Running title: WINE BACTERIA PRODUCING VOLATILE PHENOLS

Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids

BLANCA DE LAS RIVAS, HÉCTOR RODRÍGUEZ, JOSÉ ANTONIO CURIEL, JOSÉ MARÍA LANDETE, ROSARIO MUÑOZ*

Departamento de Microbiología, Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid

*Corresponding author:

Address: Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain. Phone.: +34 91 5622900; Fax: +34 91 5644853.

E-mail: rmunoz@ifi.csic.es

Abstract

The potential to produce volatile phenols from hydroxycinnamic acids was investigated for lactic acid bacteria (LAB) isolated from Spanish grape must and wine. For the detection of LAB that potentially produce volatile phenols we developed a PCR assay. Synthetic degenerate oligonucleotides for the specific detection of the *pdc* gene encoding a phenolic acid decarboxylase were designed. The *pdc* PCR assay amplifies a 321 bp DNA fragment from phenolic acid decarboxylase. The *pdc* PCR method was applied to 85 strains belonging to the six main wine lactic acid bacteria species. *Lactobacillus plantarum*, *Lactobacillus brevis,* and *Pediococcus pentosaceus* strains produce a positive response in the *pdc* PCR assay; whereas *Oenococcus oeni*, *Lactobacillus hilgardii*, and *Leuconostoc mesenteroides* strains did not produce the expected PCR product. The production of vinyl and ethyl derivatives from hydroxycinnamic acids in culture media was determined by high performance liquid chromatography. A relation was found between *pdc* PCR amplification and volatile phenols production, so that the lactic acid bacteria strains that gave a positive *pdc* PCR response produce volatile phenols, whereas strains that did not produce a PCR amplicon neither produce volatile phenols. The proposed method could be useful for a preliminary identification of lactic acid bacteria strains able to produce volatile phenols in wine.

KEYWORKS: Wine; lactic acid bacteria; LAB; *pdc* gene; volatile phenols; hydroxycinnamic acids; PCR detection method

INTRODUCTION

Hydroxycinnamates are the major phenols in grape juice and the major class of phenolics in white wine (*1*). These acids are also the first to be oxidized and subsequently initiate browing, a problem in white wines. There are three common hydroxycinnamates in grapes and wine, *p*-coutaric acid, caftaric acid, and fertaric acid, based on *p*-coumaric, caffeic, and ferulic acids, respectively. In grape berries the simple hydroxycinnamic acids noted above are not found. Instead these acids exist as esters of tartaric acid, *p*-coutaric acid, caftaric acid, and fertaric acid, respectively. These substances are found in the flesh of the fruit, and thus are found in all grape juices and consequently in all wines. The naturally occurring esters are susceptible to hydrolysis, and this occurs in the aqueous acidic solution of wine, releasing the simple hydroxycinnamic acids (*1*).

In terms of wine sensory qualities, the hydroxycinnamates appear to have no perceptible bitterness or astringency at the levels found in wine. However, some of their derivatives (e.g. volatile phenols) greatly influence the aroma of wine. The most important molecules in this class are 4-vinylphenol and 4-ethylphenol originated from *p*-coumaric acid, and 4-vinylguaiacol and 4-ethylguaiacol originated from ferulic acid. In wine, these compounds are potential contributors to aroma, because of their low sensory thresholds and are responsible of characteristics of some wines such as "traminer" (*2*). Above certain levels, these compounds negatively affect wine quality, imparting animal, leather, and "horse sweat" odors.

The origin of volatile phenols involves the sequential action of two enzymes on a hydroxycinnamic acid (*p*-coumaric, caffeic or ferulic acid). It is generally assumed that, first hydroxycinnamate decarboxylase decarboxylates these hydroxycinnamic acids

into their vinyl derivatives, and then by a reductase they are reduced to ethyl derivatives.

Previous works described that only certain yeasts can form important concentrations of ethylphenols in the presence of hydroxycinnamic acids (*3*). The presence of ethylphenols in wine seems not to be linked to the incidence of malolactic fermentation (*4*); although certain bacteria may posses hydroxycinnamate decarboxylate activity, none is capable of forming significant quantities of ethylphenols in the wines (*3*, *5*). The ability of wine lactic acid bacteria (LAB) to produce volatile phenols had been studied (*3*, *5*, *6*, *7*, *8*, *9*), and *L. plantarum* has been shown to synthesize an inducible phenolic acid decarboxylase (PDC), which decarboxylate *p*-coumaric, caffeic and ferulic acids into their vinyl derivatives (*10*, *11*, *12*, *13*). In addition, van Beek and Priest (2000) (*14*), established the distribution of similar *pdc* genes in various strains of *Lactobacillus* isolated from whisky fermentations. Since no molecular detection methods have been applied to different wine LAB genera to detect their potential to produce volatile phenols, the aims of this study were i) to establish the presence of the gene encoding PDC in wine LAB, ii) to correlate the presence of the *pdc* gene to the production of volatile phenols in laboratory media, and iii) to propose the detection of the *pdc* gene in a LAB strain as a molecular method to determine its potential to produce volatile phenols in wine.

MATERIALS AND METHODS

Strains and growth conditions. The strains analysed in this study are showed in **Table 1**. Most of these strains were isolated from grape must and wine samples at the Instituto de Fermentaciones Industriales (*15*). These strains were formerly named

"BIFI", and later renamed as "RM". By sequencing their 16S rDNA some of these strains were reclassified, e. g. *Oenococcus oeni* BIFI-28 that was currently classified as *Lactobacillus plantarum* RM28 (*16*), and *Lactobacillus buchneri* BIFI-77 that was reclassified as *Lactobacillus hilgardii* RM77 (*17*). Some of the strains that were not classified previously by biochemical methods could be classified by the sequencing of their 16S rDNA, such as *Lactobacillus* sp. BIFI-62, BIFI-63, BIFI-66, and BIFI-79, which were classified as *L. hilgardii* strains (*17*).

Pure cultures of LAB control strains were purchased from the Spanish Type Culture Collection (CECT). Strains of *Oenococcus oeni* were grown on medium for *Leuconostoc oenos* (MLO medium) (*18*) supplemented with 10% tomato juice. The other LAB tested were routinely grown in MRS broth (Difco, France). All bacteria were incubated at 30 ºC in microaerophilic conditions.

Bacterial DNA extraction. Bacterial chromosomal DNA was isolated from overnight cultures using a protocol previously described (*16*). Briefly, LAB cells grown in liquid culture media were pelleted by centrifugation, and resuspended in TE solution (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) (*19*) containing 10 mg/ml of lysozyme (Sigma, Germany). Cells were lysed by adding SDS (1%) and proteinase K (0.3 mg/ml). Crude DNA preparation was purified by performing two phenol/chloroform/isoamyl alcohol (25:24:1) and one chloroform/isoamyl alcohol (24:1) extractions. Chromosomal DNA was precipitated by adding two volumes of cold ethanol. Finally, the DNA precipitate was resuspended in an appropriate volume of TE solution to achieve, approximately, 1 mg/ml concentration.

PCR amplification of phenolic acid decarboxylase. The *pdc* gene encoding the phenolic acid decarboxylase was amplified by PCR using 10 ng of chromosomal DNA. PCR reactions were performed in 0.2 ml microcentrifuge tubes in a total volume of 25 µl containing 1µl of template DNA (aprox. 10 ng), 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U of Ampli*Taq* DNA polymerase, and containing 1 µM of each primer. The PCR reactions were performed using the degenerate primers 49 (5´- GANAAYGGNTGGGARTAYGA) encoding the PDC sequence (D/E)NGWEYE, and primer 50 (5´-GGRTANGTNGCRTAYTTYT) encoding EKY(A/E)TYP, where R: G or A, Y: G, C, or A, and N: G, A, C, or T. These degenerate primers were based on well-conserved domains approximately 100 amino acids apart of the PDC proteins (**Figure 1**). The reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer, USA) using the following cycling parameters: initial 5 min denaturation at 94 ºC followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 50 ºC for 30 sec and extension at 72 ºC for 30 sec. The expected size of the amplicon was 321 bp. Fragments of the expected size were resolved on a 2% agarose gel.

Hydroxycinnamic acids degradation assay. Selected bacteria belonging to different species of wine LAB were cultivated in MRS broth. The selected *O. oeni* strain, *O. oeni* CECT 4100^T, was grown in MLO media. For the degradation assays, the media was supplemented with filter sterilized hydroxycinnamic acid to a 1mM final concentration. The inoculated media were incubated at 30 ºC, in darkness, under microaerophilic conditions, without shaking, for 10 days. Incubated media with cells and without phenolic compound and incubated media without cells and with phenolic compounds were used as controls. From the supernantants, the phenolic products

obtained were extracted twice with ethyl acetate. The hydroxycinnamic acids assayed were *p*-coumaric acid (Sigma C9008), caffeic acid (Sigma C0625), or ferulic acid (Sigma F3500).

The hydroxycinnamic acids derivatives 4-vinylphenol (Lancaster L10902), 4-vinylguaicol (Lancaster A13194), 4-ethylphenol (Fluka 04700), 4-ethylcatechol (Lancaster A12048), 4-ethylguaiacol (Lancaster A12048), phloretic acid (Sigma H52406), hydrocaffeic acid (Lancaster A12069), and hydroferulic acid (Lancaster A12069) were used as standard for the identification of the degradation compounds.

High-Performance Liquid Chromatography-Diode Array Detector-Electrospray Mass Spectrometry (HPLC-DAD/ESI-MS) analysis. Samples were inyected in a Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatograph as described previously (*8*). The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers or by high-performance liquid chromatography-diode array detector-electrospray mass spectrometry (HPLC-DAD/ESI-MS) as reported previously (*8*). The ESI parameters were as follows: drying gas (N_2) flow and temperature 19 l/min at 340 ºC; nebulizer pressure, 40 psi; capillary voltage, 4000 V. The ESI was operated in negative mode, scanning from 100 to 3000 *m/z* using the following fragmentator voltage gradient: 100 V from 0 to 200 *m/z* and 200 V from 200 to 3000 *m/z*.

RESULTS AND DISCUSSION

Presence of a phenolic acid decarboxylase encoding gene (*pdc*). Recently, Couto et al. (2006) screened 35 strains of wine LAB for their ability to produce volatile phenols in culture medium from the corresponding phenolic acids, *p*-coumaric and ferulic acid (*7*). Since van Beek and Priest (2000) established the wide distribution of the gene (*pdc*) encoding a phenolic acid decarboxylase (PDC) in various strains of *Lactobacillus* isolated from whisky fermentations (*14*), the aim of this study was determine the presence of the *pdc* gene in LAB strains isolated from wine and grape must as well as in control collection strains. The 85 LAB strains analysed belonged to the six most frequently isolated species from wine and must, such as *Oenococcus oeni* (42 strains), *Lactobacillus plantarum* (12 strains), *Leuconostoc mesenteroides* (18 strains), *Lactobacillus hilgardii* (7 strains), *Lactobacillus brevis* (5 strains), and *Pediococcus pentosaceus* (1 strain) (**Table 1**).

Previously, primers PDC 489F and PDC 813R, coding for NGWEY and VVPEF respectively, based on the alignment of three decarboxylase genes (*pdc* from *L. plantarum*, ferulate decarboxylase from *Bacillus pumilus*, and phenolic acid decarboxylase from *Bacillus subtilis*) were designed by van Beek and Priest (2000) (*14*). These primers amplified a *pdc* DNA region coding from amino acid residues 22 to 134. However, these primers are only one nucleotide sequence, and, taking into account that amino acid residues could be determined by several codons, in this study new and degenerate oligonucleotides primers were synthesized. To design primers to amplify the *pdc* gene encoding the phenolic acid decarboxylase, sequences included in the database from the recently sequenced LAB genomes were aligned. Two conserved domains were selected to design the degenerate oligonucleotides 49 and 50 (**Figure 1**). These oligonucleotides amplified a 321 bp DNA region encoding from amino acid 21 to 127.

DNA extracted from the 85 LAB strains analyzed was used as template in PCR reactions using oligonucleotides 49 and 50. As showed in **Table 1**, all the strains belonging to the same species showed an identical PCR response. Strains belonging to the *L. brevis*, *L. plantarum*, and *P. pentosaceus* species gave a 321 bp amplicon on the PCR assay, suggesting the presence of the corresponding PDC protein (**Figure 2**). These results are in agreement with those obtained previously in relation to volatile phenols production by these LAB species (*3*, *5*, *6*, *7*, *14*).

However, *pdc* amplification was not obtained from strains belonging to *O. oeni*, *L. mesenteroides*, and *L. hilgardii* species (**Table 1, Figure 2**). The results obtained from *O. oeni* and *L. mesenteroides* strains were expected since in previous studies strains form these LAB species were not able to produce volatile phenols (*5*, *6*, *7*).

These results were in agreement with the information obtained from the complete genome sequences of representative strains from these LAB species. The genome sequence of *L. plantarum* WCFS1, *L. brevis* ATCC 367, and *P. pentosaceus* ATCC 25745 strains revealed the presence of a *pdc* gene copy. In addition, the genome sequence from *O. oeni* PSU-1 and *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 strains, revealed the absence of a *pdc* gene copy on their complete genomes.

However, unexpected results were obtained with *L. hilgardii* strains. Previously, van Beek and Priest (2000) (*14*) in a *L. hilgardii* strain, isolated from malt whisky fermentation, amplified a fragment of the *pdc* gene copy and included its sequence on the databases (accession number AF257158). However, unexpectedly, this strain was unable to decarboxylate *p*-coumaric acid as well as ferulic acid. Later, Couto et al. (2006) (*7*) assayed eight *L. hilgardii* strains and none of them showed decarboxylation or activity. The absence of decarboxylase activity was in agreement with the absence of

a *pdc* gene copy as revealed in our study. The availability of the complete genome sequence from a *L. hilgardii* strain will help to solve this issue.

Degradation of hydroxycinnamic acids by wine LAB strains in culture media. So far, the results obtained in this work seem to indicate that the molecular screening for the presence of a *pdc* gene copy could result in an adequate method to detect the potential production of volatile phenols by wine LAB. In order to ascertain this finding, one strain from each one of the species screened by the PCR assay was selected. Therefore, *L. brevis* CECT 4121^T, *L. hilgardii* CECT 4786^T, *L. plantarum* CECT 748^T, *L. mesenteroides* CECT 912^T, *O. oeni* CECT 4100^T, and *P. pentosaceus* CECT 4695^T strains, were selected for the degradation assay.

The ability to decarboxylate hydroxycinnamic acids has been mostly tested on *p*-coumaric and ferulic acid; however, it has been demonstrated that the PDC decarboxylase from *L. plantarum* is also able to decarboxylate caffeic acid, producing vinylcatechol (*11*, *13*). Therefore, selected strains were grown in culture media containing one of the three hydroxycinnamic acids, *p*-coumaric, caffeic, or ferulic acids. **Figure 3** and **Table 2** showed the results obtained. As expected from the *pdc* PCR assay, the selected *L. hilgardii*, *L. mesenteroides*, and *O. oeni* strains were not able to decarboxylate any of the hydroxycinnamic acid assayed (**Figure 3**). These results confirm the results obtained by previous authors (*6*, *7*) who described that strains belonging to these species did not decarboxylated hydroxycinnamic acids. However, *L. hilgardii* CECT 4786^T was able to reduce partially the *p*-coumaric acid present to produce phloretic acid.

As described previously, *L. plantarum* CECT 748^T strain decarboxylates the three hydroxycinnamic acids to their vinyl derivatives. Vinylphenol and vinylcatechol

were subsequently reduced to their ethyl derivatives, ethylphenol and ethylcatechol. The chromatograms obtained from each hydroxycinnamic acid in *L. plantarum* showed the presence of both derivatives simultaneously (**Figure 3, A, and B**). As described previously, *L. plantarum* partially decarboxylates ferulic acid to vinylphenol (*8*, *9*). However, the vinylguaiacol produced from ferulic acid was not reduced to ethylcatechol, and a partial reduction of ferulic acid to hydroferulic acid was observed (**Figure 3, C**). This result was in disagreement with our previous results that indicated that ethylguaiacol is produced from the reduction of vinylguaiacol (*9*); however, the different behaviour observed could be due that, in both experiments, *L. plantarum* cultures were grown in different culture conditions that might affect gene expression. It have been reported that the presence of glucose in the media, as in the media used in this work, could induce a carbon catabolite repression mechanism on the aromatic degradative pathways in *Acinetobacter baylyi* (*21*). Therefore, the effects of different culture conditions need be investigated to determine their influence on the *pdc* gene expression. It has been previously described that *L. plantarum* PDC showed activity on *p*-coumaric, caffeic and ferulic acids (*9*, *13*, *22*), and that *L. plantarum* displays acid phenol reductase activity to produce the ethyl derivatives (*22*).

L. brevis and P. pentosaceus strains showed a similar metabolism; *p*-coumaric and caffeic acids were completely decarboxylated and only their vinyl derivatives were detected (**Fig. 3, A and B, and Table 2**); however, similarly to *L. plantarum* and possibly due to a lower activity of the PDC on ferulic acid, the *L. brevis* and *P. pentosaceus* cultures showed vinylguaiacol formation although undecarboxylated ferulic acid still could be observed (**Fig. 3, C**). The observed results could indicate a different growth rate, the presence of a less active reductase enzyme, or that the reduction step was absent in both strains. Previously, Couto et al. (2006) found that the

reduction step was only found in the *Lactobacillus* genus, however in their study *L. brevis* LMG7934 was not able to form 4-ethylphenol (*7*), similarly to the *L. brevis* type strain assayed in this work.

In summary, an easy and fast PCR method to detect LAB possessing a phenol acid decarboxylase encoding gene was described. In this work, it was also demonstrated that the amplification of a *pdc* DNA fragment is an useful method to preliminarily identify the LAB strains able to produce volatile phenols in wine.

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FIGURE CAPTIONS

Figure 1. Comparison of amino acid sequences of PDC fragments from lactic acid bacteria. Multiple alignments were done using the program BioEdit after retrieval of sequences from the UniProtKB/trrEMBL database. The partial amino acid sequences are: *Lactobacillus plantarum* WCFS1 (LPL) (Q88RY7), *Lactobacillus fermentum* (LFE) (Q9KHI8), *Lactobacillus paracasei* (LPA) (Q9KHJ0), *Lactobacillus pentosus* (LPE) (Q9KHI9), *Lactobacillus crispatus* (LCR) (Q9KHJ1), *Lactococcus lactis* ssp. *lactis* IL1403 (LLL) (Q9CEB3), *Lactococcus lactis* ssp. *cremoris* MG1363 (LLC) (A2RN76), *Lactobacillus reuteri* 100-23 (LRE) (Q1UAS9), *Pediococcus pentosaceus* (PPE) (Q9F3X2), *Lactobacillus sakei* 23K (LSA) (Q38UX6), *Enterococcus faecium* DO (EFA) (Q3Y2T7), *Lactobacillus brevis* ATCC 367 (LBR) (Q03TU3), and *Lactobacillus hilgardii* (LHI) (Q9KHJ2). Asterisks indicated amino acid identity; dashed, gaps introduced to maximize similarities. The sequence encoded by oligonucleotides 49 and 50 is indicated in bold letters, and showed by an arrow.

Figure 2. PCR amplification of the *pdc* gene from wine lactic acid bacteria. Chromosomal DNA from the following strains was used for PCR amplification with oligonucleotides 49 and 50: (a) *L. plantarum* CECT 748^T; (b) *L. plantarum* RM28; (c) *L. plantarum* RM35; (d) *L. plantarum* RM72; (e) *L. mesenteroides* CECT 912^T; (f) *L. mesenteroides* RM54; (g) *P. pentosaceus* CECT 4695^T; (h) *L. hilgardii* CECT 4786^T; (i) *L. brevis* CECT 4121^T; (j) *L. brevis* RM84; (k) *O. oeni* CECT 4100^T; (l) *O. oeni* RM4; (m) *O. oeni* RM17; (n) *O. oeni* RM25; and (o) *O. oeni* RM46. Products were subject to agarose gel electrophoresis and stained with ethidium bromide. Left lane, 50-bp

molecular weight ladder. Numbers indicate some of the molecular sizes (in bp). The amplicon size (321 bp) is indicated by an arrow.

Figure 3. HPLC chromatograms of the degradation of hydroxycinnamic acids by wine LAB strains. Chromatograms of supernatants from *L. mesenteroides* CECT 912^T, *L. brevis* CECT 4121^T, and *L. plantarum* CECT 748^T grown for 10 days in presence of *p*-coumaric (A), caffeic (B), or ferulic acid (C). The HPLC chromatograms were recorded at 280 nm. pCA, *p*-coumaric acid; CA, caffeic acid; FA, ferulic acid; VP, vinylphenol; VC, vinylcatechol; EP, ethylphenol; EC, ethylcatechol; HFA, hydroferulic acid.

Table 1

Table 1. Presence of a *pdc* gene in the wine LAB strains studied as determined by the PCR assay

Species	Strain number	PCR
Lactobacillus brevis	CECT 4121^T (ATCC 14869^T), CECT 5354 (ATCC 367), CECT 4669 (ATCC 8287), CECT 216 (DSMZ 1268), RM84	+
Lactobacillus hilgardii	CECT 4786^T (ATCC 8290^T), RM42, RM62, RM63, RM66, RM77, RM79	-
Lactobacillus plantarum	CECT 748^T (ATCC 14917^T), RM28, RM31, RM34, RM35, RM38, RM39, RM40, RM41, RM71, RM72, RM73	+
Leuconostoc mesenteroides	CECT 912^T (ATCC 19255^T), RM43, RM44, RM45, RM47, RM48, RM49, RM50, RM51, RM52, RM53, RM54, RM55, RM57, RM60, RM61, RM70, RM74	-
Oenococcus oeni	CECT 4100^T (ATCC 23279^T), CECT 218, CECT 4725, CECT 4721, CECT 4028 (DSMZ 20255), CECT 4029 (DSMZ 20257), CECT 4728, CECT 4758, RM1, RM2, RM3, RM4, RM5, RM6, RM7, RM8, RM9, RM10, RM11, RM12, RM13, RM14, RM15, RM16, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM24, RM25, RM26, RM27, RM29, RM46, RM69, RM80, RM81, RM82, RM83	-
Pediococcus pentosaceus	CECT 4695^T (ATCC 33316^T)	+

^{T}, type strain

Table 2

Table 2. Degradation of hydroxycinnamic acids by wine lactic acid bacteria

	*p*CA	CA	FA
Lactobacillus brevis CECT 4121^T	VP	VC	VG
Lactobacillus hilgardii CECT 4786^T	ND[1]	ND	ND
Lactobacillus plantarum CECT 748^T	VP, EP	VC, EC	VG[2]
Leuconostoc mesenteroides CECT 912^T	ND	ND	ND
Oenococcus oeni CECT 4100^T	ND	ND	ND
Pediococcus pentosaceus CECT 4695^T	VP	VC	VG

*p*CA, *p*-coumaric acid; CA, caffeic acid; FA, ferulic acid; VP, 4-vinylphenol; VG, 4-vinylguaiacol; EP, 4-ethylphenol; VC, 4-vinylcatechol; EC, 4-ethylcatechol. ND, non degraded.
[1], phloretic acid was observed
[2], hydroferulic acid was observed

Figure 1

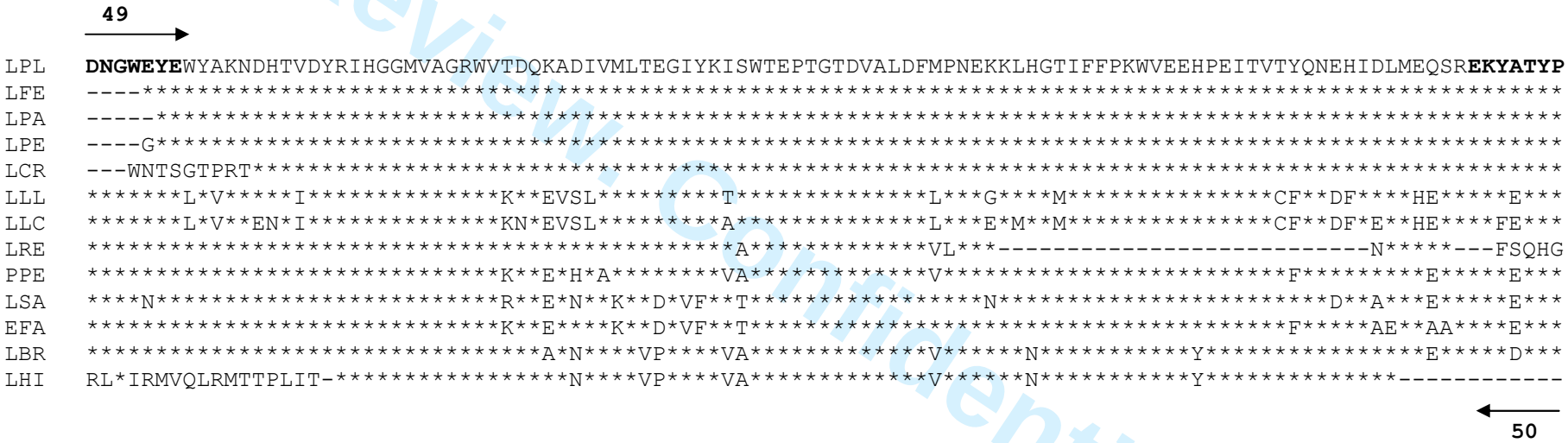


Figure 2

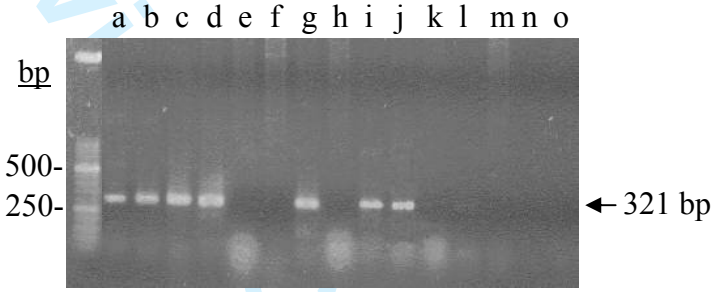
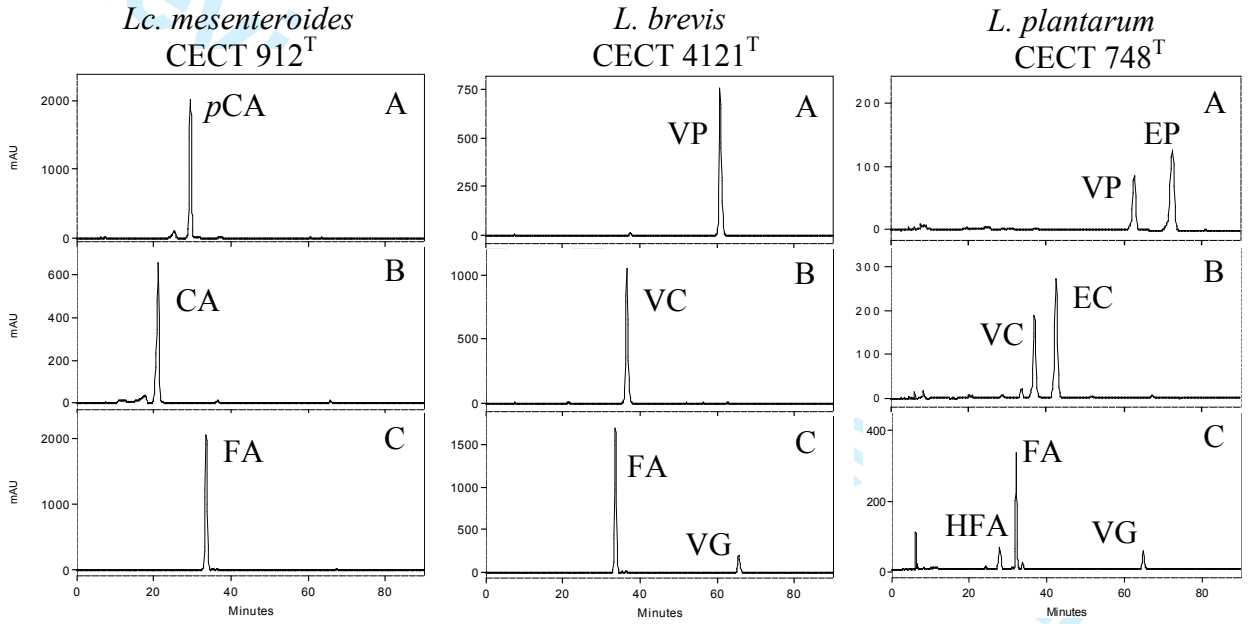


Figure 3


Lc. mesenteroides
CECT 912T
L. brevis
CECT 4121T
L. plantarum
CECT 748T
pCA
A
CA
B
FA
C
VP
A
VC
B
FA
VG
C
VP
EP
A
VC
EC
B
HFA
FA
VG
C
mAU
Minutes