A abordagem multiplex qPCR-HRM demonstrou eficácia na detecção de microrganismos que contaminantes de cerveja em reações multiplex. No entanto, variações de sensibilidade entre os primers ressaltam a importância de um design cuidadoso para reações multiplex com primers dentro da mesma faixa de sensibilidade. Nossa pipeline é altamente adaptável e pode ser aplicado não apenas na detecção de vários microrganismos cervejeiros, mas também em outros segmentos da indústria alimentícia, indústria farmacêutica, óleo & gás, dentre outros, melhorando efetivamente medidas de controle de qualidade com custo competitivo com os métodos tradicionais.

Palavras-chave: Microrganismos contaminantes de cerveja. Controle de qualidade. qPCR multiplex. HRM. Genes ortólogos.

SUMMARY

1. INTRODUCTION

Beer stands as the most widely consumed alcoholic beverage globally, accounting for approximately 60% of the total volume of alcoholic beverages and a market reaching US$ 570 billion in 2022. Projections for 2023 anticipate a growth rate of 5.4%, with the beer industry expected to surge to a remarkable US$ 754 billion by 2027 (Statistica, 2023).

Beer is considered a microbiologically stable beverage due to its intrinsic properties. Low pH (3.8 to 4.7), ethanol concentration (0.5 to 10 % w/v), hop antimicrobial compounds (iso-α acid, 17 to 55 ppm), low oxygen concentration (less than 0.1 to 0.3 ppm) and high concentration of CO_2 (0.5% w/v) creates an inhospitable environment for the proliferation of most microorganisms (Larson & Morton, 1991; McDonnell & Russell, 1999; Jespersen & Jakobsen, 1996). Nevertheless, it is worth considering that microbiological contamination does happen in beer (Lin et al., 2008; Kanta & Wil, 2003; Priest & Campbell, 2003; Van der Aa Kuhlle & Jespersen, 1998; Baiano, 2021). According to Schneiderbanger (2018), among 13,802 beer samples analyzed from 2010 to 2016 in European breweries, 11.8% showed positive results for microbial contamination.

Microbial contamination in the brewing industry can lead to a spectrum of negative effects on beer quality, ranging from subtle alterations in flavor and aroma to the production of off-flavors, such as the unwelcome diacetyl (buttery odor), turbidity, changes in viscosity, attenuation (resulting in reduced sweetness due to the consumption of fermentable sugars), and reduce fermentation efficiency (March et al., 2005; Walkling-Ribeiro et al., 2011).

Levilactobacillus brevis (formally *Lactobacillus brevis*) is one of the main beer-spoilage microorganisms in the brewing industry. In a study carried out from 1980 and 2002, *Lactobacillus* genus alongside *Pediococcus* accounted for 60 to 90% of registered cases of contamination. *Levilactobacillus brevis*, *Lactobacillus lindneri* (now *Fructilactobacillus lindneri*) and *Pediococcus damnosus* are the most frequent (70 to 80% of cases). *L. brevis* is one of the main brewing contaminants, accounting for 50% of the cases (Back, 1981, 1994, & 2005; Suzuki, 2011 & 2012, Priest & Campbell, 2003). The main problems caused by these genera to the brewing industry are acidification, increase in turbidity and super attenuation of the wort. In some cases,

there is also the production of off-flavor diacetyl by *L. casei* and *P. claussenii* and the production of exopolysaccharides, leading to a gelatinous appearance of the beer (Van Nierop et al., 2006; Vriesekoop et al., 2012).

Species of the genus *Bacillus* are also able to persist throughout all stages of beer production, due to their ability to produce endospores. Nonetheless, they normally do not become a problem in the brewing industry, as they do not change the characteristics of beer (Suzuki, 2015). This is attributed to growth inhibition caused by low pH of the beverage and by compounds with antimicrobial activity present in hops (Takahashi et al., 2015). However, *B. cereus*, which has already been isolated from beers by our laboratory, is a foodborne pathogen that can produce toxins, causing two types of gastrointestinal illness in humans: the emetic (vomiting) syndrome and the diarrheal syndrome (McDowell, Sands & Friedman, 2023).

Among acetic acid bacteria, *Acetobacter is* the most frequently found in contaminated beer. Its presence was reported in 13% of samples (Lin et al., 2008). Among the most common species are *A. aceti*, *A. liquefaciens*, *A. pastorianus*, and *A. hansii* (Van Vuuren & Priest, 2003; Priest, 2006). One of the main problems caused by this genus is beer acidification and the production of off-flavor vinegar. The anaerobic gram-negative bacteria from the genera *Pectinatus* and *Megasphera* are other known spoilage microorganisms, responsible to produce off-flavors such as H_2S and short-chain fatty acids. Wild yeasts are another source of contamination in the brewery and the emergence of new species are frequently being reported and are expected to increase due to different types of beers (e.g., non-alcoholic beer) (Esmaeili et. al, 2015; Suzuki, 2020). Most recently, biogenic amine formation by beer spoilage microorganisms have been reported (Yu et al., 2021).

Renowned worldwide institutions as the European Brewery Conventional (EBC), American Society of Brewing Chemists (ASBC), the Master Brewers Association of the Americas (MBAA), the Brewery Convention of Japan (BCOJ), and the Institute of Brewing and Distilling (IBD) have focused on developing methods for modern quality control procedures in brewing. However, specific regulations and standardized analytical determination methods for all identified contaminants are currently lacking, with variations in maximum tolerable levels between countries that predominantly focus on raw materials, such as grains, and not specifically to microbial

contamination, especially in the final product (Stewart, Russell, & Anstruther, 2017; Ciont et al., 2022).

While numerous methods have been developed for the rapid identification of beer-spoilage microorganisms such as PCR, LAMP, antibody-based techniques, fluorescence *in situ* hybridization and ribotyping (Suzuki et al., 2020, Hutzler et al., 2015; Siegrist et al.,2015, Yasuhara et al., 2001; Tsuchiya et al., 2000), the quality control in brewing often relies on traditional microbiological analyses, especially the enumeration of contaminating microorganisms in culture media, which is time-consuming and, in most cases, do not provide species or genus-level identification (de Melo Pereira et al., 2010; Condina et al., 1970; Jespersen & Jakobsen,1996). The PCR technique has been reported as a reliable, sensitive, and a rapid technique for detecting contaminating microorganisms. Although the result obtained in the end-point PCR only indicates the presence or absence of the microorganism and gel electrophoresis is required after PCR, some brewers have been adopting it for quality control (Juvonen & Satokari, 1999; Motoyama & Ogata, 2000; Suzuki, Koyanagi & Yamashita, 2004; Tshuchiya et al., 1992 and 1993; Asano et al., 2008, Zendeboodi et al, 2020). Furthermore, real-time PCR (qPCR) kits have been developed enabling the identification and quantification of target species and genera linked to beer contamination (Invisible Sentinel, 2015; Pall Corporation, 2015; Biotecon Diagnostics, 2015).

In this study, we introduce the concept of multiplex real-time PCR (qPCR), where quantification and identification of various microbial species can occur simultaneously in a single reaction tube. This approach offers advantages in terms of time efficiency, labor, reagent consumption, and cost savings. Among the molecular techniques, qPCR probably has the best performance in terms of sensitivity, specificity, and rapidity (Heid et al., 1996; Mackay, 2004 & Carvalho et al., 2007). Furthermore, when combined with High Resolution Melting (HRM) analysis, it becomes an attractive alternative for the identification of food pathogens and beer-spoilage microorganism. (Jin et al., 2012; Nadai et al., 2018; Erdem et al., 2016).

HRM is a technique employed in the genotypic characterization of samples, including food, and is based on the displacement profile of the melting curve of PCR products. After PCR amplification, temperature gradually increases and melting curves profiles are generated accordingly with DNA denaturation by monitoring the fluorescent

intercalating DNA dye (Reed et al. 2007). With this technique, even a single nucleotide polymorphism can be detected by monitoring melting temperature (Tm) (Saptarshi et al., 2023). Thus, the qPCR multiplex combined with HRM (qPCR-HRM) has the advantage of detecting multiple contaminants by the multiple melting curves profile (Chedid; Rizou; Kalaitzis, 2020; Combes; Joët; Iashermes, 2018; Druml; Cichna-markl, 2014; Nunziata; Cervelli; Benedetti, 2018; Reed; Kent; Wittwer, 2007). In other words, identification and quantification of microorganisms are achieved based on the melting properties of the double-stranded DNA and have been shown to be suitable for the discrimination of closely related beer-spoilage species (Nadai et al., 2018, Erdem et al., 2016; Juvonen et al., 2008; Kao et al., 2007; Iacumin et al., 2015). Besides, when HRM does not give clear results, the PCR product can still be analyzed on gel (Vossen e al., 2009). The advantages of using HRM are clear: melting is faster than electrophoresis, no sequencing is needed, multiple targets can be detected at once with high sensitivity and data analysis can be performed automatically. Therefore, we hypothesize that qPCR coupled to HRM is suitable for the detection of multiple beer-spoilage microorganisms using a multiplex reaction due to its sensitivity, specificity, and reproducibility, being able to overcome some of the drawbacks pointed out in the conventional PCR analysis and other methods.

Orthologs genes is a type of homologs (e.g., a gene inherited in two species from a common ancestor) evolving from a common ancestor by speciation (Fitch 1970, 2000). In comparative genomics, clustering orthologous genes can highlight the divergence and conservation of gene families (Yoshizaki et al., 2014), therefore being a good strategy for primer design aiming to discriminate microorganisms. OrthoMCL is an algorithm that uses all-v-all BLAST and Markov clustering to select the reciprocal best similarity pairs between proteomes (Li, Stoeckert & Roos, 2003; Salichos & Antonis, 2011) and was applied in this work to identify orthologs groups for beer spoilage genus to select regions for primer design.

Real-time multiplex PCR assay associated with HRM (qPCR-HRM), targeting orthologous sequences, was developed to detect beer-spoilage microorganisms from the genus *Acetobacter*, *Bacillus* and *Levilactobacillus*. The SYBR Green real-time PCR assay revealed itself as a quick, reliable, cost-effective method for beer-spoilage genus detection assay.

2. MATERIAL AND METHODS

2.1 Strains and growth conditions

Bacterial and yeast strains used in this work for the validation of the designed primers and qPCR-HRM analysis are listed in Table 1. All beer-spoilage microorganisms were obtained from the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (LBBMA/DMB/UFV)), where bacterial isolates from different breweries are deposited. For the experiments with yeasts, the commercial lager yeast (SafAle™ US-05) was purchased from Fermentis (France). Latic acid bacteria were grown in capped tubes containing MRS medium (Thermo Scientifi34c™) for 3 to 5 days at 30ºC, 200 *rpm* (De Man et al., 1960). Yeasts were rehydrated in sterile distilled water (1:10 m/v yeast to water ratio) for 30 min at 30 °C prior to inoculation in YPD medium (Thermo Scientific™) at 30 ºC for 48 h (Treco & Lundblad, 1993) and all other aerobic bacteria species were grown in Tryptic Soy Broth (BD Bacto™). Strains were also cultivated in capped tubes containing commercial lager beers (5% ethanol) for 5 to 7 days to check whether beer compounds could interfere in qPCR performance. Strains were cultivated in Petri dishes containing solid culture medium and stored at 4 ºC. Weekly, an isolated colony was transferred to a new solid medium for maintenance. All pure cultures were stored at - 80 ºC with its respective culture medium, supplemented with glycerol 20%.

2.2 DNA extraction, quantification and sequencing

DNA was extracted from liquid cultures using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), as described by the manufacturer, and stored at - 20 ºC. DNA was stained with GelRed™ (Biotium) and analyzed by gel electrophoresis in 0.8% (w/v) agarose gel with 0,5 X TAE buffer. The 1 kb DNA Ladder (Promega) was used as reference. Gels were visualized by UV transilluminator Fire-Reader (UVITEC Cambridge) to ensure that DNA was present and intact. DNA quantification was performed in a Qubit ® 2.0 Fluorometer (Invitrogen™) using the Qubit DNA Assay kit, as described by the manufacturer.

PCRs were performed at a final volume of 25 µL. Each reaction contained 5 µL of the 5X GoTaq ® Buffer, 2.5 µL $MgCl_2$ (25 mM), 0.125 µL GoTaq® DNA Polymerase (5U/µL), 0.5 µL of each primer (10 $\mu mol.l^{-1}$), 2 µL of extracted DNA (20 ng), 0.5 µL of dNTP (10 mM) and 13.875 µL H_2O. All PCR reagents were obtained from Promega.

PCR reactions were performed on a Mastercycler Gradient 5331 thermocycler (Eppendorf) using the following protocol: i) an initial denaturation step of 95ºC for 2 min, followed by ii) 35 cycles of 95ºC for 1 min, 53ºC for 1 min, and 72ºC for 1 min and iii) final extension step of 72ºC for 5 min. For bacterial strains, the target DNA was amplified using universal primers for the housekeeping gene 16S rRNA 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1100R (5' GGGTTNCGNTCGTTG 3') (Lane DJ., 1991). For yeasts, the target DNA sequence was the internal transcribed spacer region (ITS), using primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (Pham et al., 2011). All PCR amplification products were analyzed in agarose gels following the protocol previously described.

PCR products were purified using the Illustra™ GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), following the manufacturer's instructions and samples were sent to the Instituto Renê Rachou (Fiocruz, Brazil) for sequencing, using a capillary electrophoresis in an ABI 3730XL sequencer. Sequences were analyzed in the (FinchTV, 2006) to assess its quality score (Q score). Quality control of the sequences was set up as follows: Q score equal to or greater than 20 (99% of inferred base accuracy) for long sequences (more than 10 nucleotides). Nucleotides at the beginning and in the end of each sequence that did not meet the defined pattern were excluded. Good-quality sequences for both primers (forward and reverse) were then opened in the Bioedit (Hall TA., 1999) for each isolate and the reverse complement of the reverse primer sequence was made and the consensus sequence was generated. The consensus sequence obtained was used in the BLASTn algorithm to compare with those deposited in the GenBank database for microorganism identification. The obtained DNA was used for the validation of primer specificity, sensibility and as a control for real-time PCR.

2.3 Definition of genetic markers

Our approach was to seek for potential marker genes that i) only occur in the species of each analyzed genus and ii) its identification could cover the maximum number of species within that genus. Our pipeline was performed as it follows:

I. Local alignment between genes of species associated with beer spoilage: proteomes from *A. pasteurianus* (CICC 22518), *B. cereus* (BC33) and *L. brevis* (NPS-QW-145) were retrieved from NCBI and used as a reference for its own genus on the

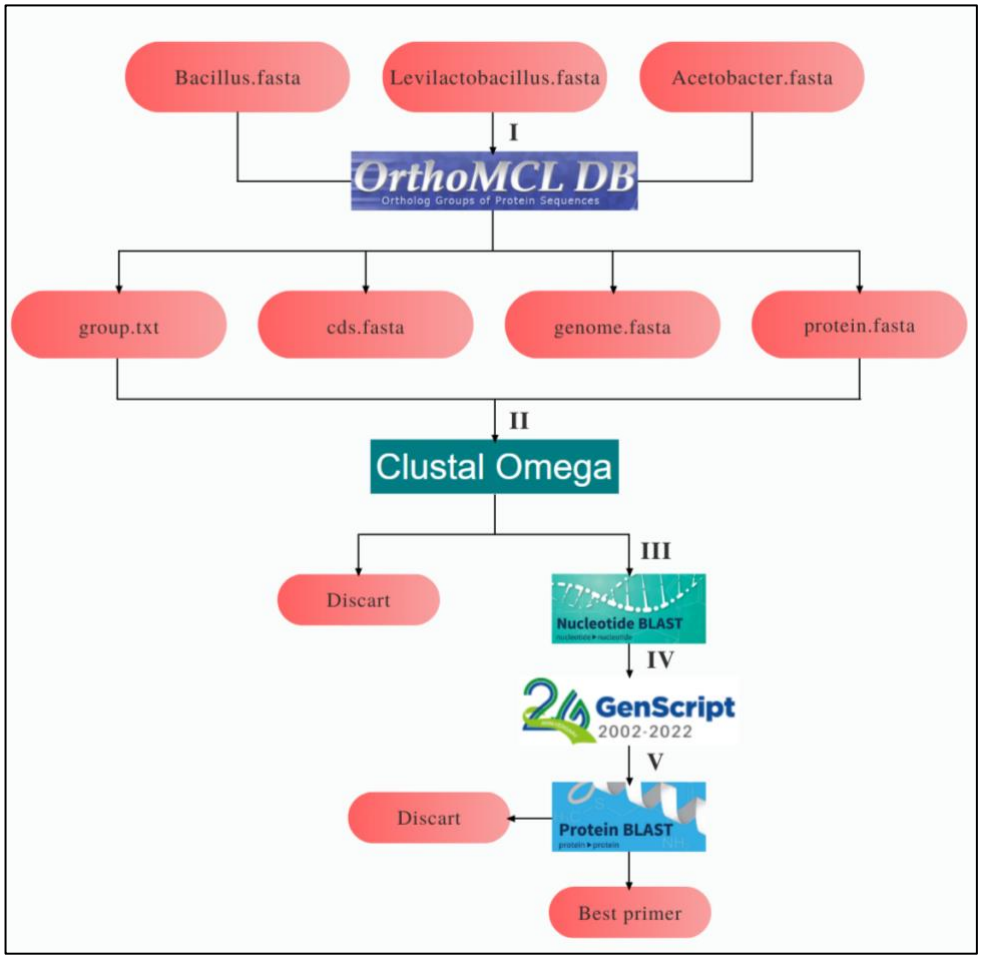
OrthoMCL pipeline (Li et al., 2003), where an all-v-all BLAST (v. 2.11.0) was performed within each proteome and sequences were filtered based on the following quality parameters: maximum 20% stop codons and maximum protein length of 10 amino acids in size. As a result, an output file (group.txt) was generated by OrthoMCL containing clusters of orthologous genes for each genus.

II. Orthologous clusters identification for each beer-spoilage species in a genus level: cluster's sequences were aligned in Clustal Omega (McWilliam H., 2013). Orthologous clusters showing a sequence identity higher than 70% between their protein sequences were selected and submitted to BLAST analysis.

III. Identification of genus-specific targets: in order to identify the best suitable gene sequences for primer design, able to discriminate microorganisms in a genus level, we checked: 1) sequences that had matches to the reference species used in the analysis and/or related species for the same target genus and 2) sequences that had no match to all other 18 beer-spoilage genera: *Pediococcus*, *Megasphaera*, *Pectinatus*, *Selenomonas*, *Gluconobacter*, *Obesumbacterium*, *Zymomonas*, *Dekkera*, *Saccharomyces*, *Kluyveromyces*, *Brettanomyces*, *Torulaspora*, *Pichia*, *Zygosaccharomyces*, *Lactobacillus*, *Paenibacillus*, *Micrococcus* and *Sphingomonas*. Clusters sequences having the highest percentage identity score, BLAST hits for the desired genus and no hits for non-target genera were manually selected for primer design (Table 2).

IV. Primer design: qPCR primers were designed to discriminate between species belonging to the genera *Levilactobacillus*, *Acetobacter* and *Bacillus* following the requirements for qPCR and HRM analysis: amplicon lengths (50 - 200 bp), CG content (40 – 60%) and Tm (58 – 60 ºC) (ThermoFisher, 2010, 2016). Primer design was performed on GenScript (Stothard P., 2000) using the selected cluster's sequences (Appendix A) as reference and the following parameters were customized to generate the primers: amplicon from 50 to 200 bp and optimum Tm = 60 ºC. All primers were tested *in silico* using Primer-Blast software (Ye et al., 2012), available on the NCBI website.

Figure 1. Pipeline to identify exclusive genes from the genera *Acetobacter*, *Bacillus* and *Levilactobacillus* for primer design. In **I** (OrthoMCL), the proteomes from *A. pasteurianus*, *B. cereus* and *L. brevis* were submitted to OrthoMCL to obtain a file with the orthologous sequences specific for each genus. The output file (group.txt) with the cluster's identification were generated and its protein sequences were accessed in the file (protein.fasta). In **II** (Clustal Omega), the cluster sequences were aligned in the Clustal Omega software to access percentage identity within sequences of each cluster. Sequences with percentage identity higher than 70% were selected. In **III** (Nucleotide BLAST), the previous selected sequences were submitted to BLASTn searching for a match to its target genus and no hits for non-target genera. In **IV** (GenScript), sequences showing hits for the target genus in the previous step were submitted to GenScript for primer design. The designed primers were submitted to **(V)** (Protein BLAST), searching for primers exhibiting hits for its target genera and no hits for the non-target ones. The best primers were selected to be further tested in PCR reactions.



Source: The authors.

2.4 End-point PCR

End-point PCR reactions were performed with all designed primers to check their ability to amplify the target DNA and its specificity for the desired bacterial genus. Their specificity and sensitivity were checked using pure DNA from relevant target and non-target strains (Table 1). PCR reactions contained 5 µL of the 5 X GoTaq ® Buffer, 2.5 µL MgCl2 (25 mM), 0.125 µL GoTaq® DNA Polymerase (5U/µL), 0.5 µL of each primer (10 µmol.l-1), 2 µL of extracted DNA (20 ng), 0.5 µL of dNTP (10 mM) and

13.875 µL H2O. All PCR reagents were obtained from Promega. PCR reactions were performed on a Mastercycler Gradient 5331 thermocycler (Eppendorf) at the following conditions: i) initial denaturation step of 94 ºC for 2 min, followed by ii) 35 cycles of 94 ºC for 15 s, 57 ºC for 30 s, and 68 ºC for 1 min. The amplification products were visualized by electrophoresis in 0.8% agarose gel and stained with GelRed™ (Biotium). Images were obtained in an UV transilluminator Fire-Reader (UVITEC Cambridge). All reactions were carried out in duplicates. The 100 bp DNA Ladder (Promega) was used as molecular size marker.

2.5 High Resolution Melting (HRM)

PCR amplification, DNA melting, and fluorescence level measurement were performed in the Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument with EDS Software. Reaction volume was 10 µL: 5 µL SYBR Green ® I Master Mix (Promega), 2 µL genomic DNA template (20 ng), 0.5 µL of each primer (10 mM), 0.1 µL dye, and 1.9 µL of DNA-free water (Sigma-Aldrich). An optimized qPCR reaction for the HRM method was conducted in a 48-well plate as follows: initiation at 95 °C for 10 min, then 35 cycles (95 °C for 30 s and 60 °C for 1 min). After amplification, a melting step was performed followed by a gradual denaturation of DNA for the elaboration of the melting curve, with an increment of 1 °C per minute until the temperature reached 95 °C. Fluorescent signals with temperature changes were recorded in the green channel. Data analysis was performed using StepOne Software v2.2.2 (Applied Biosystems, Thermo Fisher Scientific). Cycle threshold (Ct) values (the number of cycles required to get a positive PCR result differentiated from the background noise) were obtained with the same software (Livak & Schmittgen, 2001). Non-normalized fluorescence data for SYBR Green ® were used from HRM studies using the first derivative (df/dt) to estimate the values of melting temperatures (Tm). For this, the qPCR package was used in an R environment (Spiess, 2018; R Core Team, 2022). The calculated df/dt, as well as the Tm values, were used for construction of the graphic plots also in the R environment, with the support of the plot_ly {plotly} function (Sievert, 2020). After determination of the temperature shift, the different melting curves of the several amplification plots were generated with the data points before and after the melting curve. All real-time assays included negative and positive controls and were performed in duplicates.

To evaluate the specificity of the designed primers, a total of 14 strains from the following genera were tested: Acetobacter, Bacillus, Levilactobacillus, Micrococcus, Paenibacillus, Saccharomyces (Table 1). The specificity of the primers was first based on the Ct value. Therefore, we looked for primers showing low Ct values for the target genus (higher amount of nucleic acid sequence generated in few cycles) and none or high Ct values for non-target genus. After this first screening, the best primers were analyzed by the HRM method, based on the normalized melting peaks.

To evaluate the limit of detection of the selected primers for their respective genus, the HRM method was further validated using serial dilution of the extracted genomic DNA in the following concentrations: 10, 5, 2.5, 1 and 0.5 ng in a 10 µL PCR reaction.

To validate the ability of the mixture of primers in the HRM, we run a qPCR in three conditions:

I. Mixture of primers + pure DNA of each genus: we kept the same volume and concentrations of the primers as in the previous reactions, although having all primers combined. This was done to test whether this mixture would affect the overall performance of DNA amplification and melting curve profile. Each mixture of primers was tested with pure DNA from each genus.

II. Mixture of DNAs + pure primer: this assay was performed to check whether the mixture of different DNAs in a qPCR reaction would affect primer specificity and sensibility. Therefore, each set of primers were evaluated individually in qPCR reactions containing a mix of DNA from the species *L. brevis* (6.66 ng), *B. cereus* (6.66 ng) and *A. pasteurianus* (6.66 ng), reaching a final DNA concentration as described above (20 ng).

III. Mixture of primers + mixture of DNAs: we combined in one qPCR reaction both mixtures (primers and DNAs) to validate whether the mixture of both reagents would influence in the qPCR amplification and HRM analysis.

3. RESULTS

3.1 Strain Identification

The 16S rRNA gene sequencing of the 25 isolates belonging to the LBBMA culture collection resulted in five genera: *Levilactobacillus* (12), *Bacillus* (6), *Acetobacter* (9), *Paenibacillus* (3) and *Micrococcus* (2) (Table 1).

Table 1. Identification of isolates by partial gene analysis 16S rRNA with its access number from the sequences deposited in the Genbank database.

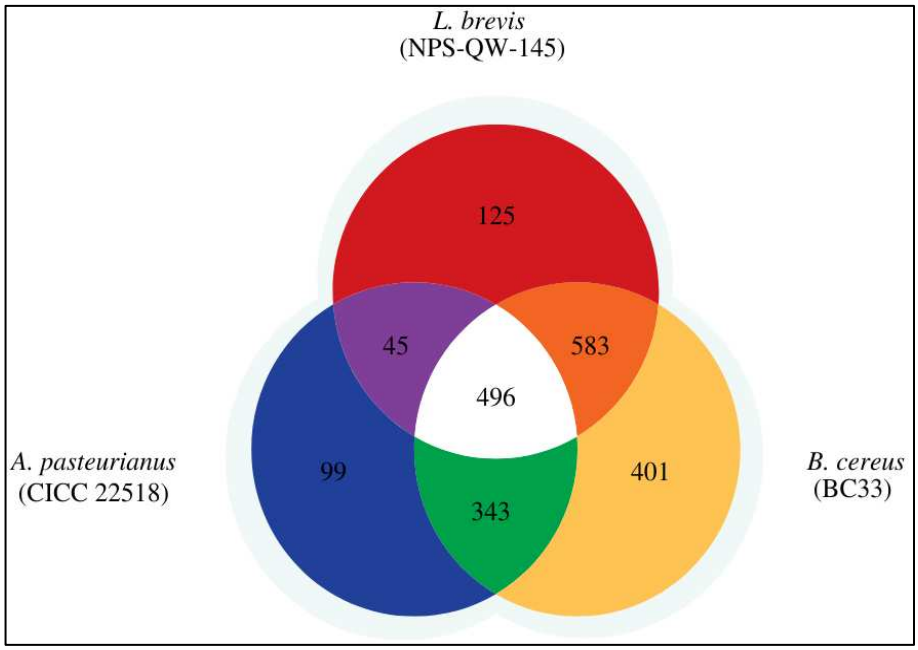
Isolate	Microrganism	Accession
P01	*Levilactobacillus brevis*	LC716666.1
P03	*Paenibacillus chibensis* *	MN918426.1
P02	*Levilactobacillus brevis*	OQ552721.1
P04	*Lactobacillus brevis*	YJ11B (GQ289391.1)
P05	*Bacillus cereus*	OR232325.1
P08	*Lactobacillus brevis*	SC13 (FJ532364.1)
P09	*Paenibacillus chibensis*	BGRI EBC SK18-28(MK332542.1)
P10	*Bacillus subtilis**	30L2-1 (JN366756.1)
P11	*Levilactobacillus brevis*	LC716666.1
P12	*Levilactobacillus brevis**	OQ552721.1
P15	*Paenibacillus chibensis**	ZYb3 (FJ432004.1)
C03	*Levilactobacillus brevis*	CP031198.1
C04	*Micrococcus* spp.*	30B.4 (MK456434.1)
C05	*Lactobacillus brevis**	KM668048.1
TUG1	*Bacillus cereus**	LKT 1/1 (AJ577278.1)
TUG3	*Acetobacter* spp.	(MK256695.1)
TUG4	*Acetobacter* spp.*	YJ11B (GQ289391.1)
TUG5	*Acetobacter malorum*	HWW3 (MH424758.1)
TUG6	*Bacillus megaterium*	N9 (MG430354.1)
TUG7	*Acetobacter malorum**	MT573608.1
TUG8	*Acetobacter cerevisiae**	SCMA40 (KJ469783.1)
TUG9	*Acetobacter malorum.**	MT573608.1
CVIC1	*Micrococcus yunnanensis*	SML_M164 (MG937693.1)
CVIC2	*Bacillus cereus*	OR232325.1
CVIC03	*Bacillus* spp.	PVL04 (KF648904.1)

*Beer-spoilage microorganisms used in the PCR assays. Source: The authors.

3.2 Comparative genomics study and target selection

OrthoMCL resulted in 2,408 proteins for Levilactobacillus, 2,211 for Acetobacter, and 5,278 for Bacillus. All proteins passed the quality test. Next, accessing OrthoMCL results (e.g., called groups.txt file) we identified 2,092 orthologous clusters, from which 99 were exclusive for Acetobacter, 125 for Levilactobacillus and 401 for Bacillus. All protein clusters is shown in Figure 2.

Figure 2. Venn diagram of orthologous genes in the three species used in this study with quality parameters as a maximum of 20% of stop codons and proteins length with more than 10 amino acids in size, used as thresholds in the all-against-all BLAST alignments. Exclusive genes of *L. brevis* (red), *B. cereus* (yellow) and *A. pasteurianus* (blue). Numbers of genes overlapping between all three species are given in their respective colors and circle.



Source: The authors.

After completing steps I-III (section 2.3), a total of 11 clusters were selected for primer design: *Acetobacter* (cluster1339, cluster1990, cluster2017, cluster2028, cluster1964), *Bacillus* (cluster2242, cluster2250), *Levilactobacillus* (cluster2354, cluster2310, cluster2340, cluster2341) (Appendix A). As a result, 12 pairs of primers showing the best parameters were selected for PCR assays, targeting sequences with amplicons ranging from 82 to 186 bp (Table 2).

Table 2. List of primers used in this work.

Genus	Primer	Sequence 5' --> 3'	Tm (ºC)	Size (bp)	Target region
Acetobacter	1247	TGGATCGGGCTTCATTTATGCG CGGGTCAGACCAACCCACAT	59.25 59.80	130	Hypotetical protein
	1269	CCACCCTGACGGTCGATCTG AACCCGCTGACAGGGTTGAA	60.3 60.55	130	Carbohydrate porin
	1077	CCACGTGCAGCCGGTCTATC AAAGGCCTGACCACCGGAGA	60.3 60.27	136	Energy transducer TonB
	1077_2	ACATTGAAGGCCGCGTGACA CGGGCCTTGTGCACGTAATC	60.06 59.1	130	Energy transducer TonB
	2110	TGGAAACAGGGCACCATCCG CGTCACAGGCAGCGTCAAGA	59.78 60.13	159	Hypothetical protein

Bacillus	887	TCTCATCAAAGGTTGCGAATGCTG AGCCAACCATCTGTTGTTCCATCT	59.67 59.98	82	C40 family peptidase
	1143	CATGAACTACAAGAATGTGCAACGACA GGACGTGTATTCGCTACTGATGC	59.30 59.56	98	Spore coat protein CotY
Levilactobacillus	2319	GGCGAGTACGAGGACTTCCA TTAGCTGGCGTCCGCATCTT	59.38 59.29	180	Hypotetical protein
	592	CCGGGCTTACCCAACGAAGA GTGTGCGCTGGCATGGTATT	59.21 59.74	189	5-dehydro-4-deoxy-D-glucuronate isomerase
	2283	CGCATATGGCGCACTGTGG CGCGGACAAACCGACCAATTC	59.49 59.68	122	Hypotetical protein
	2284	GAGTGACCGCCATCGCTCAG GCCGTTGCCGATCAACTGGT	60.34 60.76	153	Hypotetical protein
	2284_1	CCATCTTTCAGCTCGGCTGGT GCCATTGCTTCTCGCGTATTGG	59.59 59.57	183	Hypotetical protein

Source: The authors.

Primer specificity was first tested in an optimized end-point PCR reaction for selecting the best primers. Primers were tested against different beer-spoilage bacteria and the lager yeast *Saccharomyces pastorianus* (Table 1). A first screening was done by checking the amplification products of each set of primers in gel electrophoresis (data not shown). A second confirmation was done in a qPCR reaction to determine the Ct value for each tested species and its melting curve pattern. All strains in Table 1 (*) were tested in both types of PCR reactions. Among all primers, one resulted in no amplification (1247), five were genus-specific (1269, 1077, 1077_2, 2319, and 887) and six resulted in amplifications of more than one genus (2110, 592, 2283, 2284, 2284_1, and 1143) (Table 3).

Table 3. Ct values for the primers used in this work in qPCR reaction performed with 35 cycles.

Species	Strain	*Acetobacter*					*Levilactobacillus*					*Bacillus*	
		1247	1269	1077	1077_2	2110	2319	592	2283	2284	2284_1	887	1143
A. malorum	TUG7	-	29	26	26	25	-	27	26	26	26	-	-
A. cerevisiae	TUG8	-	29	26	26	25	-	23	28	25	25	-	28
Acetobacter spp.	TUG9	-	29	26	27	27	-	-	-	28	27	-	-
Acetobacter spp.	TUG4	-	29	25	25	26	-	24	25	24	24	-	-
Paenibacillus spp.	P03	-	-	-	-	24	-	-	-	26	-	-	21

P. chibensis	P15	-	-	-	-	24	-	-	-	25	-	-	26
L. brevis	C05	-	-	-	-	-	-	8	9	8	9	-	-
L. brevis	P12	-	-	-	-	-	-	9	9	8	9	-	-
Micrococcus spp.	C04	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	TUG1	-	-	-	-	-	26	25	26	26	26	27	28
B. subtilis	P10	-	-	-	-	-	27	25	25	25	25	-	24
S. pasteurianus	US_05	-	-	-	-	-	-	-	-	-	-	-	-

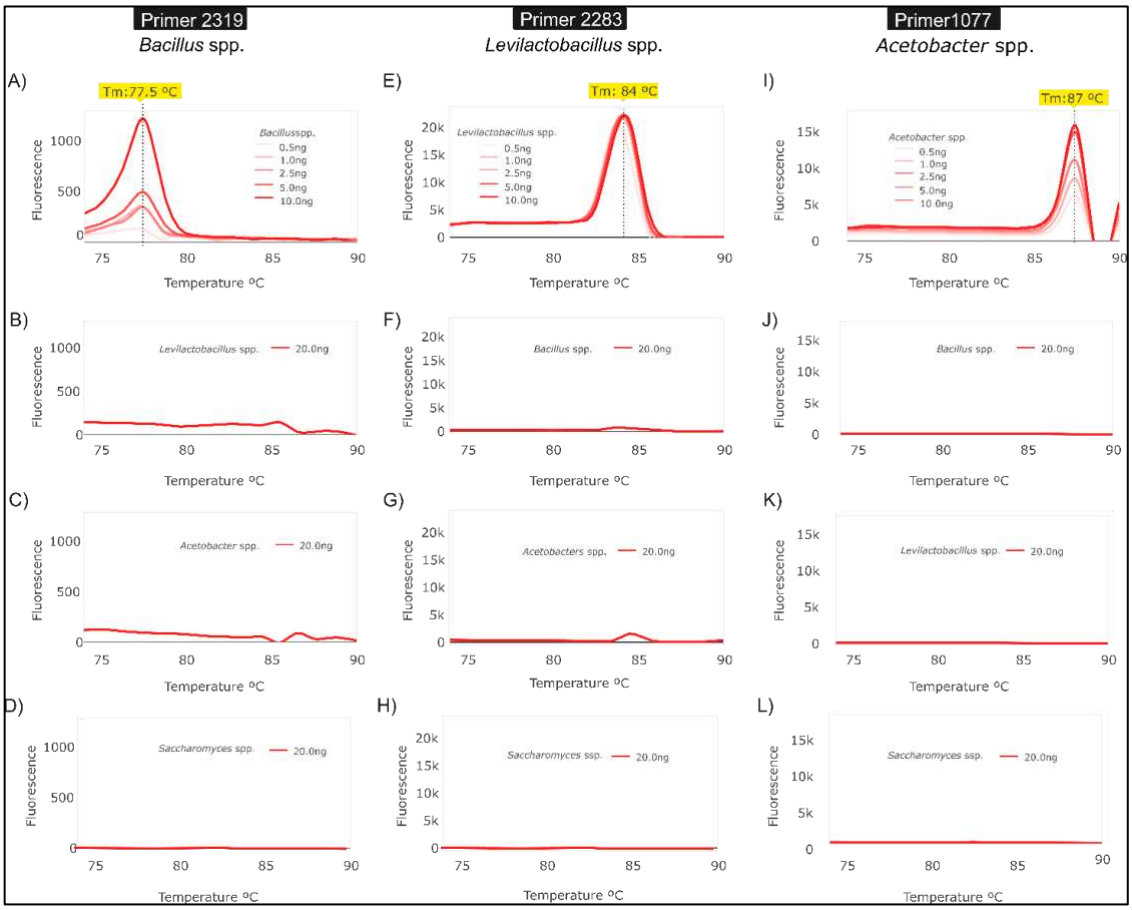
Source: The authors.

Primers showing the best results were selected to compose the multiplex reaction in qPCR. Primer 1077 was chosen as a representative for *Acetobacter* since it only amplifies this genus and had the lowest Ct value in comparison to the other primers. Primer 2319, despite being selected by OrthoMCL for *Levilactobacillus*, only amplified *Bacillus* and, therefore, was selected for this genus. Lastly, primer 2283 was selected for *Levilactobacillus*. Although all primers designed for this genus showed positive Ct values for the non-target genus in later cycles (Ct = 25 or higher), the amplification signal with primer 2283 for the genus of interest starts in the first cycles (Ct = 9) and, therefore, it was chosen for HRM analysis.

3.3 High Resolution Melting

Primer sensitivity, specificity and melting curves profiles were first analyzed individually for each set of primers, using DNA of its respective genus and non-target genera in PCR reactions (Table 1). All selected primers (2319, 1077, and 2283) exhibited different melting temperatures and specificity for the target genus (Fig. 4). Only one melting curve was obtained for each primer, indicating the specificity of the primer for its designed sequence. No melting curves were obtained with DNA from non-target genera for primer 2319 (Fig. 4. B, C, D) and 2283 (Fig. 4. F, G, H) and 1077 (Fig. 4. J, K, L). Furthermore, it was possible to detect the presence of DNA in the smallest concentration (0.5 ng), with less accentuated signal for primers 2319 and 1077 (Fig. 4. A, E, I). For these primers, the increase in fluorescence was linear with DNA concentration. On the other hand, for primer 2283, the smallest amount of DNA (0.5ng) the signal does not seem to differ from those obtained with higher DNA concentration (Fig. 4. E).

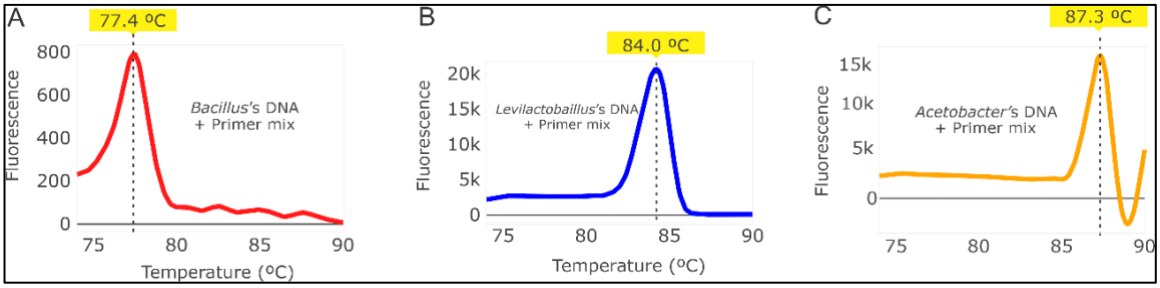
Figure 4. Melting curve plots showing the specificity and sensitivity of the designed primers for its target region in a SYBR Green real-time PCR assay. (a), (e) and (j) primer specificity and sensibility for its target genus in different DNA concentrations. (b), (c) and (d) specificity of the primer 2319 for the non-target genus, followed by primer 2283 (f), (g) and (h) and primer 1077 (j), (k) and (l).



Source: The authors.

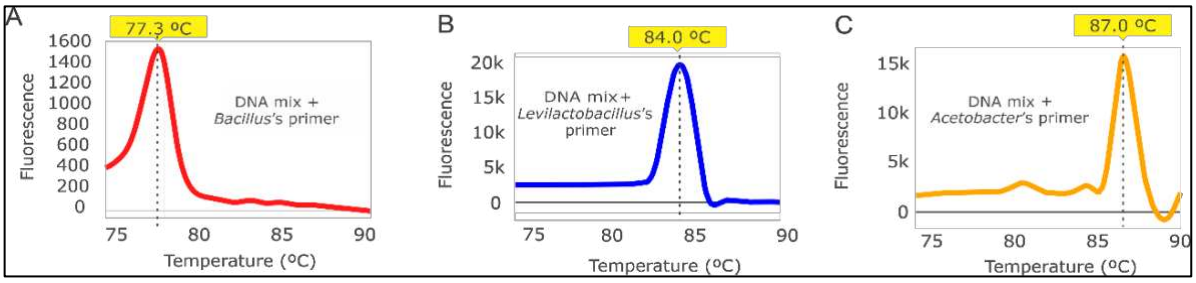
Each primer maintained its specificity for the target genus in all multiplex qPCR conditions: i) the mixture of primers with pure DNA (Figure 5), ii) mixture of DNA with pure primer (Figure 6) and iii) mixture of primers with mixture of DNA (Figure 7).

Figure 5. Melting curve plots showing the sensitivity of the SYBR Green real-time PCR assay performed with the mixture of primers with pure DNA (20 ng) from target microorganisms: (a) *Bacillus*, (b) *Levilactobacillus* and (c) *Acetobacter.*



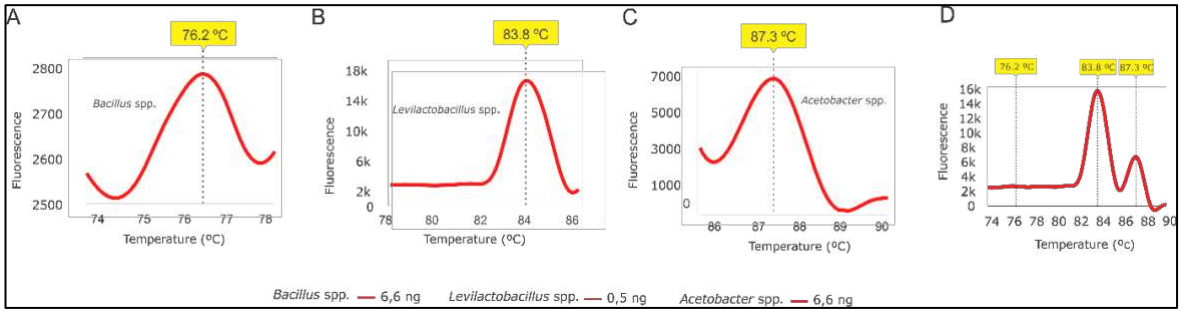
Source: The authors.

Figure 6. Melting curve plots showing the sensitivity of the SYBR Green real-time PCR assay performed with the mixture of DNA (6,66 ng of each genus) with each selected set of primers targeting is respective genus (a) *Bacillus*, (b) *Levilactobacillus* and (c) *Acetobacter.*



Source: The authors.

Figure 7. Melting curve plots showing the sensitivity of the SYBR Green real-time PCR assay performed with the multiplex reaction. Melting curve plots data points with 3º C before and after the melting curve for the genus (a) *Bacillus*, (b) *Levilactobacillus* and (c) *Acetobacter*. Melting curve of all primers together in one graph (d).



Source: The authors.

4. DISCUSSION

A crucial aspect in developing a reliable assay based on qPCR to detect and discriminate beer-spoilage microorganisms at the genus level is the selection of primers able to amplify only the target sequence (specificity of primers in targeting a given sequence of interest) and with sensitivity (minimum concentration of nucleic acid or number of cells which always gives a positive PCR result) (Nutz et al., 2011). Besides, primers must have different melting temperatures, enabling distinction between contaminants when performing HRM.

All selected primers were specific, being able to generate a single melting peak for their target genus (Figure 4a, 4e, 4i) and none for the non-target ones (Figure 4b, 4c, 4d, 4f, 4g, 4h, 4j, 4k, 4l, 4h), including the beer-spoilage genera *Micrococcus* and *Paenibacillus* (data not shown). Primer 2283 (*Levilactobacillus*) showed a positive Ct value (Ct = 26) for *Acetobacter* and *Bacillus* (Table 2). However, Ct values were higher for the non-target genera in comparison with the target genus (Ct = 9), which resulted

in a weak amplification of the non-target templates. Consequently, no melting curves were obtained for the non-specific genera *Acetobacter* and *Bacillus* (Figure 4f, 4g).

Amplicons of all sets of primers had different melting temperatures, making their differentiation easier after qPCR amplification (Figures 4-7). Since Tm is positively correlated with sequence length and GC content (% G+C) (Tong & Giffard, 2012), it was expected to have distinct melting temperatures, since the targets for amplification are regions with different lengths and GC content. Besides, amplification occurred even at the lowest DNA concentration tested (0.5 ng), indicating the high sensitivity of the designed primers for their targets (Figure 4a, 4e, 4i).

The perfect alignment of the primers with their target region is well known to be a crucial factor for primer sensitivity, since it impacts PCR amplification (Ye et al., 2012). In BLASTn analysis, the set of primers 2283 showed total alignment with its target genus. In this respect, the high sensitivity of the set of primers 2283 can be assigned with the perfect alignment of this primer with its target sequence. Set of primers 1077 showed mismatches ranging from 0 to 2 in each primer (forward and reverse) (Appendix B). Lastly, the set of primers 2319 did not show any hit for *Bacillus in silico*, although amplification for this genus occurred in PCR assays. This outcome emphasizes the complexities inherent in primer design, which do not always align with *in silico* tests. Discrepancies may arise from variations in taxonomic compositions, differences in template quality between reference databases and the actual biological community, and disparities between simulation conditions and the actual dynamics of primer binding during qPCR (Yu & Zhang, 2011).

Besides that, the number of copies of the target sequence in the genome is also a crucial factor affecting sensitivity (Bastien & Reischl, 2008). Set of primers 2283 and 2319 target a sequence related to a hypothetical protein in those genomes. Set of primers 1077 targets a sequence gene for the Energy transducer TonB, an energy transduction protein involved in active transport of nutrients, including iron, through the outer membrane. In our analysis, the target region of *Acetobacter* seems to occur in more than one copy, since its alignment occurred in up to three regions on the genome, while for *Levilactobacillus* just one region is presented (Appendix B). Therefore, since both factors (copy number in the genome and the quality of primer alignment to its target genus) influence primer sensibility, further experiments are encouraged to clarify

the impact of both factors together on primer sensitivity and sequence copy number in the genome.

Other factors influencing PCR sensitivity include DNA extraction (Yera et al., 2009), PCR inhibitors, (Bessetti J., 2007; Opel K.L., Chung D., & McCord B.R., 2010; Wilson I.G, 1997), primer content and magnesium concentration. Therefore, changing these factors could be a good option to improve the efficiency of primers 1077 and 2319. Despite this, all primers designed in this work are reliable in detecting and discriminating their target genus.

Primer-primer interactions, cross-reaction targets formation and competition between real target and cross-reacting species are some of the major concerns when performing multiplex reactions, leading to the exhaustion of reaction components and reducing assay robustness, specificity, and sensitivity (Kubista, 2006). Here, the mixture of primers did not affect the qPCR performance with pure DNA (Figure 5a, 5b, 5c). The same occurs when the mixture of DNA is combined with specific primers of each target-genus (Figure 6a, 6b, 6c). Therefore, when analyzed individually, the mix of all three sets of primers, as well as the mixture of DNAs, did not affect the identification of the target genus. Conclusively, the set of primers designed in this work can discriminate their target genus when performed in conditions that will normally occur when analyzing real samples (i.e., presence of more than one species or genus).

The last assay involved the simultaneous combination of all mixtures to create the final multiplex reaction, which includes the mixture of all primers and pure DNA from all three genera. In this experiment, the primers effectively amplified their target genus, and no other melting profiles were observed, indicating the absence of primer dimers. However, the efficiency of the primers significantly influenced the final multiplex reaction (Fig. 7a, 7b, 7c). In earlier experiments (data not shown), when all genera were present at the same DNA concentration (6.66 ng), the melting curve pattern was primarily influenced by the fluorescence level due to *Levilactobacillus* amplification, resulting in only its melting curve being generated. Nevertheless, by reducing the DNA concentration of *Levilactobacillus* to 0.5 ng, melting curves for all genera became visible (Fig. 7a, 7b, 7c). Nonetheless, due to the substantially higher fluorescence signal generated by primer 2283, the melting curve for *Bacillus* was masked when *Levilactobacillus* was present. The *Bacillus* melting curve can only be visualized when restricting the melting curve graph to its specific melting temperature

range (73-79 ºC). It's worth noting that all primers remained specific to their target genera, but for *Bacillus*, visualizing its melting curve requires careful consideration of the specified temperature range.

PCR reagent exhaustion did not pose a problem in this assay, as all the intended targets were successfully amplified. However, a 2-fold decrease occurred in the final multiplex reaction fluorescence for *Acetobacter*. (Fig. 7c), compared to its amplification when only its primers were present in the reaction (Fig. 5c). Therefore, higher DNA concentration of *Levilactobacillus* in the multiplex assay could lead to the reduction of PCR components affecting the other primers, since its amplification starts after 9 cycles, leaving primers 2319 and 1077, that starts in later cycles with less reagents to amplify their respective target sequences.

L. brevis is the most frequently beer-spoilage microorganism in beer. Thus, having the primer 2283 as the most sensitive one is very useful in detecting this genus in the brewing industry, enabling its detection in beer since the very early stages of beer spoilage. Primers 2319 and 2283 also amplify the target DNA at the lowest concentration tested in this work (0.5 ng). Nonetheless, DNA amplification for those genera starts in later cycles, what could be a concern in a real scenario, in which DNA is extracted from beers. In these beverages, the presence of PCR-inhibitors, such as high concentrations of brewing yeasts and their autolysis by-products, polyphenols, polysaccharides, alcohol, and proteins could be a limiting factor for detection of their respective target genera (Kumar et al., 2008; Schrader et al., 2012), since these factors could delay DNA amplification, with a Ct value exceeding the limit of those tested in this work (35 cycles). Despite this, the DNA extracted directly from microorganisms cultivated on the artificial contamination experiment in beer did not show any interference in our qPCR assay. Further, appropriate DNA extraction protocols seem to be a quick solution for problems regarding PCR-inhibitors (Pereira et al., 2018). Nevertheless, low DNA concentrations were proven to not be a problem for this set of primers in the conditions established in this work. Moreover, most of the protocols for DNA extraction from beer involves membrane filtration prior to extraction, aiming to concentrate bacteria cells before lysis, thus increasing DNA yield in the sample.

In a real scenario, controlling DNA concentration of beer-spoilage microorganisms prior PCR analysis in a brewery is not worth since the amount of each spoilage-genus in beer is unpredictable. Despite the potential for optimizations (e.g.,

increasing *Bacillus*'s primer concentration or $MgCl_2$ in the reaction), the high sensitivity of primer 2283 could still lead to dubious results. In this case, we recommend performing a multiplex reaction within primers with similar efficiency. In this work, primers 2319 and 1077 can be performed together in a multiplex reaction resulting in a consistent result, while primer 2283 should be used in a single reaction or in a multiplex reaction with other primers of similar sensitivity.

5. CONCLUSION

In this study, we have successfully developed a real-time multiplex PCR assay coupled to HRM analysis targeting orthologous sequences for the detection of beer-spoilage microorganisms belonging to the genera *Acetobacter*, *Bacillus*, and *Levilactobacillus*, across a range of DNA concentrations (0.5, 1, 2.5, 5, and 10 ng in 10 mL reaction volume). Our findings demonstrate that the SYBR Green real-time PCR assay is a swift, reliable, and cost-effective method for this purpose.

The multiplex reaction with primers 2319, 2283, and 1077 proved effective in identifying the target genus. However, it's essential to consider the varying efficiency of these primers when designing multiplex reactions. Depending on the specific requirements of the assay, it may be prudent to employ them in single reactions or in combination with other primers of similar sensitivity.

The methodology employed in this study can be readily adapted to design primers for the detection of different beer-spoilage microorganisms. We encourage further exploration of multiplex reactions involving three or more primers to optimize method cost-effectiveness. This approach is not limited to the field of brewing but can be extended to diverse applications across various domains for the detection of spoilage microorganisms.

In summary, our research provides a robust foundation for the development of PCR-based assays to target and identify beer-spoilage microorganisms, showcasing the adaptability and cost-efficiency of this approach while emphasizing the importance of primer efficiency considerations in assay design.

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7. APPENDIX

Appendix A – List of clusters selected for primer design in this work

Genus	Cluster	Identity (%)	Sequence
Acetobacter	Cluster1339	100.00	GTGGTGTTGAGAGTATCTGTTGCAAGGGAGTTTGTGGATCGGGCTT CATTTATGCGGCGCTGCGATAGCGGCACGTATGGGTGTATGTCTG GTGTTGATCTTTGTCAGTGTGAATCGGTTGGTGCGCGTCTGGGCGT ATCGGAAATGTGGGTTGGTCTGACCCGTATTGAGCAAAGAGATTTG CTCATGCGTTAA
Acetobacter	Cluster1990	95.88	ATGCAAAAATACCGTAAAAATAATAGAAAATTTCTAAAGTTTTTTTGT CTTTACGCGACAATTCTAACTGCTCCCGCCCTAGCGCATGGTGAAG AGACGCAGGTTGAGCAGGATCGTCGTCGTTTAGGCAATTCAGGGC CATTGATTGAACAGATTGATCCGGCCACGGTGCGGGATTCCCAGC GTGCGGCAGAGGCCGCGGCTTCTGAGAAAAAAGGCGATGACACGC CAGACATGAGTGACCACCTGCTTGGCAACATGTGGGGCGCGCGGG ATTGGATGGCCAGACACGGCATCAGCTTTGACATTCAGGAAGTGGA TGAACTGTGGGGCAATGCCACAGGGGGCACGGCCTCGGGGGCGG ATGGCGCCAGTGGCTCGGGCACCGGCCCCGCCTATGATGGTGTGA CCATGCCCACCCTGACGGTCGATCTGGAAAAGCTGATCGGCCTGA AGGGCGGCACATTCAATGTCAGCGCCCTGCAGCTGCGCGGGCGCT CCATCTCGCAGGATCATCTGGCCAACTTCAACCCTGTCAGCGGGTT CGAGGCCGACCGTTCCACACGCCTGTTCGAGCTGTGGTATCAGCA GTCCTTTTTGGACGGCAAGCTGGACGTCAAGATCGGGCAGCAGGA TCTGGATACCGAGTTCCTGATCAGTGATTACGGGGCTTTGTATCTG AACTCCAACTTCGGCTGGCCCATGGCGCCATCGGTCAACCTGTATG CCGGTGGCCCGTCCTGGCCGCTGTCTTCTCCGGCCATTCGCATCC GGTATCGTCCGTCAGACAAGTTCACCTTCATGTTTGCCGCAGCGGA TGACAACCCGCCGGGCAATCGCAACAACTCCTTTGGCATCCAGAAC GGCGGCAACAGTGCAGATCCCACCAATCAGAATACTCATGATGAAG ATGGCGCCAACTTCAACATGGGCACCGGTGCGCTGCTGATTACCG AACTGCAATATGCCCTCAACCCCCAACCCGATGACATGTCGCATGT CACTCAGGACCCCGGTCTGCCGGGTATCTACAAGCTGGGTGGCTA TTACGATACGGCCAAATTCCCTGATTACCGCTACAACAATCAGGGC AAGGCTTTAGGGAGTGCGGCGGACACTACGGGCATTCCGCGGTGG GATCGGGGTAACTGGATGGTCTATGGCATTATTGACCAGATGATCT GGCGGCCCTCGCTCCAGTCCCCTCAATCTGTAGGTGTTTTTGCGC GTGCCACAGGCAATGGGGGAGATCGCAACATGATCAGCTTTGCTAT TGATGCGGGCATCAACCTCAAGGCGCCCTTCAAGGGGCGTGACAA TGACACGGTGGGTCTGGGCTGGGGCATTGGCCGGGCCTCTTCTGG CCAGCGGCGGTATGACCGTAACTCCGGTGCACCTGTGCAGGGCAA TGAGAACCATCTGGAACTCACCTATCAGGCACAAGTGATGCCGTGG TGGGTCATGCAGCCGGACTTCCAGTATGTCTGGCATCCCTCTGGC GGTGTGACTGACTGGACAGGTAACCGTCTCGTCGGGAACGAAGCC ATCTTCGGCCTCCACTCCAATATCACTTTCTAG

	Cluster2017	75.20	ATGCAGAATATTTCAGAACATTCCGAAAAGCTGGAAAGTGACGCTG CTCAACAGACAGAGGGAAGGAGCGTTATGTTCTTCGCGCTGGCGG TGCGGTTGGAGCTGGAGCGCTTATAGCGGTTCTTCTTGCATGGAG CTGGTGTGTGGGGGCTGATGTATGGGATGGGCACGAAGGCGAGC GTGCCTGTCGAGCATCCGCCGATCAAGGCTGAGATGCTGCCACCA CCTCAGCCGCCACCGCCGCCACCTCCCCGCCGCCACCACCGCCG GTGATGGCCGAGCCGCCGCCGCCATATATTCCGCCACCCAAAATC AGTGCCACCGCCACCCAAGCCGCCCATCAAGCACGTGGCCAAGGC GCCGCCCAAACACCCGGCGCCCCCACAAACCAAAACGGCGAAGG CCCCACCAGCCAACACCGAGCCCGCAGCCAGCGCACCCCCATCC GATGCGCCGGACACCACGGCCGGCACCGCGCCGCTCAACCACGT GCAGCCGGTCTATCGCCAGAAATGGAGGAAGACAACATTGAAGGC CGCGTGACAGTAGCCTGCGATGTGGAGCCTACGGGCATGACCAGC AACTGCCAGGTGCAGTCGGTCTCCGGTGGTCAGGCCTTTGCGCAG GCGGCGTTGGATTACGTGCACAAGGCCCGCTATCGCCCGGCCACA CGCAATGGCGCACCGGTGAAGGAACTGCACAAGGTCTACGTCATT CGCTTTAGGCTGGATGACTGA
	Cluster2028	75.46	ATGGATGGCGAACGCATTTCCGGGGATGAAGATGCAGCTGTTGGC GGTATTGCCGCAGATCGGCTGAGAAGCATTATTGAGCGTGTTGAGC GCCTGGAAGAAGAGCGCAAGGCTCTGGCTGGAGATATCAAGGATA TCTTTACAGAAGCAAAGTCTGCCGGGTTTGATGTAAAGGTTATCCG CCAGATCATCCGTCTGCGGAAGCAGGAACCGGCCGAAGTGGAAGA GCAGGAAACACTGCTGGATATCTACCGTCGCGCATTGGGTATGTAA
	Cluster1964	77.00	TTGAAGGAGCCCGATCATGAACAACATGACACAGGCTTCCCAGAAG CTTCCGATCTGGAAACAGGGCACCATCCGGCTCATGAAGATCTGTT GCAGATCCGCGATGCGCTTTTGCCAGCACGGCAGGGCATGCGCGA TGGAAAAAACCACCATGTGGACGGGATCATCCTGCTGACGGAGCG TCTTGTAAAAGATCTTGACGCTGCCTGTGACGCGCTGGATGCGGCA TCGCGAAGCTGA
Bacillus	Cluster2242	83.00	ATGTTTAAGAAAAGTATAGGAATTTTATTGTTTTTAATTCTATCTATCT CGACATTTAGTATAGTAACTCACGCTGCTAGTAATTCAGAATCCGTG AATCAATCATTCTATGGGTATAAGGAGCCATCTTTTAATTCAGCTAA AACAAATGGCGGATCAGAGTATGGTGCTCAAAATGTAGGAGTCGTG GAAAAAAGAGATAATGGCTGGTGGAAGATTGAAACATGGGAAGGTC CAGTTTGGATTAACTTAAATGGGGAAGAACGTGTCATGGGAGATTT CTATGGATATGATGAACCTTCTCTCTCATCAAAGGTTGCGAATGCTG GAGCGAAGTACGGGCGACAAACATTTAGAATAGTAGATGGAACAAC AGATGGTTGGCTTAAATTTAAGACGTGGGAAGGCGAAAAATGGATG AATCCTGCGGCTGAACAAATTACCGTCAACAAAACGATTTATGCATA TAATGAGCCTTCATTTAATGCAAAGAAAGCAAATTATGGAGCACCAT TTAACCCACAAAATTGGGGAGTAGTAGAAAAAAAAGAAAATGGCTG GATGAAAGTAAGTACTTATGAAGGTTATAAGTGGATCAATCCAGATG GAGAAGAAAAATTCATAAACAAATCATTCTATGCGTATAACGAAGCC TCATTTAATGCAGCAAAAGCAAATGCTGGAGCATTATATAATCCGCA GAATTTTAGAGTTGTGGATGGGACAACCAGTGGATGGTTAAAGGTT AAAACATGGGAAGGCGAGAAATGGATGAATCTAGATGGAGAAGAAA GATTTATAAATAAATCTTTCTATGCGTATAATGAACCTTCATTTAGTT CAGGAAAAGCAAACGCTGGAGCATTATATAGTCCGCAGAATTTTAG AGTTGTGGATGGGACAACTAGTGGATGGTTGAAGATTAAAACATGG GAAGGCGACAAGTGGATTAATCTTAACCAAACAGATTCTGGTAATTC TGGAGTGGTTGACCTTGCTTTAAAACAATTAGGAAAACCATATGTAT TTGGAAATAGTGGACCTAATTCATTTGATTGTAGCGGTTTTATTTATT ATGTATTTAAAAATAATGGATACAATATTGGAAGAACAAGTGTAG CTGGATATTGGGGAATGGTTACAAAAATAAGTGATCCTCAACCAGG AGATTTAGTATTCTTACAGAATACATATAAAGCGGGTCCTTCTCATTT AGGAATATATTTAGGGAATGGTGAATATATTCATGCAGCTGACGAAA CAACAGGTGTAATTAAAAGTAAGATAAATAGTTCATACACTCAAAAG CACTTCTTGGGGTATGCTAGATTTTCAAAATA

Levilactobacillus	Cluster2250	82.00	ATGAGCTGCAATTGTAATGAAGATCATCAACATGAGTGTGATTTCAA CTGTGTATCGAATGTTGTTCGTTTTATACATGAACTACAAGAATGTG CAACGACAACATGTGGATCTGGTTGCGAAGTTCCATTTTTAGGTGC ACATAACAATGCATCAGTAGCGAATACACGTCCTTTTATTTTATACA CAAAAACTGGAGAACCTTTTGAAGCATTTGCACCATCTTCAAGCCTT ACTAGCTGCAGATCTCCCATTTTCCGCGTAGAAAGTATAGATGATG ATGACTGTGCTGTATTGCGTGTATTAACTGTAGTTTTAGGTGATGGT ACCGCTGTACCACCTGGTGACGACCCAATCTGTACGTTTTTAGCTG TACCAAATGCAAGACTAATATCAACCTCCACTTGCATTACTGTTGAT TTAAGTTGCTTCTGCGCTATTCAATGCTTACGCGATGTTTCTATTTAA
	Cluster2354	81.35	TTGGCAGATAAAGAAGTTACGCAAGTTGAAATGATGAAGATTATTGC ACTGTTCCGTAAAGAAGGCTTTAAGGGCGAGTACGAGGACTTCCAA CGAGTTAATGGTACCGACCGAGAGTTCTTTGTGGTCATGAGTAATG AGCAAGGTATTAAGGCATTGTTCCGAGCTAGCTTGATGTTGAACGC TGTGGAGTTCCAGTACGTACTGGATGATAAGCATACGTTTGTGCAG GAAGATGCGGACGCCAGCTAA
	Cluster2310	77.30	ATGGCATTTAATATGGTAACGAAATATGCACATAGTCCTAAGGATAT TGATCATTACAATACGGATGAATTACGTGATCAGTTCTTAATGGAAA AGATTTTTAATCCGGGTGATATCTTACTGACCTATACTTATAACGAC CGGATGATCTTTGGTGGTGTGACGCCAACTGATCAACCACTTGAAA TTAAATTAGATAAAGAGCTAGGGGTTAAGTACTTCTTGGAACGTCGT GAATTGGGCTTCATCAACATTGGTGGTGAAGGTAAAGTTACGATCG ATGGTCATGAAGATACAATTGCACCGCACGATGGCTATTACATTAGT ATGGGAACTAAGGAAATTAAGTTTGAGTCCGTGGATACTAAGAATC CAGCCAAGTTTTATGTCGTATCGACACCAGCACACCGGGCTTACCC AACGAAGAAGTTGGCGTATAAAGATTCGATTGCGATGCCAATGGGC GATCAAGAACATATGAACAAGCGGACCATTCATAAGTACATCGATG CTTCCATCATGGATACCTGCCAGTTACAAATGGGGTACACGGTATT GGAACCAGGGAACTCTTGGAATACCATGCCAGCGCACACCCATGC CCGGCGGATGGAAACCTACCTTTATATTGAATTTGGTGCTGACGAT ACCCGAGTTGCCCACTTTATGGGGACACCAGAAAATACGAAGCACA TTTGGTTGGAGCCAGAACAGGCAGTTGTTAACCCAAGTTACTCGAT TCACTGTGGGGTTGGGACAACGAACTACGCTTTCATCTGGGCAATG TGTGGTGAGAACCAGACTTACGATGATATGGATGCAGTTGACATGC ATCAATTACGTTAA
	Cluster2340	83.00	ATGAGTAATGGCGCATATGGCGCACTGTGGGGTCTCGTTTTGGGA CTCGTATGGGTTTTCCAAAGCTTCCCCGCAATGTTGTTAGTTGCTGT GTTTGGCCTAGTGGGCTGGGGAATTGGTCGGTTTGTCCGCGTAGA TCTTTCGGCCCTAGGTAAACGGATTGAACAACTCTTATCGAGATAA
	Cluster2341	76.37	ATGCGACGAGGCAACAAAGGACTTTTGACGATTCTAGGACTGATTC TACTAGCTTTGGCCATCTTTCAGCTCGGCTGGTGGCTGCCGATTCC GGGACTTTCAGCGTGGAGTTTAGAAATGAGCTATACCCAAATGGCT TGGTTAAAATATGTTTTAGCCGGGGCCGTGATTATTACCGGAATCG TTGGTTTGGGATTAGTGGTTATCGGTATCTTCAAACCAATACGCGA GAAGCAATGGCAATTCACCAATCAGCTTGGTATGTTGGAAGTGCCA CAGG CGGCGCTAGAAAAAGCGTTACGTCACCAACTGGTTGAACAGGTGG GCTTAGTTGACCCCCGAGTAACGGTGAAATTGTTACGCCACCGGC GTGCCCGAGTGACCGCCATCGCTCAGGTCAGTGCAACGGATCAGA TTGATCTGCTGGCACAACAGGCTAGCCAAGTGATTGATGCTTATTTA CAGCAACAATTAGATTTGACGGCCGTCAAACCGGTTGTCCGGTTAT CACCAGTTGATCGGCAACGGCACGTGTCAGTCGTGTAA

Source: The authors.

Appendix B – Primer alignment analysis with its target genus

Products on target templates

Primer 1077 (*Acetobacter* spp.)

>NZ_CP039846.1 *Acetobacter pasteurianus* strain CICC 22518 chromosome, complete genome

```
product length = 136
Forward primer  1        CCACGTGCAGCCGGTCTATC  20
Template        1858092  ....................  1858111

Reverse primer  1        AAAGGCCTGACCACCGGAGA  20
Template        1858227  ....................  1858208


product length = 136
Forward primer  1        CCACGTGCAGCCGGTCTATC  20
Template        1149033  .A.T................  1149014

Reverse primer  1        AAAGGCCTGACCACCGGAGA  20
Template        1148898  ............G..A....  1148917
```

>NZ_CP042808.1 *Acetobacter oryzoeni* strain B6 chromosome, complete genome

```
product length = 136
Forward primer  1        CCACGTGCAGCCGGTCTATC  20
Template        2068714  ...T................  2068695

Reverse primer  1        AAAGGCCTGACCACCGGAGA  20
Template        2068579  ..............A....  2068598


product length = 136
Forward primer  1        CCACGTGCAGCCGGTCTATC  20
Template        1269141  ...T................  1269122

Reverse primer  1        AAAGGCCTGACCACCGGAGA  20
Template        1269006  ..............A....  1269025


product length = 136
Forward primer  1       CCACGTGCAGCCGGTCTATC  20
Template        110978  TA..................  110997

Reverse primer  1       AAAGGCCTGACCACCGGAGA  20
Template        111113  ...A...........A....  111094
```

Primer 2283 (*Levilactobacillus* spp.)

>NZ_CP015398.1 *Levilactobacillus brevis* strain NPS-QW-145 chromosome, complete genome

```
product length = 122
Forward primer  1        CGCATATGGCGCACTGTGG  19
Template        2411559  ...................  2411541

Reverse primer  1        CGCGGACAAACCGACCAATTC  21
Template        2411438  ......................  2411458
```

Source: The authors.