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Short communication

Multiplex PCR for rapid identification of major lactic acid bacteria genera in cider and other fermented foods

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ABSTRACT

Lactobacillus, *Pediococcus*, *Oenococcus* and *Leuconostoc* are the main Lactic Acid Bacteria (LAB) genera present in cider as they are able to survive this hostile environment. LAB play a significant role in cider quality, for example in the process of malolactic fermentation, even though they can also be involved in spoilage of cider (production of biogenic amines, exopolysaccharides, off-flavours…). In this context a better monitoring of the fermentation process is a matter of interest to guarantee cider quality. In the present study, we designed a genus-specific multiplex PCR for a rapid and simultaneous detection of the four main LAB genera involved in cider production. This multiplex PCR worked equally with purified genomic DNA of bacterial isolates and with colonies directly picked from agar plates. This new PCR method was also successfully extended to wine and dairy isolates, and thus constitutes an effective tool to quickly identify LAB associated with fermented foods. Moreover, many biodiversity studies would also benefit from this fast, cheap and reliable identification method.

1. Introduction

Cider (fermented apple juice) is produced in at least 25 countries worldwide. Its consumption is the most important one in Europe, and especially in England, Spain, France, Ireland and Germany, representing almost 60% of the world consumption compared with only 11% in North America (Global Data and the European Cider and Fruit Wine Association, 2018). Lactic acid bacteria (LAB) are ubiquitous or added as starters in many fermented food products, especially fermented beverages such as cider (Marsh et al., 2014). Cider represents a specific habitat in which the physiological and chemical conditions change during the fermentation process, thus allowing LAB to grow (Cousin et al., 2017). Since they are adapted to such hostile conditions, LAB influence the cider production. The role of LAB in cider is complex and still unclear. After the alcoholic fermentation has been carried out by yeasts, LAB convert L-malate into L-lactate (malolactic fermentation) which leads to a biological pH increase, and contributes to microbial equilibria and flavour modifications. LAB activity has therefore a potential positive effect on cider quality. On the other hand, several LAB can also be responsible for spoilage in cider by generating numerous off-flavours (Buron et al., 2012), biogenic amines (Coton et al., 2010; Garai et al., 2007; Ladero et al., 2011) and exopolysaccharides (Dueñas-Chasco et al., 1997, 1998; Ibarburu et al., 2007, 2015; Puertas et al., 2014).

One of the main challenges of sustainable cider production, and, to a larger extent, of sustainable fermented foods and beverages production, is the control of LAB development through the fermentation process. This strongly relies on the rapid and accurate identification of the LAB involved in cider production. Monitoring the occurrence of the main LAB throughout cider fermentation thus constitutes a keystone for monitoring the smooth running of the process. At the present time, 14 different LAB species, belonging to the genera *Lactobacillus*, *Pediococcus*, *Oenococcus*, and *Leuconostoc* have been described in cider (Cousin et al., 2017) (Cousin et al., unpublished data). Some of these species, such as *L. mali* (Carr and Davies, 1970), *L. collinoides* (Carr and Davies, 1972), *L. sicerae* (Puertas et al., 2014), and *O. sicerae* (Cousin et al., unpublished data), have been described for the first time in cider.

Identifying LAB is a real challenge, and it can be tricky. There are still no available highly selective media that allows the consistent isolation and identification of LAB, and especially of new LAB species

Abbreviations: CIRM-BIA, Centre International de Ressources Microbiennes - Bactéries d'Intérêt Alimentaire; LAB, lactic acid bacteria; SNP, Single Nucleotide Polymorphism; UCMA, Université de Caen Microbiologie Alimentaire

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(Vera et al., 2009). Future cultivation work will therefore need to focus on improved subcultivation, purification, and preservation techniques to recover and use a larger fraction of the microbial diversity found in fermented apple products (Overmann et al., 2017). Due to the major role of LAB in the quality of cider, a rapid and cheap identification of these bacteria is therefore of utmost importance. Having a better knowledge of the LAB present at the different stages of the fermentative process pave the way to find new levers of action for better controlling cider fermentative process, for example by predicting the formation of aroma compounds and anticipating quality defects that may occur. This is the reason why molecular tools, in addition to the time it usually takes to carry out phenotypical and physiological tests, have been developed to quickly identify these bacteria in fermented foods (Ben Amor et al., 2007). Among those molecular tools, multiplex PCR constitutes a rather cheap and rapid method for the identification of LAB. For example, a multiplex PCR was designed to allow the identification of seven species of probiotic *Lactobacillus* species (Kwon et al., 2004). Multiplex PCR have also been designed for the identification of LAB in fermented foods such as sourdough (Robert et al., 2009), wine (Petri et al., 2013), coffee (Schillinger et al., 2008), banana fruits (Chen et al., 2017), pickles (Yu et al., 2012), and dairy products (Plessas et al., 2017; Yu et al., 2011). So far, there is no multiplex PCR which might allow the identification of bacteria in cider, except for a few pathogenic (Li and Mustapha, 2004) and spoilage bacteria (Coton et al., 2005, 2010).

This study aimed at designing a multiplex PCR for the rapid and accurate identification of LAB isolated from cider. The detection of the four major LAB genera frequently isolated from cider was based on the design of universal and genus-specific primers, namely *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus*. This multiplex PCR was also extended to LAB isolates from wine and dairy environments. It easily and accurately identified the tested genera and species. This multiplex PCR worked equally with bacterial genomic DNA and with colonies directly picked from plates. This method constitutes an effective tool to quickly identify LAB isolates. In this regard, it offers rapid strain screening possibilities for supporting microbial quality controls, the monitoring of fermentation processes, even biodiversity studies of fermented apple products and, to a larger extent, of fermented foods.

2. Material and methods

2.1. Bacterial strains and growth conditions

Bacterial strains were provided by the UCMA's (Université de Caen Microbiologie Alimentaire, Caen, France) and the CIRM-BIA's (Centre International de Ressources Microbiennes - Bactéries d'Intérêt Alimentaire, INRA, Rennes, France) culture collections (Table 1). The strains used for this work consisted in reference LAB strains of different genera, LAB isolated from cider, wine must and dairy environment, one yeast (*Saccharomyces uvarum*), and other non-LAB (i.e. acetic acid bacteria and *Zymomonas mobilis*) isolated from the same biotopes. All LAB and acetic acid bacteria were routinely cultivated at 30 °C with 5% CO_2, in MRS (De Man, Rogosa and Sharpe, Difco) supplemented with 5 $g{\cdot}L^{-1}$ fructose and 0.5 $g{\cdot}L^{-1}$ cysteine, at pH 5.5. *Zymomonas mobilis* was cultivated in *Zymomonas* medium (Coton et al., 2005) in anaerobic conditions at 30 °C. *Saccharomyces uvarum* was cultivated in YEG medium (yeast extract 5 $g{\cdot}L^{-1}$; glucose 20 $g{\cdot}L^{-1}$) at 25 °C.

2.2. DNA extraction

Microbial genomic DNA was extracted from 1 mL of stationary phase cultures using the NucleoSpin® Microbial DNA kit (Macherey Nagel), according to the manufacturer's instructions for Gram-positive bacteria. Genomic DNA was quantified using a spectrophotometer (Thermo Scientific Nanodrop 2000) and checked for integrity in a 0.8% agarose gel containing 1× Midori green advance (Nippon Genetics Europe). The molecular size marker GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) was used. The gDNA patterns were documented by a gel-doc system (Photodoc Ebox, Vilbert Lourmat). Genomic DNA was diluted in the elution buffer to a final concentration of 5 $ng{\cdot}\mu L^{-1}$ and stored at −20 °C.

2.3. Design of genus-specific primers

The sequences of the rRNA genes (16S, 23S and 5S) of the type strains of each *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* species, and the sequences from close genera (i.e. *Weissella* sp., *Fructobacillus* sp., *Carnobacterium* sp., *Aerococcus* sp., *Enterococcus* sp., *Lactococcus* sp. and *Streptococcus* sp.) were downloaded from the NCBI GenBank database, or extracted from the corresponding genomes using the RNAmmer webtool (Lagesen et al., 2007). Universal primers targeting all bacteria were designed and used as a PCR internal control (Fig. 1, Table 2). 16S or 23S sequences were aligned with MUSCLE in MEGA7 (Kumar et al., 2016). Primers were designed manually (or slightly modified from the literature in order to standardize the annealing temperatures) on genus specific conserved regions (Fig. 1, Table 2) and purchased from Eurofins Genomics.

2.4. Multiplex PCR conditions

The multiplex PCR contained a total of 9 primers (Table 2). Multiplex PCR reactions were performed in 15 µL containing 1× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 250 nM of each primer (expect for the L_F primer which had a final concentration of 500 nM) and 5 ng template gDNA. Multiplex PCR was performed in a Veriti Thermal Cycler (Applied Biosystems) under the following conditions: 3 min at 95 °C for initial denaturation, 35 cycles consisting in denaturation for 15 s at 95 °C, annealing for 15 s at 53 °C, and elongation at 72 °C for 20 s, followed by a final 5-minute extension step at 72 °C.

PCR amplifications were analysed by gel electrophoresis on 2% agarose gels containing 1× TAE and 1× Midori green advance, in a horizontal electrophoresis system (Mupid®-ONE) for 30 min at 100 V. The molecular size marker GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific) was used. The PCR amplification patterns were documented by a gel-doc system (Photodoc Ebox, Vilbert Lourmat).

2.5. Validation of the multiplex PCR on identified strains from cider, wine and dairy environments

The multiplex PCR was validated using bacterial strains, previously identified by 16S Sanger sequencing, isolated from cider, grape must and dairy products. Each targeted biotype and genera species was represented by a single strain out of 66 (Table 1). Since PCR was performed directly on colonies picked from plates, the first denaturation step of the PCR run was extended to 10 min to allow an efficient cell lysis.

2.6. Application of the multiplex PCR to unknown isolates from a cider sample

The practicability of the multiplex PCR system for LAB identification was directly investigated on unidentified isolates from a cider sample. Lactobacilli were sought on Rogosa SL (Conda) agar, oenococci on modified MLO agar (Medium for *Leuconostoc oenos* supplemented with 10 $g{\cdot}L^{-1}$ L-acid malic, Caspritz and Radler, 1983), pediococci on PSM agar (Pediococci Selective Medium, Leuschner et al., 2003) and leuconostoc on MSE agar (Mayeux, Sandine and Elliker, Biokar). Each agar media was supplemented with 100 $mg{\cdot}L^{-1}$ pimaricin and 7 $mg{\cdot}L^{-1}$

Table 1
Bacterial strains used in this study.

Strain name	Other name	Identification	Source
UCMA[a] 15531		*Lactobacillus mali*	Cider
UCMA 7168[T]	DSM 20336[T]	*Pediococcus pentosaceus*	Dried beer yeast
UCMA 14183[T]	DSM 20343[T]	*Leuconostoc mesenteroides*	Fermenting olives
UCMA 18216[T]	DSM 20252[T]	*Oenococcus oeni*	Wine
UCMA 5972		*Aerococcus viridans*	Raw milk
UCMA 7287[T]	DSM 4847[T]	*Carnobacterium gallinarum*	Ice slush from around chicken carcasses
UCMA 10625[T]	DSM 20477[T]	*Enterococcus faecium*	
UCMA 14185[T]	DSM 20349[T]	*Fructobacillus fructosus*	Flowers
UCMA 993[T]	DSM 20481[T]	*Lactococcus lactis*	
UCMA 1000[T]	DSM 20617[T]	*Streptococcus thermophilus*	Pasteurized milk
UCMA 14184[T]	DSM 20410[T]	*Weissella viridescens*	Cured meat products
UCMA 10447		*Saccharomyces uvarum*	Cider
UCMA 12998[T]	DSM 20515[T]	*Lactobacillus collinoides*	Cider
UCMA 15818		*Lactobacillus hordei*	Cider
UCMA 16955		*Lactobacillus diolivorans*	Cider
UCMA 15387		*Pediococcus ethanolidurans*	Cider
UCMA 15901		*Pediococcus parvulus*	Cider
UCMA 18596		*Leuconostoc mesenteroides*	Cider
UCMA 18597		*Leuconostoc pseudomesenteroides*	Cider
UCMA 14597		*Oenococcus oeni*	Cider
UCMA 15228[T]	DSM 107163[T]	*Oenococcus sicerae*[b]	Cider
UCMA 16846		*Acetobacter orleanensis*	Cider
UCMA 15501		*Gluconobacter oxydans*	Cider
UCMA 10408[T]		*Zymomonas mobilis*	Cider
CIRM–BIA[a] 2232		*Lactobacillus brevis*	Grape must
CIRM–BIA 2081		*Lactobacillus buchneri*	Grape must
CIRM–BIA 2239		*Lactobacillus coryniformis*	Grape must
CIRM–BIA 2123		*Lactobacillus hilgardii*	Grape must
CIRM–BIA 2238		*Lactobacillus kunkeei*	Grape must
CIRM–BIA 2241		*Lactobacillus mali*	Grape must
CIRM–BIA 2240		*Lactobacillus casei*	Grape must
CIRM–BIA 2180		*Lactobacillus pentosus*	Grape must
CIRM–BIA 2115		*Lactobacillus plantarum*	Grape must
CIRM–BIA 2242		*Lactobacillus satsumensis*	Grape must
CIRM–BIA 2243		*Pediococcus pentosaceus*	Grape must
CIRM–BIA 2124		*Leuconostoc mesenteroides*	Grape must
CIRM–BIA 2244		*Oenococcus oeni*	Grape must
CIRM–BIA 2246		*Acetobacter aceti*	Grape must
UCMA 2946		*Lactobacillus casei*	Raw milk
UCMA 2976		*Lactobacillus rhamnosus*	Raw milk
UCMA 2988		*Lactobacillus curvatus*	Raw milk
UCMA 3226		*Lactobacillus paracasei*	Raw milk
UCMA 3274		*Lactobacillus plantarum*	Raw milk
UCMA 6033		*Pediococcus pentosaceus*	Milking parlour
UCMA 4462		*Leuconostoc mesenteroides*	Raw milk
UCMA 4507		*Leuconostoc citreum*	Raw milk
UCMA 4524		*Leuconostoc lactis*	Raw milk
CIRM–BIA 447		*Lactobacillus acidophilus*	Fermented milk
CIRM–BIA 875		*Lactobacillus brevis*	Cheese
CIRM–BIA 667		*Lactobacillus casei*	Cheese

(*continued on next page*)

Table 1 (*continued*)

CIRM–BIA 154		*Lactobacillus coryniformis*	Cheese
CIRM–BIA 573		*Lactobacillus crispatus*	Cheese
CIRM–BIA 1366		*Lactobacillus delbrueckii*	Cheese
CIRM–BIA 678		*Lactobacillus helveticus*	Cheese
CIRM–BIA 871		*Lactobacillus johnsonii*	Cheese
CIRM–BIA 1440		*Lactobacillus paracasei*	Cheese
CIRM–BIA 779		*Lactobacillus paraplantarum*	Cheese
CIRM–BIA 1490		*Lactobacillus pentosus*	Cheese
CIRM–BIA 845		*Lactobacillus plantarum*	Cheese
CIRM–BIA 929		*Lactobacillus reuteri*	Whey from Comté cheese
CIRM–BIA 776		*Lactobacillus rhamnosus*	Cheese
CIRM–BIA 575		*Pediococcus pentosaceus*	Cheese
CIRM–BIA 1453		*Leuconostoc citreum*	Cheese
CIRM–BIA 1451		*Leuconostoc lactis*	Cheese
CIRM–BIA 435		*Leuconostoc mesenteroides*	Cheese
CIRM–BIA 1824		*Leuconostoc pseudomesenteroides*	Cheese

[T]: type strain; [a] Culture collections: UCMA: Université de Caen Microbiologie Alimentaire; CIRM-BIA: Centre International de Ressources Microbiennes - Bactéries d'Intérêt Alimentaire. [b] *Oenococcus sicerae* sp. nov. is currently being described for publication (Cousin et al., unpublished data). The 12 strains highlighted in grey have been used for developing the multiplex PCR.

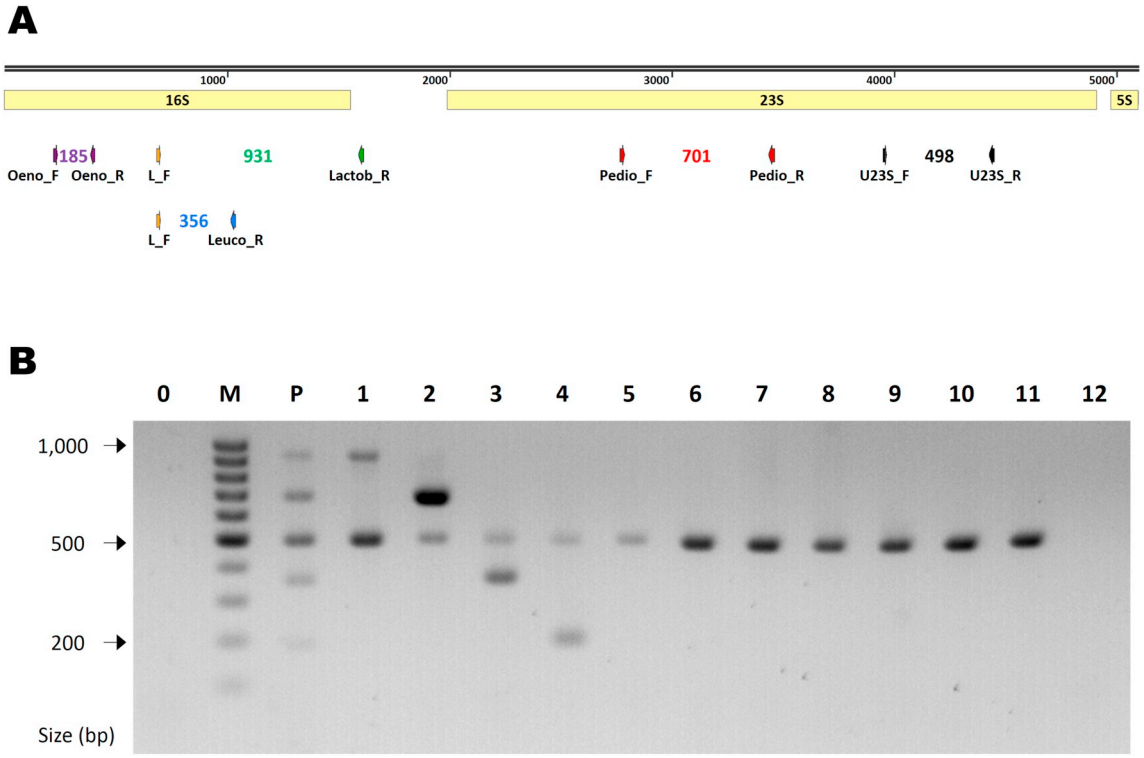

Fig. 1. (A) Position of universal and genus-specific primers on the bacterial ribosomal operon. Schematic representation of the ribosomal operon and the position of the different primers used in the multiplex PCR. Numbers indicates the theoretical length of the PCR amplicons. PCR amplicon sizes were estimated on *Lactobacillus mali* for All bacteria and *Lactobacillus* sp. PCR, on *Pediococcus pentosaceus* for the *Pediococcus* sp. PCR, on *Leuconostoc mesenteroides* for the *Leuconostoc* sp. PCR and on *Oenococcus oeni* for the *Oenococcus* sp. PCR; L_F primer: non-specific primer, used as forward primer for both *Lactobacillus* sp. and *Leuconostoc* sp. amplifications. (B) Multiplex PCR with universal and genus-specific primers. Lane 0: negative control without template DNA; Lane M: GeneRuler 100 bp DNA Ladder; Lane P: Pool of PCR products; Lanes 1: *Lactobacillus mali* UCMA 15531, 2: *Pediococcus pentosaceus* UCMA 7168[T], 3: *Leuconostoc mesenteroides* UCMA 14183[T], 4: *Oenococcus oeni* UCMA 18216[T], 5: *Aerococcus viridans* UCMA 5972, 6: *Carnobacterium gallinarum* UCMA 7287[T], 7: *Enterococcus faecium* UCMA 10625[T], 8: *Fructobacillus fructosus* UCMA 14185[T], 9: *Lactococcus lactis* UCMA 993[T], 10: *Streptococcus thermophilus* UCMA 1000[T], 11: *Weissella viridescens* UCMA 14184[T], 12: *Saccharomyces uvarum* UCMA 10447. Agarose gel 2%.

Table 2
List of the primers used in this study.

Name	Locus	Genus targeted	Sequence (5′-3′)	Size[a] (bp)	Source
U23S_F U23S_R	r23S	All bacteria	TTGTCGGGTAAGTTCCGAC GTAGCTTTTATCCGTTGAGCGA	498	This study
Pedio_F Pedio_R	r23S	*Pediococcus* sp.	GAACTCGTGTACGTTGAAAAGTGCTGA GCGTCCCTCCATTGTTCAAACAAG	701	Modified from (Pfannebecker and Fröhlich, 2008)
L_F[b] Lactob_R	r16S r16S/23S ITS	*Lactobacillus* sp.	AGTGGAACTCCATGTGTAG CYCTCAAAACTAAACAAAGTTTC	931	This study Modified from (Dubernet et al., 2002)
L_F[b] Leuco_R	r16S	*Leuconostoc* sp.	AGTGGAACTCCATGTGTAG CACTTCTATCTCTAAAAGCTTCAAAG	356	This study
Oeno_F Oeno_R	r16S	*Oenococcus* sp.	GTCCTTTTGGATCGCTAGAG CGTCACACTTTCGTGCATTGC	185	This study

[a] PCR amplicon sizes were estimated on *Lactobacillus mali* for All bacteria and *Lactobacillus* sp. PCR, on *Pediococcus pentosaceus* for the *Pediococus* sp. PCR, on *Leuconostoc mesenteroides* for the *Leuconostoc* sp. PCR, and on *Oenococcus oeni* for the *Oenococcus* sp. PCR.
[b] Non-specific primer, used as forward primer for both *Lactobacillus* sp. and *Leuconostoc* sp. amplifications.

cycloheximide to limit yeast and fungi growth. MRS, MLO and PSM plates were incubated at 30 °C with 5% CO_2, and MSE plates were incubated at 30 °C in aerobic conditions. After two to three days (depending on the medium), 32 colonies (8 from each medium) were picked from plates and subjected to the designed multiplex PCR, as already described in this section.

3. Results

3.1. Design of the multiplex PCR

In this study, one pair of universal primers was generated to target all bacteria, as well as 4 pairs of genus-specific primers, to identify LAB in cider, i.e. *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus*. The primer sequences were based on the bacterial ribosomal operon (Fig. 1). Primer specificity was tested against closely related LAB genera, *Weissella* sp., *Fructobacillus* sp., *Carnobacterium* sp., *Aerococcus* sp., *Enterococcus* sp., *Lactococcus* sp. and *Streptococcus* sp., and the yeast *S. uvarum* as negative control (Table 1). Primers were designed in such a way that the amplification products of the different genera varied in length (Fig. 1, Table 2). Furthermore, these primers had similar melting temperatures and no complementarity to each other. These characteristics made it possible to use all primer sets in a multiplex PCR system. Different primer concentrations (250–500 nM), annealing temperatures (48–58 °C) and elongation times (15–45 s) have also been tested in order to obtain strong and specific signals for the multiplex PCR (data not shown). The best PCR conditions were 250 nM for the primers (expect for the L_F primer with 500 nM), 53 °C for the annealing temperature and 20 s for the elongation. The multiplex PCR (amplification and migration) was reached in less than 2 h.

The multiplex PCR clearly identified the four targeted genera (Fig. 1B, lanes 1 to 5). All other LAB only gave back the 500 bp-signal related to bacteria (Fig. 1B, lanes 6 to 12), and this specific band was absent from the yeast *S. uvarum* corresponding lane, as expected (Fig. 1B, lane 13).

3.2. Validation of the multiplex on cider isolates

The multiplex PCR was initially tested on already identified strains isolated from cider of the genera *Lactobacillus*, *Pediococcus*, *Oenococcus* and *Leuconostoc*. For each genera, one strain per species of interest was used. As before, the multiplex PCR assigned all tested species to the four

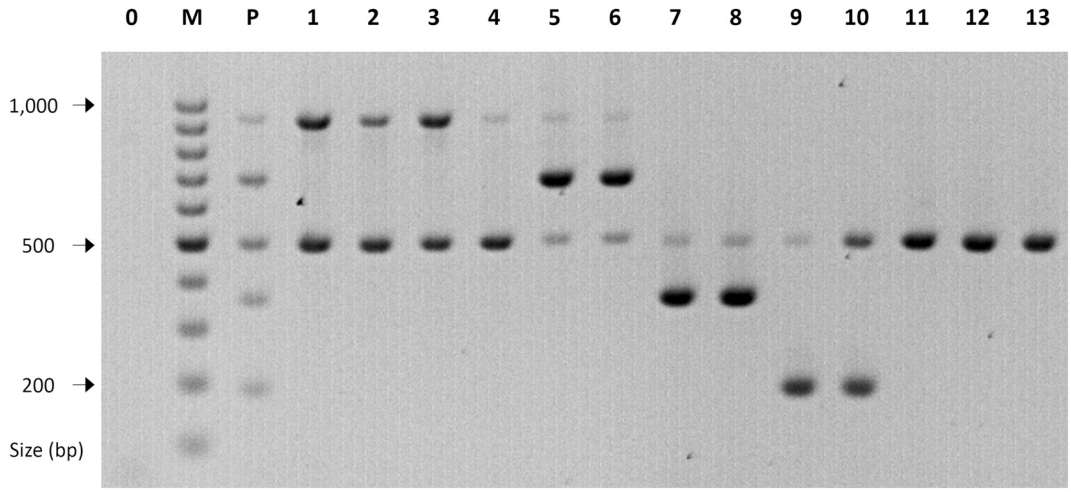

Fig. 2. Validation of the multiplex PCR on cider isolates. Lane 0: negative control without template DNA; Lane M: GeneRuler 100 bp DNA ladder; Lane P: Pool of PCR products; Lanes 1: *Lactobacillus mali* UCMA 15531, 2: *Lactobacillus collinoides* UCMA 12998^T, 3: *Lactobacillus hordei* UCMA 15818, 4: *Lactobacillus diolivorans* UCMA 16955, 5: *Pediococcus ethanolidurans* UCMA 15387, 6: *Pediococcus parvulus* UCMA 15901, 7: *Leuconostoc mesenteroides* UCMA 18596, 8: *Leuconostoc pseudomesenteroides* UCMA 18597, 9: *Oenococcus oeni* UCMA 14597, 10: *Oenococcus sicerae* UCMA 15228^T, 11: *Acetobacter orleanensis* UCMA 16846, 12: *Gluconobacter oxydans* UCMA 15501, 13: *Zymomonas mobilis* UCMA 10408^T. Agarose gel 2%.

targeted genera (Fig. 2, lanes 1 to 9). This included four species of *Lactobacillus* sp., two of *Pediococcus* sp., *Leuconostoc* sp. and *Oenococcus* sp. To exclude false-positive results obtained using the multiplex PCR, some other cider associated bacteria such as *Zymomonas mobilis*, a well described cider alteration bacterium, and the acetic acid bacteria *Acetobacter orleanensis* and *Gluconobacter oxydans*, were used as negative controls. The multiplex PCR on these control strains only gave back the 500 bp-signal related to bacteria, as expected (Fig. 2, lanes 10 to 12).

The only difference with the multiplex PCR development assays (see Section 3.1) was the presence of a supplemental weak signal at 931 bp, corresponding to the specific *Lactobacillus* sp. band, in pediococci species, *P. ethanolidurans* and *P. parvulus*, isolated from cider (Fig. 2, lanes 6 and 7). This extra *Lactobacillus* sp. band of weak intensity compared to the strong intensity of the specific *Pediococcus* sp. band of 701 bp did not prevent the unambiguous identification of these isolates as members of *Pediococcus* sp.

In second phase, the multiplex PCR was tested on 32 unidentified isolates from a cider sample (Fig. 3). All 32 isolates were confirmed to be bacterial with a positive signal for the internal control at 500 bp. The multiplex PCR successfully classified 21 isolates out of the 32 collected into the four targeted genera. All 8 isolates from MSE were identified as *Leuconostoc* sp., 5 of 8 isolates from Rogosa SL as *Lactobacillus* sp., 3 of 8 isolates from PSM as *Pediococcus* sp., and 3 of 8 isolates from MLO as *Oenococcus* sp. (Fig. 3). Two other isolates from MLO were also identified as *Lactobacillus* sp. (Fig. 3).

3.3. Validation of the multiplex on wine and dairy isolates

In order to assess the possibility to apply this new multiplex PCR for the identification of LAB from other fermented food products, 41 LAB strains, including one species of *Pediococcus* sp., one species of *Oenococcus* sp., 24 species of *Lactobacillus* sp. and 4 species of *Leuconostoc* sp., isolated from wine or dairy environments have also been tested (Table 1). But first and foremost, the multiplex PCR assigned all tested species to the targeted genera *Pediococcus* sp., *Oenococcus* sp. *Lactobacillus* sp., and *Leuconostoc* sp. (Figs. S1–S2). Only *L. brevis* and *L. kunkeei* did not show any specific amplification of the *Lactobacillus* specific 931-bp band.

To sum up, the new multiplex PCR efficiently and accurately assigned the tested species from cider, wine and dairy environments, i.e. three species of *Pediococcus* sp., two species of *Oenococcus* sp. and four species of *Leuconostoc* sp., to the targeted genera *Pediococcus* sp., *Oenococcus* sp. and *Leuconostoc* sp. Regarding the genus *Lactobacillus* sp., the multiplex PCR accurately identified 22 species out of the 24 tested in this study.

4. Discussion

4.1. Development of the multiplex PCR

Lactic acid bacteria are present in many fermented food products, such as fermented fruit juices, and especially cider. *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* are genera of special interest as they are able to survive difficult cider environments (low pH and nutrient concentrations, presence of ethanol). LAB play an important role during cider production, and of other fermented food products and beverages. They have a critical impact on cider quality as they play a crucial part in the process but can also cause defects (Cousin et al., 2017). Therefore, the identification of these microorganisms during the fermentation process and their control in the long run is of prime importance. Microbiological identification methods based upon the physiology of the isolates often lead to ambiguous results and the identification by 16S Sanger sequencing is time and money consuming. The development of alternative identification methods of LAB in cider is thus primordial. The multiplex PCR developed in this study can be performed directly on colonies picked from plates, the whole procedure

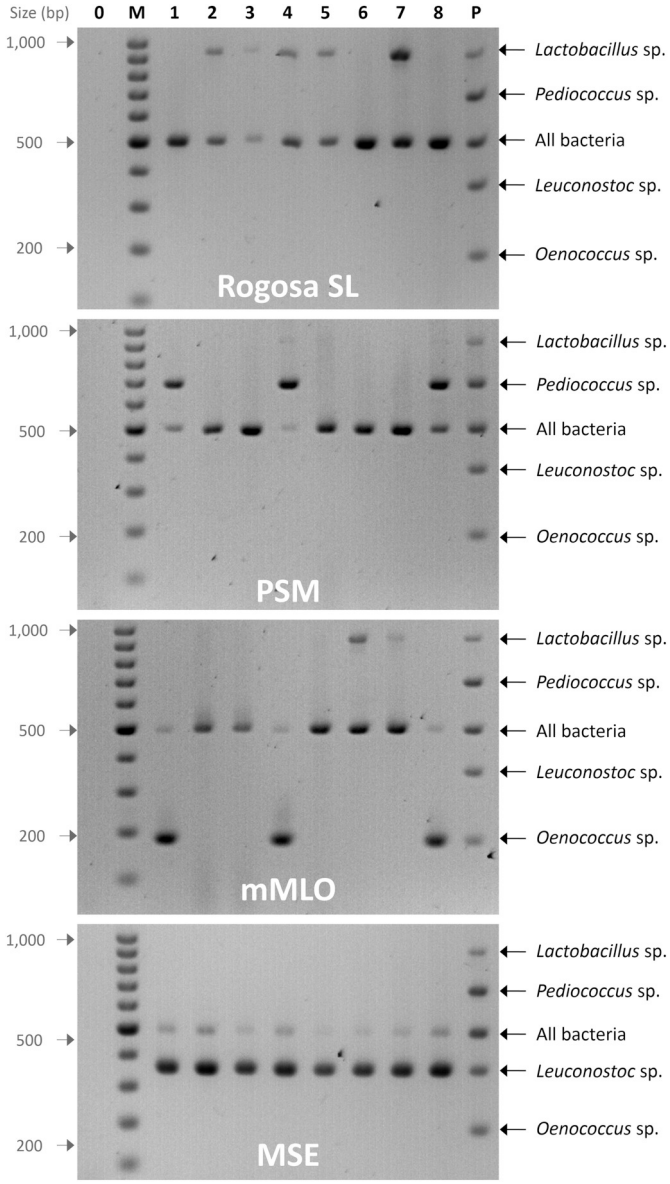

Fig. 3. Application of the multiplex PCR to identify cider isolates. Colony forming units of bacterial cells were determined by plating serial dilution of a cider sample on Rogosa SL, modified MLO, PSM and MSE agar as described in the Material and Methods section. After a few days, 32 colonies (8 from each medium) were used for testing the new multiplex PCR on colonies. Lane 0: negative control without template DNA; Lane M: marker GeneRuler 100 bp DNA ladder; Lanes 1–8: Colony isolated on the different medium; Lane P: Pool of PCR products. Agarose gel 2%.

- including the PCR run and the result analysis - taking less than 2 h and having a limited cost. This multiplex PCR allows a fast classification at the genus level of LAB isolates from cider. Other multiplex PCR targeting bacterial isolates from cider have been designed before. Contrary to the present multiplex PCR, these multiplex methods have been designed for safety purposes only, targeting biogenic amine-forming LAB (Coton et al., 2010) or spoilage bacteria such as *Zymomonas mobilis* (Coton et al., 2005). Some other PCR have been set up to differentiate yeasts, such as *Saccharomyces* - the main yeast responsible for ethanol production in cider (De Melo Pereira et al., 2010; Josepa et al., 2000; Sharpe et al., 2014) - or *Brettanomyces/Dekkera*, involved in cider and wine spoilage (Cocolin et al., 2004; Hulin et al., 2014). All these studies confirmed the use of a multiplex PCR as a powerful identification tool for food isolates.

Since LAB are closely related to each other, 16S rRNA gene sequences of different species often have a similarity rate higher than 97%. Even if complete genome sequences of big sets of lactobacilli (Sun et al., 2015) and *O. oeni* (Sternes and Borneman, 2016) have recently been published, only few cider related LAB species have been studied for their genome structure so far. The rRNA genes are therefore the easiest sequences available online. This eliminates the need to identify and sequence specific areas between targeted bacteria, as performed in previous studies (Petri et al., 2013; Rodas et al., 2003). In the current study, universal and genus-specific primers for the detection of the four major LAB genera frequently isolated from cider were obtained from the ribosomal operon sequences. These primer sets were manually designed after sequence alignments or derived from the existing literature. The specificity of the new primers was checked against closely non-targeted LAB and other cider related bacteria or yeast (Cousin et al., 2017), in order to exclude false-positive or undesired cross reactions.

4.2. Identification of bacterial isolates from cider and other food environments

This multiplex PCR rapidly and accurately identified all tested isolates from cider, previously identified by 16S Sanger sequencing. The multiplex PCR also identified 21 out of 32 isolates from four isolation media targeting members of the *Lactobacillus*, *Pediococcus*, *Oenococcus* and *Leuconostoc* genera. The 11 remaining isolates were identified as bacteria by the bacterial universal primers of the new multiplex PCR. They might be acetic acid bacteria, also associated with cider environments (Cousin et al., 2017).

Previous multiplex PCR have been designed for wine isolates (Petri et al., 2013) or probiotic lactobacilli strains (Kwon et al., 2004). In the present study, in addition to the cider isolates, the multiplex PCR was successfully extended to LAB strains isolated from wine and dairy environments. This further emphasizes the power and usefulness of this new multiplex PCR in the rapid and accurate identification of LAB in various fermented food environments, and therefore extends its scope of application.

In a nutshell, the multiplex PCR assigned all tested species to the targeted genera *Pediococcus* sp., *Oenococcus* sp. and *Leuconostoc* sp. Only two *Lactobacillus* species, *L. brevis* and *L. kunkeei*, did not show any positive signal for the *Lactobacillus* sp. specific band pattern. This is probably due to the presence of Single Nucleotide Polymorphisms (SNPs) between the Lactob_R primer and the r16S/23S ITS sequence of these species. The presence of 3 SNPs in *L. brevis* has already been described (Dubernet et al., 2002). The presence of three SNPs for *L. brevis* and seven SNPs for *L. kunkeei* was confirmed after mining the genomes of these species (Sun et al., 2015). Because of this great variability in *Lactobacillus* sequences and of the continuously increasing number of new *Lactobacillus* species, designing universal *Lactobacillus* sp. primers seemed to be a difficult task to carry out. Indeed, the number of *Lactobacillus* species at the time of the present study almost reached 200 species. The inability of the multiplex PCR to identify *L. brevis* and *L. kunkeei* does not greatly impact the reliability of the method as these two species are not found in cider products (Cousin et al., 2017). When using this multiplex PCR with LAB from other fermented foods, this limit should nonetheless be taken into account. An alternative approach to further identify unknown isolates, and especially *L. brevis* and *L. kunkeei*, can be MALDI-TOF MS. This technique has already been used to identify LAB in dairy fermented foods (Nacef et al., 2017). As interesting as this method may be, MALDI-TOF mass spectrometry is not accessible to every lab, whereas the current multiplex PCR is, and the implementation of a comprehensive MALDI-TOF database can prove laborious, especially when it comes to the multitude of LAB species.

5. Conclusions

This multiplex PCR allows a fast, cheap and simultaneous identification of the four main LAB genera found in cider. It also identified LAB from other fermented foods such as wine or dairy environments. The rapid and reliable detection of LAB genera will permit an easy monitoring of the fermentation process in the long run, bringing rapid first insights into the lactic microflora involved throughout the process. For complete, knowledge of the LAB involved during cider fermentation, this multiplex PCR approach have to be completed with other identifications methods, especially when it comes to species identification. Thus, this new multiplex PCR could be further applied to biodiversity studies or microbial quality control, constituting a rapid first screening method of the lactic flora involved in fermentation processes.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.004.

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