Presence in *Lactobacillus plantarum* of an esterase active on a broad-range of phenolic esters

María Esteban-Torres\textsuperscript{a}, José María Landete\textsuperscript{b}, Inés Reverón\textsuperscript{a}, Laura Santamaría\textsuperscript{a}, Blanca de las Rivas\textsuperscript{a}, Rosario Muñoz\textsuperscript{a,*}

\textsuperscript{a} Laboratorio de Biotecnología Bacteriana, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

\textsuperscript{b} Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Carretera de La Coruña km 7.5, 28040 Madrid, Spain

Running Title

*Lactobacillus plantarum* esterase active on phenolic esters

Address correspondence to Rosario Muñoz, r.munoz@csic.es
*Lactobacillus plantarum* is the lactic acid bacteria species most frequently found in the fermentation of food products from plant origin on which phenolic compounds are abundant. *L. plantarum* strains showed a great flexibility to adapt to different environments and growth substrates. From 28 *L. plantarum* strains analyzed, only cultures from seven strains were able to hydrolyze hydroxycinnamic esters, such as methyl ferulate or methyl caffeate. As revealed by PCR, only these seven strains possessed the *est_1092* gene. When the *est_1092* gene was introduced into *L. plantarum* WCFS1 or *L. lactis* MG1363 strains, their cultures acquired the ability to degrade hydroxycinnamic esters. These results supported that Est_1092 is the enzyme responsible for the degradation of hydroxycinnamic esters on the *L. plantarum* strains analyzed. The Est_1092 protein was recombinantly produced and biochemically characterized. Surprisingly, Est_1092 was not only able to hydrolyze hydroxycinnamic esters since all the phenolic ester assayed were hydrolyzed. Quantitative PCR experiments revealed that the expression of *est_1092* was induced on the presence of methyl ferulate, an hydroxycinnamic ester, but was inhibited on methyl gallate, an hydroxybenzoic ester. As Est_1092 is an enzyme active on a broad-range of phenolic esters, possessing simultaneously feruloyl esterase and tannase activity, its presence on some *L. plantarum* strains will provide them additional advantages to survive and growth on plant environments.
INTRODUCTION

*Lactobacillus plantarum* is a highly versatile lactic acid bacteria species found in many different ecological niches such as vegetables, meat, fish, and dairy products as well as in the gastro-intestinal tract (1). The genome of *L. plantarum* strain WCFS1 was the first to be fully sequenced, and it was in fact the first of any *Lactobacillus* genomes to be published (2). When the genome diversity of *L. plantarum* on a full genome scale was analyzed, it revealed that *L. plantarum* strains were predicted to lack 9-20% of the genes of the reference genome *L. plantarum* WCFS1, and about 50 genes appeared to be specific for strain WCFS1, as they were not found in any other strain (1). This variability confirms the flexibility of *L. plantarum* to adapt to different environments and growth substrates.

Phenolic compounds are important constituents of food products of plant origin, as they are related to the sensory characteristics of the food, and beneficial to the consumer health (3). Therefore it is interesting to know the metabolic pathways of biosynthesis or degradation of these compounds in bacteria. *L. plantarum* is the lactic acid bacteria most frequently found in the fermentation of food products of plant origin, being the bacteria model for the study of phenolic compounds metabolism (4). Among these compounds, the metabolism of phenolic esters is greatly relevant as they are widely spread throughout the plant kingdom (3). Esters of phenolic acids belong mainly to two distinguishing constitutive carbon frameworks: the hydroxycinnamic and the hydroxybenzoic structures (3) (see Fig. S1 in the supplemental material). In relation to hydroxybenzoic esters, two esterase enzymes able to hydrolyze them have been described in *L. plantarum*. The TanA<sub>Lp</sub> and TanB<sub>Lp</sub> esterases, also known as tannases, hydrolyzed the ester bonds of gallic and protocatechuic acids (5, 6). TanB<sub>Lp</sub> is an
inducible enzyme present in all the *L. plantarum* strains, whereas TanALp is not inducible by methyl gallate and is rarely present among the strains of this species (6). Subsequently, the hydroxybenzoic acids formed by tannase action are decarboxylated by a decarboxylase enzyme recently described (7).

In relation to the metabolism of hydroxycinnamic esters, the decarboxylase enzyme involved in their metabolism (PAD) has been characterized (8, 9), however, the knowledge about the esterases (feruloyl esterases) implicated is still limited. Feruloyl esterases are the enzymes involved in the release of phenolic compounds from plant cell walls, and constitute an interesting group of enzymes with a potentially broad range of applications in the food, fuel, pharmaceutical, and paper-pulp industries (10-13). The potential of feruloyl esterases for opening up the plant cell wall is significant for designing processes for improved biomass utilization (13). Ferulic acid released from the plant cell wall is an effective industrial component by virtue of its antioxidant and photoprotectant properties (11). In human and rumial digestion, feruloyl esterases are important to de-esterify dietary fiber, releasing hydroxycinnamates and derivatives, which have been shown to have positive effects, such as antioxidant, anti-inflammatory, and antimicrobial activities (13).

The availability of the *L. plantarum* WCFS1 genome allows the application of bioinformatics tools to predict function of the genes, and to reconstruct metabolic pathways and regulatory networks. However, understanding protein function is always a major goal in biology. In sequenced genomes, most of the genes are annotated on the basis of sequence similarity to other proteins that have already been characterized (14). However, the definite approach to assigning a molecular function to a predicted open reading frame is to isolate and biochemically characterize the corresponding protein (14). In this regard, a wide study to dissect the complex array of esterase activities in *L.*
plantarum WCFS1 cells was designed by our group (15-23). From the esterases
assayed, only Lp_0796 was able to hydrolyze hydroxycinnamic acids, being therefore
considered as a feruloyl esterase. Given the industrial significance of feruloyl esterases
and taking into account the great variability present on the L. plantatum pangenome, in
this work the metabolism of esters from hydroxycinammic acids was studied in several
L. plantarum strains, and the enzyme involved on this metabolism was genetically and
biochemically characterized.

MATERIALS AND METHODS

Strains and growth conditions. In this study 28 L. plantarum strains were analyzed. L.
plantarum WCFS1, NC8, and LPT 57/1 strains were kindly provided by M.
Kleerebenzem (NIZO Food Research, The Netherlands), L. Axelsson (Norwegian
Institute of Food, Fisheries and Aquaculture Research, Norway), and J. L. Ruiz-Barba
(Instituto de la Grasa, CSIC; Spain), respectively. Eight strains were purchased from the
Spanish Type Culture Collection (CECT): L. plantarum CECT 220 (ATCC 8014),
CECT 221 (ATCC 14431), CECT 223, CECT 224, CECT 749 (ATCC 10241), CECT
4185, CECT 4645 (NCFB 1193), and the type strain L. plantarum subsp. plantarum
CECT 748T (ATCC 14917, DSMZ 20174). Seven strains were purchased from the
German Collection of Microorganisms and Cell Cultures (DSMZ): L. plantarum DSM
1055, DSM 2648, DSM 10492, DSM 12028, DSM 13273, DSM 20246, and the type
strain of L. plantarum subsp. argenteratensis DSM 16365T. Eleven strains were isolated
from must grape or wine of different wine-producing areas of Spain over the period
RM41, RM71, RM72, and RM73) (24). In addition, two Lactobacillus paraplantarum
strains purchased from the DSMZ, DSM 10641 (ATCC 10776) and DSM 10667T, were
also analyzed. From these strains, the complete genome sequence of WCFS1
(GCA_000203855.3), NC8 (GCA_000247735.2), and ATCC 14917T
(GCA_000143745.1) strains is available.

*L. plantarum* strains were routinely grown in MRS medium adjusted to pH 6.5
and incubated at 30 ºC. For the degradation assays, *L. plantarum* strains were cultivated
in a modified basal and defined medium described previously for *L. plantarum* (18, 25).

*Lactococcus lactis* MG1363 was used as a host for heterologous gene expression
in the pNZ:Tu plasmid (26). *Escherichia coli* DH10B was used for DNA manipulations.

*E. coli* BL21(DE3) was used for expression in the pURI3-Cter vector (27). The pGro7
vector (TaKaRa) overexpressing GroES/GroEL *E. coli* chaperones was also used. *E.
coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and 140 rpm. When
required, ampicillin and chloramphenicol were added to the medium at a concentration
of 100 or 20 μg/ml, respectively.

**Hydrolysis of esters from phenolic acids by *L. plantarum* cultures.** The
sterilized modified basal media was supplemented at 1mM final concentration with the
filter-sterilized ester of phenolic acids. *L. plantarum* WCFS1 transformed with pNZ:Tu
plasmid harbouring *est_1092* gene was grown in RPM media containing
chloramphenicol (5 μg/ml). *L. lactis* was cultivated in M17 media supplemented with
glucose (0.5% final concentration) and *L. lactis* strains harbouring pNZ:Tu derivatives
were grown in chloramphenicol added media (5 μg/ml). The *L. plantarum* or *L. lactis-
inoculated media were incubated in darkness without shaking, at 30 ºC for 7 days.

Incubated media with cells and without phenolic compound and incubated media
without cells and with phenolic compounds were used as controls. The reaction
products were extracted twice with one third of the reaction volume of ethyl acetate
PCR detection of est_1092 gene. Bacterial chromosomal DNA was isolated from overnight cultures as described previously (28). The est_1092 gene (accession NC_012984.1) was amplified by PCR using 10 ng of chromosomal DNA. PCR reactions were performed in 0.2 ml centrifuge tubes in a total volume of 25 μl containing 1 μl of template DNA (approximately 10 ng), 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 200 μM of each dNTP, 1 U of AmpliTaq Gold DNA polymerase, and 1 μM of each primer. The reactions were performed using oligonucleotides 1230 and 1231 to amplify the est_1092 gene in a Personnel Eppendorf thermocycler using the following cycling parameters: initial 10 min at 98 ºC for enzyme activation, denaturation at 94 ºC for 1 min, annealing at 50 ºC for 30 s, and extension at 72 ºC for 1 min. The expected size of the amplicon was 0.9 kb. PCR fragments were resolved on 0.7% agarose gel.

Heterologous expression of est_1092 in L. lactis. The gene encoding est_1092 from L. plantarum DSM 1055 was amplified by PCR using F-1092 (5´-CCATGGTATCAAAGAAATTGAGTCGGTCAATAATTG) and R-1092 (5´-TCTAGATCATCAGGCCATATGTTCCTGCAA) oligonucleotides. The forward F-1092 primer introduced an NcoI site around the initiation codon of the est_1092 gene, and the reverse R-1092 primer introduced an XbaI site downstream the stop codon. The PCR product was digested with the two restriction enzymes and ligated into the corresponding restriction sites of vector pNZ:Tu (26). The ligation mixture was transformed into L. lactis MG1363 by electroporation (29) and the transformants containing the recombinant pNZ:Tu-1092 plasmid were checked by restriction mapping and sequencing of the inserted fragment.
Production and purification of Est_1092 from *L. plantarum* DSM 1055. The gene *est_1092* from *L. plantarum* DSM 1055 was PCR-amplified by Prime STAR HS DNA polymerase (TaKaRa) by using the primers 1230 (5´- TAACCTTTAAGGAGATATACATatgatatcaaaagaattgagtcggt) and 1231 (5´- GCTATTAATGATGATGATGATGATGggccatatgcttcctgcaaaaagcg) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lp_1092* gene sequence are written in lowercase letters). The 0.9 kb purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy (27). The vector produce recombinant proteins having a six-histidine affinity tag in their C-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.

*E. coli* BL21(DE3) was cotransformed with the recombinant plasmid pURI3-Cter-1092 and pGro7 plasmid (TaKaRa), a vector overexpressing GroES/GroEL chaperones. *E. coli* was grown in LB medium containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 2 mg/ml arabinose until an optical density at 600 nm of 0.4 was reached and then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.4 mM final concentration. Following induction, the cells were grown at 22 ºC for 20 h and collected by centrifugation (8000 g, 15 min, 4 ºC). The cells were disrupted and the Est_1092 protein (accession YP_003062676.1) was purified by affinity chromatography as described previously (18) except that the bound enzyme was eluted using 150 mM McIlvaine buffer (pH 5.0) (30).
Spectrophotometric assays for esterase activity and substrate specificity.

Esterase activity was determined by a spectrophotometric method previously described but using \( p \)-nitrophenyl butyrate (Sigma-Aldrich) as substrate (18).

The substrate specificity of Est_1092 was determined by using different \( p \)-nitrophenyl esters of various chain lengths (Sigma-Aldrich): \( p \)-nitrophenyl acetate (C2), \( p \)-nitrophenyl butyrate (C4), \( p \)-nitrophenyl caprylate (C8), \( p \)-nitrophenyl laurate (C12), \( p \)-nitrophenyl myristate (C14) and \( p \)-nitrophenyl palmitate (C16) as substrates as described previously (18, 31) but using 50 mM McIlvane buffer, pH 5.0.

The enzymatic substrate profile of purified protein was determined by using an ester library described previously (18, 32) on which two additional esters of hydroxybenzoic acids were included, ethyl protocatechuate (ethyl 3,4-dihydroxybenzoate) and methyl gallate. \( p \)-Nitrophenol was used as pH indicator to monitor ester hydrolysis colorimetrically (18). Blanks without enzyme were carried out for each substrate and data were collected in triplicate and the average activities were quantified. Results are shown as means ± standard deviations.

Esterase activity on gallate esters (tannase activity) was determined using a rhodanine assay specific for gallic acid (33). Gallic acid analysis in the reaction was determined as described previously (5). One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions.

HPLC analysis of Est_1092 activity on phenolic esters. The activity of Est_1092 against 20 potential substrates was analyzed by HPLC. The substrates assayed were esters derived from benzoic and cinnamic acids. Among benzoic acids, gallic esters (methyl gallate, ethyl gallate, propyl gallate, and lauryl gallate), benzoic esters (methyl benzoate, ethyl benzoate, and vinyl benzoate), hydroxybenzoic esters...
(methyl 4-hydroxybenzoate, and ethyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), and dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, methyl 2,5-dihydroxybenzoate, ethyl 3,4-dihydroxybenzoate or ethyl protocatechuate, and ethyl 3,5-dihydroxybenzoate) were analyzed. In relation to hydroxycinnamic acids, ferulic esters (methyl ferulate and ethyl ferulate), caffeic ester (methyl caffeate), \( p \)-coumaric ester (methyl \( p \)-coumarate), and sinapic ester (methyl sinapinate). In addition, epicatechin gallate was also assayed as potential substrates. Est_1092 (100 μg), in McIlvane buffer pH 5.0 (50 mM), was incubated at 30 °C in presence of the substrate (1 mM). As controls, McIlvane buffer containing the reagents but without the enzyme were incubated in the same conditions. The reaction products were extracted twice with ethyl acetate; the solvent fractions were filtered through a 0.45 μm PVDF filter and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, USA) chromatograph equipped with a P4000 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C\(_{18}\) cartridge (25 cm x 4.0 mm i.d., 4.6 μm particle size) at room temperature as described previously (18). 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 LC-DAD/ESI-MS.

**Biochemical characterization of Est_1092.** The effects of pH and temperature on the esterase activity of Est_1092 were studied by using buffers of different pH ranging from 3 to 9. The buffers (100 mM) used were acetic acid-sodium acetate (pH 3 to 5), sodium phosphate (pH 6 to 7), Tris-HCl (pH 8), and glycine-NaOH (pH 9). The optimal temperature was assayed by incubating purified Est_1092 esterase in 50 mM...
McIlvane buffer (pH 5.0) at different temperatures (5, 20, 30, 37, 40, 45, 55 and 65 ºC). For temperature stability measurements, the recombinant esterase was incubated in 50 mM McIlvane buffer pH 5.0 at 20, 30, 37, 45, 55 and 65 ºC for 5, 15, and 30 min and 1, 2, 4, 6, and 20 h. Aliquots were withdrawn at these incubation times to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%). The analyses were performed in triplicate.

To study the effect of metals and ions on Est_1092 activity, the enzyme was incubated in the presence of the different additives at a final concentration of 1 mM for 5 min at room temperature. Then, the substrate was added, and the reaction mixture was incubated at 30 ºC. The residual esterase activity was measured after the incubation of the purified enzyme with each additive. The additives analyzed were MgCl2, KCl, CaCl2, HgCl2, ZnCl2, CuCl2, NiCl2, MnCl2, Triton-X-100, Tween 20, Tween 80, SDS, urea, EDTA, DMSO, cysteine, DTT, PMSF, DEPC, and ß-mercaptoethanol. Esterase activity measured in the absence of any additive was taken as control (100%). Experiments were done in triplicate.

**RNA isolation, RT-PCR and qPCR.** For RNA isolation, L. plantarum MRS cultures were grown up to an OD 600nm of 0.8-09 and then supplemented with methyl ferulate or methyl gallate at 30 mM final concentration. After 10 min incubation the cultures were immediately processed for RNA extraction as previously described (34). After DNaseI treatment, the DNA-free RNA was retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer instructions. From the DNA obtained, quantitative gene expression was analyzed in an AbiPrism 7500 Fast Real Time PCR system (Applied Biosystems). The SYBR Green method was used and each assay was performed in triplicate using SYBR Green real-time PCR Master Mix (Applied Biosystems). Amplification was initiated at
95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Control PCRIs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme negative control). Specific primer pairs were designed with the Primer Express 3.0 program to amplify internal regions of \textit{lp\_0796} and \textit{est\_1092} esterase genes. Oligonucleotides 977 (5’- GCCAACATGCCGTCATTTTA) and 978 (5’- CCGCACATCATTGGCACTT) were used to amplify 56 bp of \textit{lp\_0796}, and primers 1031 (5’-TCCTCGCGGGCATGTT) and 1032 (5’-CCGTCGCTTGTTGTGCTAATT) a 59 bp fragment of \textit{est\_1092}. The expression level of the endogenous control gene \textit{(rRNA16S)} was assayed by primers 597 (5-GGGTAATCGGCCACATTGG) and 598 (5’-CTGCTGCCTCCCGTAGGA). Amplifications were performed in triplicate. All real-time PCR assays amplified a single product as determined by melting curve analysis and by electrophoresis. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNA and used to determine the efficiency (E) as E=10^{1/slope}. The expression levels of target genes were normalized. The Bestkeeper analysis (35-36) was applied, and the geometric mean of the most stably expressed housekeeping gene (\textit{16SrRNA}) was used as a normalization factor.

Results were analyzed using the comparative Ct method (also named double delta-delta Ct, $2^{\triangle\triangle Ct}$ method). Relative expression levels were calculated with the 7500 Fast System relative quantification software using \textit{L. plantarum 16SrRNA} gene as endogenous gene and the growth in the absence of compound as growth condition calibrator. In order to measure \textit{L. plantarum} gene expression, amplification of the endogenous control gene was performed simultaneously and its relative expression compared with that of the target gene.
Statistical analysis. The two-tailed Students t test performed using GraphPad InStat version 3.0 (GrapPad Software, San Diego, CA) was used to determine the differences between means. The data are representative means of at least three independent experiments.

RESULTS AND DISCUSSION

Metabolism of esters from hydroxycinnamic acids by L. plantarum strains.

Previously it has been described that L. plantarum WCFS1 cultures were unable to hydrolyze any of the four esters from hydroxycinnamic acids assayed (methyl ferulate, methyl caffeate, methyl p-coumarate, and methyl sinapinate) in spite that this strain possess Lp_0796, an enzyme exhibiting feruloyl esterase activity (18). In order to know if this is a general behaviour among L. plantarum strains, 27 additional strains were assayed. L. plantarum cultures were grown for 7 days in the presence of the four model substrates for feruloyl esterases at 1 mM final concentration. After incubation time, the phenolic compounds present in the supernatants were analyzed by HPLC. Similarly to L. plantarum WCFS1, most of the strains analyzed (20 strains) were unable to hydrolyze the esters assayed. However, seven strains (L. plantarum DSM 1055, CECT 220, CECT 221, CECT 223, CECT 224, RM35, and RM73) partially hydrolyzed methyl ferulate, methyl caffeate, and methyl p-coumarate (Figure 1), being unable to hydrolyze methyl sinapinate.

Currently, the complete genome of almost 20 L. plantarum strains is available. From the strains analyzed in this study, it is available the genome of three strains which were unable to hydrolyze the esters assayed (L. plantarum WCFS1, ATCC 14917^T, and NC8 strains). Initially, it could be possible that the gene encoding the esterase involved
in the metabolism of these esters from hydroxycinnamic acids is absent on these three genomes. Therefore, the presence or absence of proteins annotated as “esterase” among the \textit{L. plantarum} sequenced genomes was studied. Only one protein annotated as “esterase/lipase” was absent in \textit{L. plantarum} WCFS1, ATCC 14917$^\text{T}$ and NC8 genomes, and present in the genome of the JDM1 (JDM1\_1092 protein, accession YP\_003062676.1), ZJ316 (Zj316\_1310 protein, accession YP\_007413995.1), 2025 (N876\_10330 protein, accession ERJ63142), EGD-AQ4 (N692\_11190 protein, EQM54850.1) and WHE92 (O209\_09595 protein, accession EYR71161.1) \textit{L. plantarum} strains. This is a 295 amino acid-residue protein, having 33.5 kDa of expected molecular weight, which exhibited the conserved motif Gly$^{142}$-X-Ser-X-Gly$^{146}$ typical of serine hydrolases. Two conserved domains were found in Est\_1092, pfam00135 of carboxyl esterase family, and pfam07859, alpha/beta hydrolase fold, a catalytic domain found in a very wide range of enzymes, including esterases. In spite that the ability to hydrolyze esters from hydroxycinnamic acids of the sequenced \textit{L. plantarum} strains possessing this protein is unknown, this protein could be a potential candidate to be the wanted esterase. In order to corroborate this hypothesis, the presence of this protein (denominated Est\_1092 from now) was analyzed among the strains assayed previously in this study. DNA from these strains was used to amplify the 0.9 kb DNA fragment encoding the \textit{est\_1092} gene. A clear association was observed among the presence of the \textit{est\_1092} gene and the ability to degrade hydroxycinnamic esters by the \textit{L. plantarum} cultures. The gene \textit{est\_1092} was only present in CECT 220, CECT 221, CECT 223, CECT 224, RM35, RM73, and DSM 1055 strains, the same strains which possess hydroxycinnamic esterase activity on cultures (Figure S2). Other lactobacilli species presented on their genomes proteins similar to Est\_1092. Identity degrees higher than 50% were found among Est\_1092 and proteins
annotates as esterase/lipase in *L. pentosus* IG1 (G0M163 protein, 82.4% identity),

triacylglycerol lipase in *L. gasseri* CECT 5714 (J2Z3C4 protein, 59.8% identity),

esterase/lipase in *L. johnsonii* ATCC 33200 (C2E7B7 protein, 57.8%), lipase in *L. acidophilus* ATCC 700396 (Q5FJE2 protein, 56.3%), lipase in *L. crispatus* strain ST1 (D5GYE3 protein, 55.8%), triacylglycerol lipase in *L. helveticus* DSM 20075 (C9LZH1 protein, 54.8%), and hydrolase in *L. jensenii* JV_V16 (D6S2C7 protein, 53.4% identity). However, the biochemical activity of these proteins remains unknown.

**Identification of Est_1092 as an enzyme possessing cinnamoyl esterase activity.** An experimental procedure to ascertain the involvement of Est_1092 in the hidroxcinnamic esterase activity observed is to introduce this gene in strains devoid of this activity. As in *L. plantarum* WCFS1 the est_1092 is absent, it could be an adequate host to study Est_1092 enzymatic activity. In order to demonstrate the activity of Est_1092, its encoding gene was cloned into the replicative plasmid pNZ:Tu. Subsequently, the pNZ:Tu-1092 plasmid was introduced into *L. plantarum* WCFS1 competent cells. Contrarily to *L. plantarum* WCFS1, *L. plantarum* WCFS1 (pNZ:Tu-1092) cultures grown on the presence of the model substrates for feruloyl esterases partially hydrolyzed the substrates assayed with exception of methyl sinapinate (Figure S3). As the pNZ:Tu plasmid also replicates on *L. lactis*, its derivative pNZ:Tu-1092 plasmid was also introduced into *L. lactis* cells. Cultures of *L. lactis* MG1363 cells grown in the presence of the four feruloyl esterase substrates, were not able to hydrolyzed them, except methyl *p*-coumarate that was minimally degraded (Figure S3). The presence of Est_1092 on *L. lactis* cells confer them the ability to hydrolyze partially the four model substrates assayed. It could be observed that, in both host bacteria, the substrate most degraded was methyl ferulate followed by methyl *p*-coumarate. As only a minor hydrolysis of methyl sinapinate was observed on *L. lactis* cells, it is possible
that Est_1092 could be expressed more efficiently on this bacteria, and therefore, the activity on *L. plantarum* cells was not detected. These results confirmed that Est_1092 is an esterase able to hydrolyze hydroxycinamic esters. Apart from the feruloyl esterase Lp_0796 described previously in *L. plantarum* strains (18), among lactic acid bacteria only feruloyl esterases from *L. johnsonii* (12) and *L. acidophilus* (37) had been previously identified.

**Est_1092 is an esterase active on a broad-range of esters from phenolic acids.** Given the industrial significance of feruloyl esterases and the difficulty of distinguishing these enzymes based on sequence comparisons alone (38), the function of these proteins need to be confirmed through the biochemical characterization of the expressed protein. Once the feruloyl esterase activity of Est_1092 was confirmed, it was biochemically characterized. The *est_1092* gene from *L. plantarum* DSM 1055 strain was cloned into the pURI3-Cter expression vector (27) and transformed into *E. coli* BL21 (DE3). SDS-PAGE analysis of cell extracts showed that there was one major band of protein, of approximately 35 kDa, present as inclusion bodies in the insoluble fraction (data not shown). To obtain Est_1092 in a soluble form, plasmid pGro7, producing GroES/GroEL chaperones, was used. When pURI3-Cter-1092 and pGro7 plasmids were used simultaneously, Est_1092 appeared in the intracellular soluble fraction of the cells (Figure 2). Est_1092 was purified by immobilized metal affinity chromatography although some overproduced GroEL proteins were retained in the resin and eluted along Est_1092.

Esterase activity on pure Est_1092 protein was confirmed using *p*-nitrophenyl esters possessing different acyl chain lengths from C2 to C16. Est_1092 was active on all the substrates assayed, exhibiting a clear preference for *p*-nitrophenyl butyrate (Figure 3A) which was selected as substrate to determine its biochemical properties.
The optimal pH for Est_1092 activity was found at 5.0, although at pH 6.5 more than 80% of the maximal activity was observed (Figure S4A). In relation to the optimal temperature, Est_1092 exhibited maximum activity at 30 ºC, although in the range 5-30 ºC also presented 90% of its maximal activity (Figure S4B). This activity at low temperature could be related to the role of L. plantarum in the fermentation of food substrates which are carried out at low temperatures. Interestingly, Est_1092 also showed high thermostability since it retained up to 70% activity after incubation during 20 h at 37 ºC or 40% at 45-65 ºC (Figure S4C). Regarding the effects of several ions and additives, Est_1092 activity was slightly increased by Tween 80 but highly inhibited by SDS (Figure S4D).

A substrate library previously used to know the substrate profile of L. plantarum esterases was assayed (18, 21, 23). In this library were included two additional esters from hydroxybenzoic acids, ethyl protocatechuate (ethyl 3,4-dihydroxybenzoate) and methyl gallate, to check tannase activity. As expected, the ester library confirmed that the four model substrates for feruloyl esterases were efficiently hydrolyzed by Est_1092 (Figure 3B). Surprisingly, Est_1092 also exhibited activity against the two model hydroxybenzoic esters hydrolyzed by bacterial tannases (tannin acyl hydrolases), gallate and protocatechuate esters (5-6, 39-40). The specific activity of Est_1092 on methyl gallate hydrolysis was compared to the activity reported for the two tannase enzymes (TanA_Lp and TanB_Lp) previously described on L. plantarum (6). By using the rhodanine assay, Est_1092 showed a specific activity of 25 U/mg, similar to the activity of TanA_Lp (39 U/mg), but significantly lower than the specific activity observed for TanB_Lp (404 U/mg) (6). This result could indicate that, among the enzymes possessing tannase activity on L. plantarum, gallate esters seems to be mainly hydrolyzed by TanB_Lp action, the only of these enzyme which is present in all the L. plantarum strains.
The hydrolytic activity on esters from hydroxybenzoic acids is not a common activity on feruloyl esterases, such as *L. plantarum* Lp_0796 (data not shown) (41). To our knowledge, only an enzyme (Tan410) possessing feruloyl esterase and tannase activity has been reported from a cotton soil metagenomic library (42). The ability of an enzyme, such Est_1092 or Tan410, to hydrolyze both acid types makes them interesting enzymes for biotechnological applications.

In relation to tannase substrates, it has been described that only the esters derived from gallic and protocatechuic acid were hydrolyzed (5-6, 39-40, 43). It seems that other hydroxybenzoic acids without hydroxyl groups and with substituents other than –H or –OH at position 2 are not metabolized by bacterial tannases. Therefore, the hydrolysis of nine additional benzoic esters, including benzoic esters (methyl benzoate, ethyl benzoate, and vinyl benzoate), hydroxybenzoic esters (methyl 4-hydroxybenzoate, and ethyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, methyl 2,5-dihydroxybenzoate, and ethyl 3,5-dihydroxybenzoate) was analyzed by HPLC. Unexpectedly, and contrarily to bacterial tannases, all the benzoic esters assayed were hydrolyzed (Figure S5). Therefore, Est_1092 esterase from *L. plantarum*, is not only a feruloyl esterase which hydrolyzes esters from hydroxycinnamic acids, neither a tannase which hydrolyzes esters from hydroxybenzoic acids, Est_1092 is an esterase active on both ester types and in a broad-range of esters from phenolic acids.

**Expression of est_1092 in the presence of phenolic esters.** Esters from phenolic acids are common in foods, being phenolic acids accounting for almost one third of the dietary phenols (44). *L. plantarum* strains could be found on environmental niches on which phenolic acids are abundant. In order to gain insights into the specific physiological role of the esterases hydrolyzing hydroxycinnamic acids on *L. plantarum*...
DSM 1055, Lp_0796 and Est_1092, the relative expression of both esterase-encoding genes under a hydroxybenzoic ester (methyl gallate) or hydroxycinnamic ester (methyl ferulate) exposure was studied. *L. plantarum* DSM 1055 cultures were induced for 10 min by the presence of 30 mM methyl gallate or methyl ferulate, as potential esterase substrates. The gene expression levels obtained were substantially different among both esterase encoding genes, indicating the presence of two different expression patterns for these proteins (Figure S6). The *est_1092* gene, only present in a few *L. plantarum* strains, showed an expression level affected by the presence of both potential substrates. Upon methyl gallate exposure, the transcription level of *est_1092* was an 85-fold reduced. From this strong reduction it could be hypothesize an active role of Est_1092 in the synthesis of methyl gallate and not in its hydrolysis. The repression of *est_1092* and the low specific tannase activity showed by Est_1092 supported the hypothesis that Est_1092 did not play a relevant role during tannin degradation. Contrarily, the hydroxycinnamic ester (methyl ferulate) induces about a 2.5-fold increase in the expression of *est_1092* gene. This expression behaviour allows assuming that *est_1092* encoded an inducible feruloyl esterase in *L. plantarum*. This expression pattern differs from that observed on the feruloyl esterase Lp_0976. In *L. plantaum* DSM 1055 the expression of the *lp_0796* gene was reduced 1.4-fold in the presence of its substrate, methyl ferulate. Similarly, the expression of *lp_0796* in *L. plantaum* WCFS1 under methyl ferulate exposure was also reduced (5-fold) (data not shown). The repression observed in *lp_0796* could explain the degradation pattern presented by *L. plantaum* WCFS1 cultures on the presence of esters from hydroxycinnamic acids. In spite that *L. plantaum* WCFS1 possesses, al least, one enzyme exhibiting feruloyl esterase activity (Lp_0796), cultures from this strain were unable to hydrolyze the esters from
hydroxycinnamic acids presented on the culture media probably due to the repression of the \emph{lp\_0796} gene. The presence on \emph{L. plantarum} strains of enzymes able to metabolize phenolic compounds confers them a selective advantage for life in environments where compounds of plant origin are abundant. In addition, the flexibility of \emph{L. plantarum} genome to adapt to different environments and growth substrates has lead to the presence in some \emph{L. plantarum} strains of an enzyme able to hydrolyze a broad-range of esters from phenolic acids. This new esterase will be induced by esters from hydroxycinnamic acids providing \emph{L. plantarum} strains an additional advantage to survive and growth on plant environments where these compounds are abundant.

**ACKNOWLEDGEMENTS.**

This work was supported by grants AGL2011-22745 (MINECO) and RM2012-00004 (INIA). We are grateful to J. M. Barcenilla and M. V. Santamaría for their assistance. M. Esteban-Torres is a recipient of a JAE predoctoral fellowship from the CSIC. J. M. Landete is a recipient of a Ramón y Cajal contract, and L. Santamaria is a recipient of a FPI fellowship from the MINECO.

**REFERENCES**

Ursing B, de vos WM, Siezen RJ. 2003. Complete genome sequence of


pleomorphism of carboxylesterase Cest-2923 from *Lactobacillus plantarum*

WCFS1. FEBS J. **280**:6658-6671.


Characterization of a cold-active and salt-tolerant esterase from *Lactobacillus plantarum* with potential application during cheese ripening. Int. Dairy J. **39**:312-315.


Characterization of a halotolerant lipase from the lactic acid bacteria *Lactobacillus plantarum* useful in food fermentations. LWT-Food Sci. Tech. **60**:246-252.


esterases from *Pseudomonas fluorescens* and *Streptomyces diastatochromogenes*. Tetrahedrom. Asym. 12:545-556.


Legend to Figures

FIG 1. HPLC analysis of hydroxycinnamic ester degradation by L. plantarum cultures. Modified basal media containing 1 mM methyl ferulate (A), methyl caffeate (B), methyl p-coumarate (C) or methyl sinapinate (D) was inoculated with L. plantarum WCFS1 or DSM 1055 and incubated at 30 ºC for 7 days. A non-inoculated control medium was incubated in the same conditions. Detection was performed at 280 nm. The methyl ferulate (MF), methyl caffeate (MC), methyl p-coumarate (MpC) or methyl sinapinate (MS), ferulic acid (FA), caffeic acid (CA), and p-coumaric acid (pCA) detected are indicated. The chromatograms were recorded at 280 nm. AU, arbitrary units.

FIG 2. Purification of Est_1092 from L. plantarum DSM 1055. SDS-PAGE analysis of the expression and purification of the Est_1092. Analysis of soluble cell extracts of IPTG-induced E. coli BL21(DE3) (pURI3-Cter) (1) or E. coli BL21(DE3) (pURI3-Cter-
1092) (pGro7) (2), flowthrough from the affinity resin (3) or protein eluted after His
affinity resin (4). The arrow indicated the overproduced and purified protein. The 12.5%
gel was stained with Coomassie blue. Molecular mass markers are located at the left
(SDS-PAGE Standards, Bio-Rad).

FIG 3. Substrate profile of Est_1092 towards (A) chromogenic substrates (p-
nitrophenyl esters) with different acyl chain lengths (C2, acetate; C4, butyrate; C8,
caprylate; C12, laurate; C14, myristate; C16, palmitate) or (B) a general ester library.
The figure displays the relative specificities obtained toward different substrates, and
lines on the top of each bar represent the standard deviation estimated from three
independent assays. The observed maximum activity was defined as 100%.
Figure 1

- **Control**
  - A: MF
  - B: MC
  - C: MpC
  - D: MS

- **WCFS1**
  - A: MF
  - B: MC
  - C: MpC
  - D: MS

- **DSM 1055**
  - A: MF
  - B: CA
  - C: MP
  - D: MS
Figure 2