

**EVALUATION OF LACTIC ACID
BACTERIA, BIFIDOBACTERIA
AND THE EXOPOLYSACCHARIDES
THEY PRODUCE FOR
THE DEVELOPMENT OF
FOODS WITH FUNCTIONAL
CHARACTERISTICS**

**M^a Goretti Llamas Arriba
PhD Thesis
2018**



PhD THESIS

Evaluation of lactic acid bacteria, bifidobacteria and the exopolysaccharides they produce for the development of foods with functional characteristics

Ph. D. Thesis submitted by
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2018

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Spanish National Research Council (CSIC)
Biological Research Centre (CIB)



San Sebastián, 2018

The fulfilment of this Ph. D. thesis has been possible thanks to the benefit of a grant for the development of doctoral theses within the program “ayudas de formación a jóvenes investigadores y tecnólogos en el entorno científico-tecnológico y empresarial del sector agropesquero y alimentario vasco” from the Department of Environment, Territorial Planification, Agriculture and Fishing from the Basque Government. In addition, thanks to the recruitment as graduated within the project 77/17 from the Diputación Foral de Guipúzcoa.

Moreover, this Ph. D. thesis has been fulfilled within the following research projects, funded with public funds:

“Producción de exopolisacáridos funcionales y oligosacáridos por bacterias lácticas”. REF. AGL2012-40084-C03-03. (2013-2015, Ministerio de Economía y Competitividad, MINECO). IP M^a Teresa Dueñas.

“JAN4U – Dieta personalizada: diseño y desarrollo de productos alimenticios dirigidos a mejorar el estado de salud de sectores específicos de la población”. REF. IE13-356. (2013-2014, Proyectos de Investigación Estratégica ETORTEK-2013. Departamento de Industria, Comercio y Turismo del Gobierno Vasco-Eusko Jaurlaritz). IP M^a Teresa Dueñas.

“Ayudas para apoyar las actividades de grupos de investigación del sistema universitario vasco”. REF. IT866-13. (2013-2015, Departamento de Educación, Política Lingüística y Cultura del Gobierno Vasco-Eusko Jaurlaritz). IP M^a Teresa Dueñas.

“Desarrollo y evaluación de alimentos funcionales basados en homopolisacáridos bacterianos”. REF. AGL2015-65010-C3-1-R. (2016-2018, Ministerio de Economía y Competitividad, MINECO). IP Paloma López.

“Estudio de las características tecno-funcionales de bacterias lácticas productoras de dextranos y de sus polímeros con potencial industrial”. REF. 77/17. (2017-2018, Diputación Foral de Gipuzkoa). IP M^a Teresa Dueñas.

AGRADECIMIENTOS

El momento en el que uno acaba la carrera y decide realizar una tesis doctoral, creo que no es consciente de lo que eso significa en ese momento, al menos en mi caso. Yo les explicaba a mis padres que eran (otros) 4 años en los que realizaría experimentos en un laboratorio y obtendría el título de doctora, ¡otro título! Sin embargo, esta experiencia de al final algún que otro año más de 4, no solamente me ha servido a título profesional para ganar experiencia o aprender a interpretar y explicar resultados, si no que me ha hecho madurar y crecer como persona. Y esto, no lo hacen los resultados que se obtienen en los experimentos, sino las situaciones a las que me he enfrentado día a día y, sobre todo, las personas con las que me he ido encontrando a lo largo de esta etapa. Por ello, creo que ha llegado el momento de reconocerles su gran granito de arena aportado para la elaboración de esta tesis.

En primer lugar, me gustaría agradecer a mi directora de tesis **Maite Dueñas**, la confianza depositada en mí después de aquella primera entrevista, por elegirme para el disfrute de la beca con la que comenzó esta “aventura”. Gracias Maite por tu eterna paciencia, sobre todo en mis primeros meses, cuando recién aterrizada de la carrera comenzaba a aprender lo que era desenvolverse en un laboratorio; por atender todas mis grandes dudas (ahora insignificantes) de aquellos primeros años. También, por confiar cada vez más y delegar en mí, permitiéndome crecer dentro del laboratorio.

En segundo lugar, los agradecimientos van para mi codirectora de tesis, **Alicia Prieto**. Ali, desde la primera vez que aparecí en tu laboratorio, allá por 2013, siempre me has tratado como a una más. Me encantaba, y lo sigue haciendo, tu forma de enseñar, siempre amable y resolutiva. Después aceptaste acompañarme como directora de tesis, enseñándome de nuevo, de forma inconsciente, otra etapa del doctorado: la de las correcciones, los artículos, etc., la de escritora, en definitiva. Me he sentido muy a gusto y arropada compartiendo contigo este final de tesis.

La siguiente a la que quiero dedicar unas palabras es **Paloma López**. Paloma, oficialmente no has sido ni mi directora ni mi tutora de tesis, pero para mí siempre serás una de mis jefas de tesis. Mi segunda etapa del doctorado la realicé en tu laboratorio, donde fui otra más de “tus niños”, como tú dices. Lejos de mi familia y

Agradecimientos

mi entorno, siempre me sentí como en casa. Gracias por TODO, tanto por los días interminables de laboratorio en los que acabábamos exhaustos a altas horas de la madrugada, como por esos domingos de piscina y paella en tu casa de la Sierra. Aunque hubo momentos duros, de cansancio y frustrantes, siempre recordaré esa etapa de mi tesis en Madrid con especial cariño, y creo que una buena parte de culpa la tienes tú. También me gustaría agradecer a **Steve** su gran disposición siempre para echar un cable con las correcciones.

Con especial cariño recuerdo también el primer año de tesis en San Sebastián con **Alex** y **Eli P.** Fuisteis un gran apoyo, sobre todo, fuera del laboratorio. ¡Alex te debo una visita a Barcelona! Eli, ¿cuándo una de cortos por León?

Quiero mencionar también a la gente del laboratorio de Bioquímica, “los de abajo”, con los que he pasado muchos almuerzos de celebraciones: **Eli O., Ane, Oier, Mikel, Martzel** y **Ainara.**

Por último, de mi etapa final en San Sebastián, quiero agradecer a **Mónica** su compañía durante las, unas veces fugaces y otras distendidas, comidas, el apoyo que me brindaba siempre, aunque ella perteneciese al laboratorio de arriba, su ayuda en todas las técnicas que yo no conocía y, sobre todo, por todas las cosas vividas con ella como la Mónica persona, no la profesional: nuestros viajes, cañas, carnavales, etc. ¡Espero que por muchos más momentos POLLITO!

Durante mi etapa en Madrid, fueron varias las personas que hicieron de mi estancia, mi casa. Empezaré con el laboratorio 306. **Adrián**, a pesar de tus vaciles y bromas, creo que aprendimos a llevarnos bien. Gracias por tu apoyo dentro del laboratorio y por enseñarme tanto. Pero también, por todo lo vivido fuera, junto con **Kenza** y **Annel.** Los 3 me enseñasteis lo que es el sentimiento de equipo. Chicas, con vosotras, además, aprendí de otras culturas, e incluso algo de otros idiomas. Pasé muy buenos y malos momentos con los 3, y por eso, creo que un pedacito de esta tesis es vuestra también. Agradecer también a **M^a Luz** sus consejos durante estos años.

Al 305, **Gloria, José** y **Celes**, gracias también por acogerme como parte de vuestro laboratorio. Siempre que algo se complicaba en el 306, allí estaba el 305 como vía

de escape. Gracias por vuestros consejos, ayuda, y también por las comidas y fines de semana.

Sofi y **Ana**, gracias por hacerme siempre un huequito en vuestro laboratorio cuando iba a contaros mis frustraciones, y también, por los buenos momentos de teatro, congresos, y “scape rooms”. **Montse**, y **Salva** también, porque los dos habéis sido encantadores conmigo. Gracias por dejarme ser tu (o vuestra) compañera de piso aquellos meses en Madrid, por los consejos de laboratorio y por estar siempre dispuesta a ayudarme. ¡También por las cañas! Y, por último, también quiero mencionar a **Paula** y **Lorena**, aunque no hayamos tenido tiempo de conocernos mucho.

También querría agradecerles a **Sara** y **Judith**, el dejarme enseñarles lo que he ido aprendiendo estos años y dirigirles en sus proyectos. Y a **Chencho**, **Diego** y **Lydia**, por los buenos momentos de risas que he pasado con ellos.

Gracias también a **mis chicos de Vitoria**, por estar siempre pendientes, especialmente a **Jon**, por tu ayuda con la portada. También a mis pingüinillas, **Laura**, **Clara** y **Vir**, por estar siempre pendientes de mí y mi laboratorio, sobre todo en Madrid. Y también a mis biotecnólogos, **Ire**, **Juancar**, **Cris**, **Bea** y **Bei**, por seguir apoyándome después de tantos años, aunque sea en la distancia.

Por último, no quiero acabar sin agradecer a mis padres, **Luis** y **Elvi** todo lo que han hecho siempre por mí. También a **mis tíos** y **primos**, con los que he compartido tanto. Todos me habéis ayudado a crecer como persona y en algún momento habéis sido un referente para mí. Por los desplazamientos para verme el día de mi cumpleaños si estaba fuera de casa, por ayudarme a acondicionar todos los pisos de alquiler en los que he estado, por aguantar mi estrés, por escuchar mis presentaciones, por todo. Aunque no os lo diga a menudo, OS QUIERO.

Y como no, a ti **Edu** y a tu familia, especialmente en mi etapa en Madrid, cuando tu hermana, **Nela**, y **Alberto**, siempre se preocuparon por mí. Gracias por todo tu apoyo siempre, por cuidarme, por venir a verme, por tranquilizarme, por tanto. Mucho de esta tesis es tuyo también. TE QUIERO.

¡GRACIAS!

A ti, abuelo



« Si vous voulez être un véritable chercheur de vérité, il est nécessaire qu'au moins une fois dans votre vie vous doutiez, autant que vous le pouvez, de tout ».

René Descartes (1596-1650)

The father of modern philosophy

“We only have two things that we share in this life; we are born and we die. And what we do in between those times, we've got to be happy. I don't let the outside world determine”.

Dawn Fraser (1937-)

Swimmer

(First woman in achieving the 100-metre freestyle in less than 1 minute)

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ABBREVIATIONS

Abbreviations

A	Absorbance
Å	Armstrong
AFM	Atomic force microscopy
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BLASTn	Nucleotide basic local alignment search tool
BLASTp	Protein basic local alignment search tool
bp	Base pair
BPP	β-propeller phytase
<i>c</i>	<i>Cis</i> configuration
<i>C</i> *	Critical concentration
cfu	Colony forming unit
CIB	Biological Research Centre
CLA	Conjugated linoleic acid
COSY	Correlation spectroscopy
CP	Cysteine-tyrosine phytase
CUPV	Culture collection of the University of the Basque Country (UPV/EHU)
Da	Dalton
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dideoxy nucleotide triphosphate
DSC	Differential scanning calorimetry
DSS	Dextran sodium sulphate
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Agency
ELISA	Enzyme-linked immunosorbent assay
EMP	Embden-Meyerhof pathway
EPS	Exopolysaccharide
eV	Electronvolt
F6PPK	Fructose-6-phosphate phosphoketolase
FAME	Fatty acid methyl ester
FDA	USA Food and Drug Administration
FFC	Functional Food Centre
FT-IR	Fourier-transformed infrared spectroscopy
g	Gramme
Gal	Galactose
GC-MS	Gas chromatography/Mass spectroscopy
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLC	Gas-liquid chromatography
Glc	Glucose
GPC	Gel permeation chromatography
GRAS	Generally recognised as safe
GTF	Glycosyltransferase
h	Hour
HAP	Histidine acid phosphatase

Abbreviations

HePS	Heteropolysaccharide
HMBC	Heteronuclear multiple bond correlation
HoPS	Homopolysaccharide
HPLC-SEC	High-performance size-exclusion liquid chromatography
HSQC	Heteronuclear single quantum coherence spectroscopy
Hz	Hertz
IL-10	Interleukin 10
INIA	National Institute for Agricultural Food Research and Technology
IP _n	<i>Myo</i> -inositol phosphates
IR	Infrared
IUMBM	International union of biochemistry and molecular biology
IUPAC	International union of pure and applied chemistry
kbp	Kilobase pair
kDa	Kilodalton
kHz	Kilohertz
kV	Kilovolt
L	Litre
LA	Linoleic acid
LAB	Lactic acid bacteria
LAI	Linoleic acid isomerase
LA-MRS	Modified MRS supplemented with 0.5 mg/mL of linoleic acid
LPS	Lipopolysaccharide
M	Molar
mA	Milliamperes
MALLS	Multi-angle laser-light scattering
MCRA	Myosin cross-reactive antigen
MEM-Alpha Medium	Minimum essential-alpha medium
MES	2-(N-morpholino)ethanesulfonic acid
µg	Micrograms
µm	Micrometres
mg	Milligramme
MHz	Megahertz
min	Minute
mL	Millilitre
mM	Millimolar
M _n	Number average molecular weight
MRS	De Man, Rogosa and Sharpe medium
MRSG	MRS without dextrose and supplemented with 2% glucose
MRSS	MRS without dextrose and supplemented with 2% sucrose
MSA	Multiple sequence alignment
mTSB	Modified tryptic soy broth
M _w	Weight average molecular weight
η	Apparent viscosity
η ₀	Zero-shear (Newtonian) viscosity
NADH	Nicotinamide adenine dinucleotide hydrate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrate
NCBI	National Centre for Biotechnology Information
nm	Nanometre

Abbreviations

nM	Nanomolar
NMR	Nuclear magnetic resonance
¹³ C NMR	Carbon nuclear magnetic resonance
1D-NMR	One-dimensional nuclear magnetic resonance
¹ H NMR	Proton nuclear magnetic resonance
2D-NMR	Two-dimensional nuclear magnetic resonance
NR	Non-ropy
OD	Optical density
Pa	Pascal
PAP	Purple-acid phytase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Polydispersity index
PEP	Phosphoenolpyruvate
phy-MRS	Modified MRS supplemented with 0.65 g/L of sodium phytate and 0.1 M MES buffer
Pi	Inorganic phosphorus
PMA	Phorbol-12-myristate-13-acetate
PMA-THP-1	THP-1 monocytes differentiated to macrophages with PMA
ppm	Part per million
PPP	Phosphoketolase or 6-phosphogluconate pathway
priming-GTF, p-GTF	Priming-glycosyltransferase
PTS	Phosphotransferase system
PUFA	Polyunsaturated fatty acid
QPS	Qualified presumption of safety
rad	Radians
RCM	Reinforced clostridial medium
Rha	Rhamnose
RI	Refractive index
RNA	Ribonucleic acid
rpm	Revolution per minute
RPMI	Roswell park memorial institute medium
Rt	Retention time
RT	Room temperature
R _z	z-average radius of gyration
s	Second
SEC	Size-exclusion chromatography
SMD	Semi-defined medium
SMDS	SMD without dextrose and supplemented with 2% sucrose
spp.	Species
SSS	Specific substrate site
subsp.	Subspecies
<i>t</i>	<i>Trans</i> configuration
T _{5%}	Temperature for 5% weight loss
T _{50%}	Temperature for 50% weight loss
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
T _g	Glass transition temperature
TGA	Thermogravimetric analysis

Abbreviations

TLC	Thin-layer chromatography
T _{max}	Temperature of maximum weight loss rate
TNF- α	Tumoral necrosis factor α
U	Unit
UPV/EHU	University of the Basque Country
v/v	Concentration in volume/volume %
w/v	Concentration in weight/volume %
$\dot{\gamma}$	Shear-rate

SUMMARY

Lactic acid bacteria (LAB) include a heterogeneous group of microorganisms, whose main characteristic is the production of lactic acid as the major end-product of the fermentation of a variety of sugars. Bifidobacteria, belonging to the phylum Actinobacteria, were previously grouped as LAB because of their capacity for lactic acid production. However, not so long ago, a current school of thought classified them as a separated group due to their low genetic relationship with LAB [1], [2]. Bifidobacteria and LAB are considered as 'generally recognised as safe' (GRAS) organisms by the USA Food and Drug Administration (FDA), and several food-associated species have also the 'qualified presumption of safety' (QPS) status according to the European Food Safety Agency (EFSA). Currently, there is a high market demand for functional food, especially in developed countries that perceive it as a convenient and inexpensive solution to chronic health problems and as a manner of obtaining benefits from food, aiming to improve life quality. These bacteria are naturally present in a wide number of food matrices, where they are responsible for a large variety of food and beverage fermentations. In addition, during fermentation, some may synthesise exopolysaccharides (EPS), vitamins, gamma-aminobutyric acid, bioactive peptides, bacteriocins, conjugated linoleic acid (CLA) and enzymes, enriching the nutritional value of some fermented products and conferring health-benefits to the consumers [3]–[8].

Many of these BAL and bifidobacteria synthesise EPS, which are frequently responsible for the ropiness in alcoholic beverages or the slime formation in vacuum-packaged, sliced, cooked hams. Some of the EPS producing-LAB have been characterised as: *Pediococcus damnosus* and *Oenococcus oeni* in wines [9], [10], *O. oeni* and *Lactobacillus* and *Pediococcus* spp. in ciders [11]–[14], *Lactobacillus* and *Pediococcus* spp. in beers [15], [16] and *Lc. carnosum* and *Lactobacillus* spp. in hams [17], [18]. However, these EPS have been used in the food industry to promote thickening and gelling properties, for emulsification, stabilisation, control of crystallisation, inhibition of syneresis, encapsulation, to improve mouthfeel and rheology of fermented milks, and to improve dough rheology and bread texture [19], [20]. EPS are synthesised extracellularly or released onto the cell surface and to the environment in the form of slime during growth [19], [21], [22]. Depending on their monosaccharidic composition, EPS can be divided into

Summary

homopolysaccharides (HoPS), composed of a single, repeating monosaccharidic moiety, and heteropolysaccharides (HePS), formed by multiple copies of an oligosaccharide, which can contain between three and seven different types of residues. HoPS include glucans, fructans or galactans, which are synthesised by a sole enzyme called glycansucrase, belonging to the glycosyltransferases (GTF) family [19], [23], which can be encoded by genes located in plasmids or in the bacterial chromosome. By contrast, HePS are mainly composed by glucose, galactose and rhamnose in varying ratios, and occasionally, amino-sugars as well as polyol, glucuronic acid and phosphate are also present. The mechanism of their synthesis is more complex than that of the HoPS, and many enzymes are involved in the process. For instance, the priming- glycosyltransferase (*priming*-GTF/*p*-GTF) is the one responsible for the first step of the HePS synthesis [19], [24], and usually the *p-gtf* gene is carried on the chromosome of the host species.

Another active compound produced by LAB and bifidobacteria is the phytase enzyme, which degrades specifically phytates. Phytic acid or phytate is widely distributed in plant seeds, where it serves as the storage form of a large proportion of phosphate. It is considered an anti-nutrient because of its potent capacity for the chelation of proteins, amino acids and divalent cations, decreasing their bioavailability [25]. The incapacity of non-ruminant animals like human beings to metabolise phytate present in the diet, together with the huge demand for cereal-based fermented products as an alternative to dairies, lead to nutritional deficiencies of iron and zinc, especially in children of developing countries, whose staples are cereals, which contain phytates.

Finally, BAL and bifidobacteria are also utilised because they are able to produce CLA, a mixture of positional and geometrical isomers of linoleic acid with conjugated double bonds. The most important ones, biologically, are *cis*-9, *trans*-11 (*c9*, *t11*) or *trans*-10, *cis*-12 (*t10*, *c12*). They have been reported to have some health benefits: anti-carcinogenic, anti-atherogenic, anti-osteoporosis, anti-inflammatory, anti-oxidant, anti-cholesterolemic, anti-diabetic, lipolysis stimulators, immune system modulators, etc. [26]–[36].

In this work, EPS produced by bacteria isolated from different habitats have been characterised. *Bifidobacterium infantis* INIA P731 and *Bifidobacterium longum* INIA P132, isolated from breast-fed infant faeces, synthesised two HePS composed of glucose, galactose and rhamnose in 28:10:8 and 16:10:1 ratio, respectively. The immunomodulation capacity of these two HePS to immunomodulate the THP-1 cell line was tested, and the results showed an anti-inflammatory effect of the polymers studied. Another EPS producing-LAB, isolated from cider of Basque Country manufacturers was *Pediococcus ethanolidurans* CUPV141, the first instance of detection of this species in cider and the first characterised example of a *Pediococcus* producing HoPS and HePS, apart from being the first *Pediococcus* reported as HePS-producer [37]. It was composed of glucose, galactose, glucosamine and glycerol-3-phosphate. By contrast, the HoPS has been characterised in this work as a (1→3)-(1→2)-β-D-glucan (β-glucan), also synthesised by other species isolated from wines, ciders or beers [10], [13], [15], [38], [39]. The enzyme responsible for the synthesis of this β-glucan is the GTF glucosyltransferase, encoded by the *gtf* gene, located in plasmids [12], [40], [41] or in the chromosome [10], [40] of some species. In the case of CUPV141 strain, the *gtf* gene is located in a 34.4 kbp plasmid named pPE3. Finally, *Leuconostoc carnosum* CUPV411 and *Lactobacillus mali* CUPV271, isolated from vacuum-packed cooked ham and apple must from local manufacturers, respectively, were also characterised as producers of the HoPS dextran. In both cases, the dextran was composed of a linear α-(1→6) glucopyranose backbone, with a small percentage of branching in O-3 and O-4 positions. The average molecular weight was in the range of 1.23×10^8 g/mol and 3.58×10^8 g/mol for the dextrans produced by CUPV271 and CUPV411, respectively. Both polymers bore temperatures of 227 °C in anoxic conditions and about 220 °C in aerobiosis, they were amorphous materials which did not form crystals and their T_g rounded 226 °C. They presented a Newtonian viscosity flow when exposed to an increasing range of shear rate up to concentrations of 0.5%. At higher concentrations, a pseudoplastic viscosity flow was detected, characterised by a decrease in the viscosity as a response to increments of the shear rate. The critical concentration (C^*) was also calculated for the dextrans produced by CUPV411 (0.4%) and CUPV271 (3.8%) strains. Finally, the spatial conformation of both dextrans was studied by atomic force microscopy,

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and some differences were observed between them. The dextran produced by *L. mali* CUPV271 revealed a mixture of irregular rounded lumps with few random linear chains. By contrast, the dextran produced by *Lc. carnosum* CUPV411 showed irregular big aggregates and spherical small lumps. Therefore, these results lead to the conclusion that the differences in the supramolecular structure of both dextrans could be responsible for the different effects of the producing-bacteria *in vivo*.

Furthermore, the capacity of adhesion to intestinal epithelial cells (Caco-2 cell line) of bifidobacteria used in this work was evaluated. *Bifidobacterium infantis* INIA P731 yielded a 35.9% of adhesion, higher than that obtained for the probiotic *B. animalis* BB12, while *B. longum* INIA P132 only showed a 1.4% of adherence. Moreover, the presence or absence of the EPS on the adhesion capacity of the producing-bacteria *P. ethanolidurans* CUPV141, *L. mali* CUPV271 and *Lc. carnosum* CUPV411 was also assessed. For that purpose, the isogenic *P. ethanolidurans* CUPV141NR, characterised by an almost non-existent β -D-glucan production, was generated, and CUPV271 and CUPV411 strains were grown in the presence of sucrose, the substrate for the dextran production, or in the presence of glucose, a condition that does not support the synthesis of the HoPS. In the case of *P. ethanolidurans* CUPV141, the bacterium decreased its adherence capacity when the β -D-glucan was present, despite the fact that the β -D-glucan produced by the cider isolated *P. parvulus* 2.6 had been reported to be responsible for an increment in the bacterium-enterocyte interaction [12], [42], [43]. The HePS produced by *P. ethanolidurans* strains would be more accessible in CUPV141NR and recognised by receptors of the eukaryotic cells, as hypothesised in this work. In addition, a small assay was carried out in which the auto-aggregation capacity of the *P. ethanolidurans* strains was evaluated, showing the formation of huge aggregates by the β -D-glucan-producer CUPV141 strain, while those aggregates were virtually not formed by the isogenic CUPV141NR strain, showing that the glucan would be more related to the bacteria-bacteria interaction. Furthermore, the dextran produced by CUPV411 did not modify the capacity of the producing-bacteria to adhere to the enterocytes, whereas the dextran synthesised by *L. mali* influenced drastically in a negative way on the adherence of the bacterium to the eukaryotic

cells. These differences in the effect produced by dextrans on the adhesion capacity of the producing-LAB, could have their origin in the differences between the supramolecular structures of the dextrans, as indicated in the characterisation section.

Finally, LAB from the culture collection of the University of the Basque Country (UPV/EHU) were evaluated for their ability to produce phytases and bioactive CLA isomers. None of the LAB studied showed phytase activity, however, a phosphatase was observed, which showed non-specific phytate-degrading activities, which have been reported before for other LAB [44]–[46]. All the studied LAB were able to produce the bioactive *c*9, *t*11-CLA isomer in quantities reported for other LAB [47], [48]. In addition, the production of two other compounds was detected, one of them characterised as 10,13-dihydroxy-octadecanoic acid through the available bibliography and its mass spectrum. This acid has been reported as an intermediate in the biohydrogenation pathway of linoleic acid carried out by LAB [49], [50], and similar hydroxylated compounds seem to possess antifungal activity [51]. It was impossible to characterise the other compound obtained, although according to its mass spectrum it could be a CLA isomer different from those used in the calibration.

Currently, the food industry uses LAB and bifidobacteria for *in situ* production of bioactive compounds during the manufacture of fermented products. Also LAB EPS may be used as additives for the improvement of the sensorial properties of the products. In this work, different LAB and bifidobacteria are shown as candidates for the development of fermented products with an added value, as well as the utilisation of the EPS they produce for the improvement of the mouth-feel and texture of selected food.

RESUMEN

Las bacterias ácido lácticas (BAL) incluyen un grupo muy heterogéneo de microorganismos, cuya característica principal es la producción de ácido láctico como producto final mayoritario de la fermentación de una variedad de azúcares. Las bifidobacterias, pertenecientes al filo Actinobacteria, estuvieron previamente englobadas dentro de las BAL por ser productoras de ácido láctico. Sin embargo, hace unos años, una nueva corriente de pensamiento las clasificó como un grupo diferente por su escasa relación genética con las BAL [1], [2]. Tanto las BAL como las bifidobacterias son consideradas GRAS (*generally recognised as safe*) por la agencia americana FDA, y muchas especies también poseen el estatus de QPS (*qualified presumption of safety*) otorgado por la agencia europea EFSA. Actualmente, existe en el mercado una gran demanda de productos alimenticios con características funcionales, especialmente en los países desarrollados, que han encontrado en ellos una solución práctica y económicamente asequible para problemas crónicos de salud y un método para obtener beneficios a partir de los alimentos, con el objetivo de mejorar la calidad de vida. Estas bacterias se encuentran de forma natural en un gran número de matrices alimentarias, donde son las responsables de la fermentación de una gran variedad de alimentos y bebidas. Además, durante estas fermentaciones las BAL y bifidobacterias son capaces de sintetizar exopolisacáridos (EPS), vitaminas, ácido gamma-aminobutírico, péptidos bioactivos, bacteriocinas, ácido linoleico conjugado (CLA) y enzimas, enriqueciendo así el valor nutricional de algunos productos fermentados y confiriendo beneficios saludables a los consumidores [3]–[8].

Muchas de estas BAL y bifidobacterias producen EPS, considerados los responsables del ahilado en bebidas alcohólicas y de la formación de biopelículas en la superficie de jamones cocidos envasados al vacío. Algunas de las especies de BAL productoras de EPS han sido caracterizadas como: *Pediococcus damnosus* y *Oenococcus oeni* en vinos [9], [10], *Oenococcus oeni* y especies de lactobacilos y pediococos en sidra [11], [13], [14], [52], especies de lactobacilos y pediococos en cerveza [15], [16] y *Leuconostoc carnosum* y especies de *Lactobacillus* en jamones cocidos [17], [18]. Sin embargo, estos EPS tienen múltiples aplicaciones en la industria alimentaria como espesantes o gelificantes, emulsificantes, estabilizadores, como controladores de la cristalización o la inhibición de la

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sinéresis, en encapsulación o para mejorar la palatabilidad y reología de leches fermentadas y de masas y para la mejora de la textura del pan [19], [20]. Los EPS son sintetizados de forma extracelular o son liberados a la superficie celular durante el crecimiento [19], [21], [22]. Atendiendo a la composición de sus monosacáridos los EPS se dividen en homopolisacáridos (HoPS), formados por la repetición de un único tipo de residuo, y en heteropolisacáridos (HePS), compuestos por múltiples copias de un oligosacárido que puede contener entre 3 y 7 tipos de monosacáridos diferentes. Los HoPS pueden ser glucanos, fructanos o galactanos que son sintetizados por una única enzima glicansacarasa perteneciente a la familia de las glicosiltransferasas [19], [23], cuyos genes codificantes pueden estar localizados tanto en plásmidos como en el cromosoma bacteriano. Por el contrario, los HePS suelen estar formados fundamentalmente por residuos de glucosa, galactosa y ramnosa en diferentes ratios, aunque ocasionalmente también participan en su composición amino-azúcares, polioles, ácidos glucurónicos y fosfatos. El mecanismo por el cuál son sintetizados es más complejo que el de los HoPS y en él se encuentran implicadas varias enzimas. De éstas, la encargada de llevar a cabo la primera reacción del proceso es la llamada *priming*-glicosiltransferasa o primasa (*priming*-GTF/*p*-GTF) [19], [24], codificada habitualmente por un gen *p-gtf* cromosómico.

Otro de los compuestos bioactivos producido por las BAL y las bifidobacterias es la fitasa, enzima que degrada específicamente fitatos. El fitato o ácido fítico está presente en las semillas vegetales y sirve para almacenar grandes cantidades de fosfato. Está considerado como un factor antinutricional debido a su capacidad para quelar proteínas, aminoácidos y cationes divalentes, disminuyendo así la biodisponibilidad de los mismos [25]. Los seres humanos, como el resto de los animales no rumiantes, son incapaces de metabolizar el fitato presente en la dieta. Este hecho y la gran demanda de los cada vez más populares productos fermentados a base de cereales como alternativa a los productos lácteos, están provocando deficiencias nutricionales de minerales como el hierro o el zinc, sobre todo en niños de países en vías de desarrollo, cuyo alimento básico y principal son los cereales, portadores de fitatos.

Finalmente, las BAL y bifidobacterias también son utilizadas por la producción de CLA, o conjunto de isómeros geométricos y posicionales del ácido linoleico con dobles enlaces conjugados. Los isómeros 9-*cis*, 11-*trans* (9*c*, 11*t*) y 10-*trans*, 12-*cis* (10*t*, 12*c*), son los más activos biológicamente. Se les atribuyen algunos efectos beneficiosos para la salud como anticarcinogénico, antiaterogénico, antiosteoporosis, antiinflamatorio, antioxidante, anticolesterolémico, antidiabético, estimuladores de la lipólisis, moduladores del sistema inmune, etc. [26]–[36].

En este trabajo se han aislado y caracterizado los EPS producidos por BAL y bifidobacterias aisladas de diferentes ambientes. *Bifidobacterium infantis* INIA P731 y *Bifidobacterium longum* INIA P132, aisladas de heces de lactantes, produjeron dos HePS compuestos por glucosa, galactosa y ramnosa en ratios de 28:10:8 y 16:10:1, respectivamente. Se evaluó la capacidad de estos dos HePS para inmunomodular la línea celular THP-1 de monocitos humanos diferenciada a macrófagos, y los resultados mostraron una tendencia hacia un efecto antiinflamatorio por parte de los dos polímeros probados. Otra BAL con capacidad para sintetizar EPS y aislada de sidra de productores del País Vasco fue *Pediococcus ethanolidurans* CUPV141, la primera cepa de esta especie descrita en sidra, además de ser el primer pediococo capaz de sintetizar un HoPS y un HePS de forma simultánea, y el primer pediococo descrito como productor de un HePS [37]. Éste está compuesto de glucosa, galactosa, glucosamina y glicerol-3-fosfato. El HoPS, a su vez, ha sido caracterizado en este trabajo como un (1→3)-(1→2)-β-D-glucano (β-glucano), también sintetizado por otras especies aisladas de bebidas alcohólicas como sidra, vino o cerveza [10], [13], [15], [38], [39]. La enzima responsable de la síntesis de este β-glucano es la GTF-glucosiltransferasa, codificada por el gen *gtf*, localizado en diferentes plásmidos [40], [41], [52] o en el cromosoma [10], [40] de algunas especies bacterianas. En el caso de la cepa CUPV141 el gen *gtf* se encuentra en un plásmido de 34,4 kpb llamado pPE3. Por último, *Leuconostoc carnosum* CUPV411 y *Lactobacillus mali* CUPV271, aisladas respectivamente de jamón cocido envasado al vacío y mosto de manzana de productores locales, también fueron caracterizadas como productoras de EPS, concretamente del HoPS dextrano. En ambos casos, el dextrano resultó estar

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formado por una cadena principal de glucopiranosas unidas mediante enlaces α - $(1\rightarrow6)$, con un pequeño porcentaje de ramificaciones de glucosas en posiciones O -3 y O -4. El promedio del peso molecular en peso rondaba los $1,23 \times 10^8$ g/mol para el dextrano producido por la cepa CUPV271 y los $3,58 \times 10^8$ g/mol para el dextrano sintetizado por CUPV411. Los dos polímeros soportaban temperaturas de hasta 227 °C en condiciones anóxicas y sobre 220 °C en aerobiosis, eran materiales amorfos que no formaban cristales y su T_g se encontraba alrededor de los 226 °C. Presentaban un comportamiento de flujo de viscosidad newtoniano cuando eran sometidos a un rango creciente de velocidad de cizalla hasta concentraciones de 0.5%. A concentraciones más altas, adquirirían un comportamiento de flujo pseudoplástico, en el que la viscosidad disminuía a medida que aumentaba la velocidad de cizalla. También se calculó la concentración crítica (C^*) para ambos dextranos, dando valores de 3,8% y 0,4% para *L. mali* y *Lc. carnosum*, respectivamente. Finalmente, la conformación espacial de ambos dextranos fue evaluada mediante microscopía de fuerza atómica, indicando diferencias entre ellos. Mientras que el dextrano producido por *L. mali* CUPV271 se disponía formando pequeños abultamientos con cadenas lineales al azar, el dextrano sintetizado por *Lc. carnosum* CUPV411 formaba una mezcla de grandes agregados irregulares con pequeños abultamientos esféricos. En este trabajo, se concluye que estas diferencias en la estructura supramolecular de ambos dextranos podría ser la responsable de los diferentes efectos causados por las bacterias productoras *in vivo*.

Por otro lado, se estudió la capacidad de adhesión a células epiteliales de intestino (línea celular Caco-2) por parte de las bifidobacterias utilizadas en este trabajo. *Bifidobacterium infantis* INIA P731 mostró un 35,9% de adhesión, superando a la cepa probiótica *B. animalis* BB12, frente al 1,4% registrado para *B. longum* INIA P132. Además, se evaluó el efecto de la presencia o ausencia de los EPS en la capacidad de adhesión a la línea celular Caco-2 (enterocitos humanos) de las cepas productoras *P. ethanolidurans* CUPV141, *L. mali* CUPV271 y *Lc. carnosum* CUPV411. Para ello, se generó la cepa isogénica *P. ethanolidurans* CUPV141NR, caracterizada por una producción prácticamente nula del β -D-glucano, y se crecieron las cepas CUPV271 y CUPV411 en presencia de sacarosa, sustrato para la

producción de los dextranos, o en presencia de glucosa, condición en la que no se sintetizan los polímeros. En el caso de *P. ethanolidurans*, la bacteria perdía capacidad de adhesión a los enterocitos cuando el β -D-glucano estaba presente, al contrario que en estudios anteriores, donde se demostraba que el β -D-glucano producido por la especie *P. parvulus* 2.6, también aislado de sidra, era el responsable de aumentar la unión adhesión bacteria-enterocito [12], [42], [43]. En el trabajo se hipotetiza que este hecho sería posible porque en la cepa CUPV141NR el HePS está más descubierto y sea reconocido por receptores presentes en las células eucarióticas. Además, un pequeño ensayo en el que se estudiaba la capacidad de agregación de las cepas de *P. ethanolidurans*, mostró grandes agregados generados por la cepa CUPV141 productora de β -D-glucano, mientras que CUPV14NR era prácticamente incapaz de formarlos, resultados que indican la implicación del glucano en la interacción bacteria-bacteria. Por otro lado, el dextrano producido por la cepa CUPV411 no modificó la capacidad de la bacteria para adherirse a los enterocitos, mientras que el sintetizado por *L. mali* disminuyó drásticamente la adhesión de la bacteria productora. Las diferencias en el efecto de los dos dextranos sobre la capacidad de adhesión de las cepas productoras podrían residir en las distintas estructuras supramoleculares de los mismos, como se ha indicado en la caracterización de los dos dextranos.

Por último, se evaluó la capacidad para producir fitasas e isómeros bioactivos de CLA por BAL de la colección de cultivos de la Universidad del País Vasco (UPV/EHU). Ninguna BAL fue positiva para la producción de fitasas, sin embargo, se observó la producción de una enzima fosfatasa, con actividad degradadora de fitato inespecífica, ya descrita para otras BAL con anterioridad [44]–[46]. Por otro lado, todas las BAL evaluadas fueron capaces de producir el isómero CLA-9*c*, 11*t* en cantidades descritas para otras BAL [47], [48]. Además, se observó la producción de otros dos compuestos, uno de ellos caracterizado como ácido 10,13-dihidroxi-octadecanoico según la bibliografía disponible y el espectro de masas obtenido. Este ácido ha sido descrito como intermediario en la ruta de biohidrogenación del ácido linoleico que llevan a cabo las BAL [49], [50], y compuestos hidroxilados semejantes parecen ejercer un efecto antifúngico [51]. El

Resumen

otro compuesto obtenido no pudo ser caracterizado, aunque por su espectro de masas podría ser un isómero de CLA diferente a los utilizados para el calibrado.

El uso de BAL y bifidobacterias como productoras de compuestos bioactivos *in situ* en productos fermentados está siendo cada vez más común en la industria alimentaria, así como la inclusión de los EPS que producen como aditivos para la mejora de las propiedades sensoriales y organolépticas de los productos. En este trabajo se muestran una serie de BAL y bifidobacterias como candidatas para el desarrollo de productos fermentados con un valor añadido, así como la utilización de sus EPS para mejorar la palatabilidad y textura de determinados alimentos.

GENERAL INTRODUCTION

1. Lactic acid bacteria (LAB) and bifidobacteria: general characteristics

The term LAB includes a heterogeneous group of microorganisms, whose main characteristic is the production of lactic acid as the major end-product of the fermentation of a variety of sugars. Until recently, LAB were not related to a phylogenetic class of organisms, but rather to a group of microorganisms that shared similar metabolic capabilities. Thus, LAB were included in two distinct phyla: Firmicutes and Actinobacteria (Figure 1). The most important genera of LAB belonging to the Firmicutes phylum are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weisella*, which are low GC-content (31-49%) microorganisms within the order *Lactobacillales*. Among the Actinobacteria phylum, the *Bifidobacterium* genus is the only one belonging to the LAB group, and it is characterised by a high GC-content (58-61%) [1]. However, a current school of thought believes that bifidobacteria were wrongly considered LAB. Phylogenetically, only bacteria belonging to the order *Lactobacillales* would be recognised as LAB, since despite the similar physiological characteristics that other genera, like *Bifidobacterium* or *Bacillus*, share with them, they have little genetic relation with LAB [2].

Therefore, whether or not included within the LAB group, the current knowledge related to bifidobacteria was reviewed in this work.

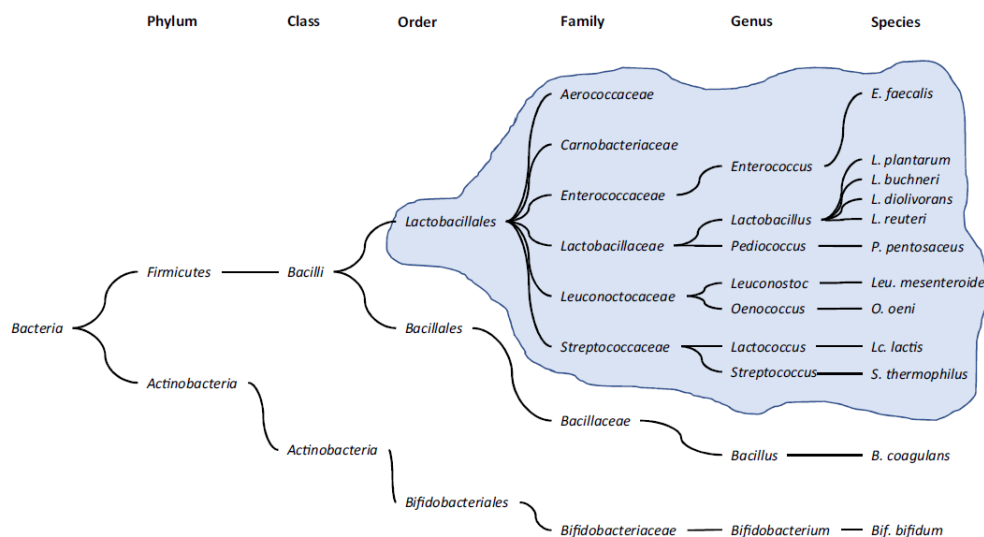


Figure 1. Schematic overview of the phylogeny of lactic acid bacteria. The orders *Lactobacillales* and *Bacillales* belong to the class Bacilli within the phylum Firmicutes, whereas the order Bifidobacteriales, commonly known as bifidobacteria, belong to the class Actinobacteria within the phylum Actinobacteria. Image reproduced from Sauer *et al.* (2017).

1.1. LAB

LAB inhabit naturally the gastrointestinal tract (GIT) of different animals, but they have also been isolated from various fermented foods or processed dairy, meat and vegetable products [1], [53]. LAB belonging to the order *Lactobacillales* are considered as non-sporulating, acid-tolerant, microaerophilic, Gram-positive bacteria, which present rod or cocci shapes and a low-GC-content genome, apart from showing a catalase negative enzymatic activity. However, they are mainly characterised by the major production of lactic acid from the fermentation of different carbohydrates, through two different pathways: (i) the glycolysis or Embden-Meyerhof pathway (EMP) and (ii) the 6-phosphogluconate or phosphoketolase pathway (PPP) [54], schematised in Figure 2. The EMP results in a homofermentation in which the lactate is the exclusive end-product. Pyruvic acid from glycolysis is reduced to the two lactic acid enantiomers (D- and L-lactic acid) in a strain-specific manner [55]. Contrarily, a heterofermentation is carried out by the PPP producing equimolar amounts of lactic acid, carbon dioxide and ethanol or acetate from either hexoses or pentoses. Strict homofermentative bacteria are unable to ferment any pentoses, due to the lack of the phosphoketolase enzyme. Nevertheless, some bacteria present a pentose-inducible phosphoketolase, which permits heterofermentation *via* the PPP; these are called facultative heterofermenters [54], [55]. These fermentations are coupled to substrate phosphorylation, which leads to low ATP yields [56]. Thus, large amounts of substrate are needed for growth, and consequently substantial amounts of end-products are obtained.

1.2. Bifidobacteria

The first *Bifidobacterium* was isolated in 1899 by Henri Tissier from the faeces of a breast-fed infant and was named as *Bacillus bifidus* [57], [58]. Bifidobacteria naturally inhabit the oral cavity or the GIT of different animals, and they have been isolated from caries (pathogens), from the faeces of humans (adults and infants), and animals and from human vagina [53], [58].

Bifidobacteria are Gram-positive, non-motile, non-spore forming, catalase-negative, acid-tolerant bacteria with a high GC content. Their name comes from the

morphology they present, which is referred to as bifid or irregular V- or Y-shaped rods resembling branches [1], [57]. Until now, the cause of this irregular shape has not been clearly understood, but the absence or low concentration of *N*-acetyl-amino-sugars [59], Ca^{2+} ions [60] or amino acids [61] has been hypothesised. They do not produce gas during their growth and they are obligate anaerobes, although the sensitivity to oxygen varies among strains [58]. Bifidobacteria, like LAB, also produce lactic acid during carbohydrate fermentation, but through the so-called 'bifid-shunt', rather than the EMP (Figure 2). This pathway centres on the activity of the key enzyme fructose-6-phosphate phosphoketolase (F6PPK), which splits hexose-phosphates into erythrose-4-phosphate and acetyl-phosphate. The final products are acetate and lactate. The bifid shunt is very advantageous for bifidobacteria, as they obtain more energy from carbohydrates compared to the EMP [57], [58], [62].

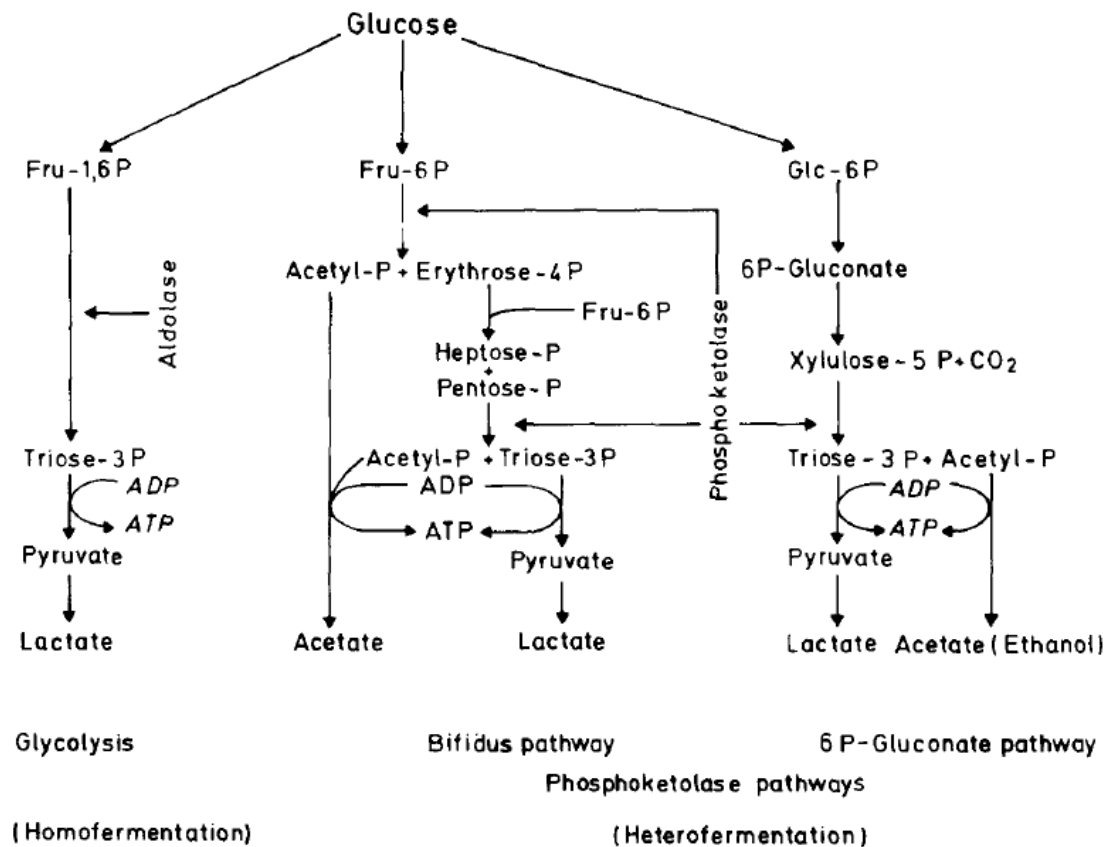


Figure 2. Schematic representation of the main pathways of hexose fermentation in LAB and bifidobacteria.

Image reproduced from Kandler, 1983.

2. Production of bioactive compounds

Functional food first originated in Japan in the 1980s. This concept has evolved from food that had 'advantageous physiological effects' to the most recent definition provided by the Functional Food Centre (FFC) as 'natural or processed foods that contain known or unknown biologically-active compounds, and which in defined, effective, and non-toxic amounts, have a clinically proved and documented health benefit for the prevention, management or treatment of chronic disease' [63]. Currently, there is a high market demand for functional food, especially in developed countries that perceive it as a convenient and inexpensive solution to chronic health problems and as a manner of obtaining benefits from food, aiming to improve life quality.

LAB and bifidobacteria are considered as 'generally recognised as safe' (GRAS) organisms by the USA Food and Drug Administration (FDA), and several food-associated species have also the 'qualified presumption of safety' (QPS) status according to the European Food Safety Agency (EFSA). LAB are naturally present in a wide number of food matrices where, due to their high metabolic versatility, are responsible for a large variety of food and beverage fermentations [4], [7], [64], [65]. In addition, LAB display a high biosynthetic capacity and therefore, during fermentation, some may synthesise exopolysaccharides (EPS), vitamins, gamma-aminobutyric acid, bioactive peptides, bacteriocins, conjugated linoleic acid (CLA) and enzymes, enriching the nutritional value of some fermented products and conferring health-benefits to the consumers [3]-[8].

Probiotics are defined as living microorganisms which, when administered in sufficient numbers, confer health benefits to the host [66] and they are mainly LAB and bifidobacteria. These bacteria have been frequently isolated from the intestinal microbiota [67] and are commercially available as food supplements or as components of functional food and beverages, mainly dairy products [68]. LAB isolated from other origins such as quinoa and amaranth fermentations have also demonstrated probiotic potential [69]. Probiotics have been evaluated as functional starter cultures in various types of fermented food products such as yoghurt, cheese, dry sausages, salami and sourdough [70]. Moreover, probiotics

have been shown to exert a wide range of strain-specific effects although the number of cells required to produce the therapeutic benefits is not known and might vary as a function of the strain and the desired health effect [70]. For instance, *Bifidobacterium animalis* subsp. *lactis* Balat_1410^{S89L} was reported to counteract an inflamed state of colitis chemically induced by dextran sodium sulphate (DSS) administration to mice, although the mechanism through which the effect was obtained could not be assessed [71]. In addition, acute infectious diarrhoea in children has also been reported to last for fewer days and be less frequent in children treated with *Bifidobacterium lactis* B94 [72] plus inulin or supplementing infant formula with *B. lactis* [73] or *Bifidobacterium longum* subsp. *infantis* CECT7210 (*B. infantis* IM1) [74] than in no treated children. This last strain was also demonstrated to potentially reduce the intestinal colonisation by pathogens and stimulate local immune response in a weaning piglet model [75]. The intake of *Bifidobacterium infantis* 35624 also resulted in positive preliminary results against depression in rats [76] and ingestion of *B. longum* IPLA20021 and IPLA20022 produced a notable increase in the levels of *Bacteroides*, other *Bifidobacterium* species and *Faecalibacterium prausnitzii* among others, modulating elderly people microbiota *in vitro* [77]. The mechanisms of action may vary from one probiotic strain to another and are, in most cases, probably a combination of activities, thus making the investigation of the responsible mechanisms a very difficult and complex task. In general, 3 levels of action can be distinguished: probiotics can influence human health by interacting with other microorganisms present in the site of action, by strengthening mucosal barriers, and by affecting the immune system of the host [78]. However, the biofunctional concept is generally used when the health-giving metabolite(s) emerge(s) in the food product during the fermentation process due to the bacterial metabolic activity. Consequently, bacteria can act as a microbial factory to enrich foodstuff, for which bacterial viability through the GIT or during the product storage is not absolutely required [8].

Therefore, LAB and bifidobacteria as food grade microorganisms with high adaptability to fermentation processes are very valuable in food biotechnology to

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widen the scope of novel functional foods and beverages, which can contribute to the GIT wellbeing and/or to the nutritional value [79], [80].

Thus, the next sections of this introduction will present the current and potential usage of LAB and bifidobacteria for the production of active components such as EPS, CLA and phytate-degrading enzymes for the contribution to the development of functional foods.

2.1. Production of exopolysaccharides (EPS)

EPS-producing bacteria are mainly isolated from food and beverage products, often characterised by being spoilt. The production of ropiness (Figure 3) is defined as a microbiological disorder common to alcoholic beverages. Moreover, the slime formation in vacuum-packaged, sliced, cooked hams, is defined as long polysaccharide ropes, which have been reported to be the consequence of the production of EPS by: *Pediococcus damnosus* and *Oenococcus oeni* in wines [9], [10], *Lactobacillus* and *Pediococcus* spp. and *O. oeni* in ciders [11], [13], [14], [52], *Lactobacillus* and *Pediococcus* spp. in beers [15], [16] and *Lc. carnosum* and *Lactobacillus* spp. in hams [17], [18].

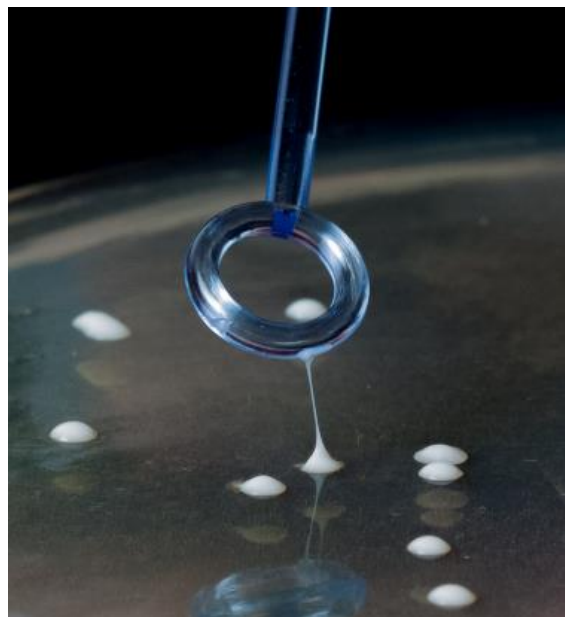


Figure 3. Macroscopic appearance of the 'ropy' phenotype of colonies of *P. ethanolidurans* CUPV141 isolated from cider (Llamas-Arriba *et al.* (2018)), on the surface of MRS-agar plates.

Alcoholic beverages with a ropy character are detected by an abnormal increase in viscosity, which sometimes makes them run thicker than oil. Furthermore, the organoleptic properties of the products change and become disagreeable to consumers, a fact which is translated into a wide economic loss. Ropy slimes in vacuum-packaged cooked meat also modify the final sensorial characteristics of the product due to souring, gas formation, unattractive odours and flavours and discolouration [18]. The production of these EPS is involved in conferring survival advantages to the producing-bacteria. They protect them from desiccation maintaining the bacterial moist, phage attacks, predation by protozoans, antibiotics, toxic compounds and osmotic stress. In addition, EPS are involved, among others, in: (i) stress resistance (alcohol, low temperatures, low pH, etc.), (ii) adhesion to surfaces and self-aggregation to form biofilms for colonisation of various niches and (iii) in cellular recognition [18], [19], [21]. Furthermore, microbiological polymers also have multiple useful applications. They have been used in the food industry to promote thickening and gelling properties, for emulsification, stabilisation, control of crystallisation, inhibition of syneresis, encapsulation, to improve mouthfeel and rheology of fermented milks, and to improve dough rheology and bread texture [19], [20]. Therefore, the chemical characterization of microbiological polymers is very important so that they can be suitably applied.

The subjects of this PhD work are long-chain polymers, which are synthesised extracellularly or released onto the cell surface and to the environment in the form of slime during growth. On the contrary, another class of polysaccharides, capsular polysaccharides, remain attached to the cell surface via covalent bonds [19], [21], [22]. Moreover, depending on their monosaccharidic composition, EPS can be divided into homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

HoPS are composed of a single, repeating monosaccharidic moiety and can be glucans, fructans or galactans. Among them, (i) dextrans are characterised by a linear backbone of α -(1 \rightarrow 6) linked glucosidic residues with variable degrees of branching (strain-specific) of α -(1 \rightarrow 3) and less frequent α -(1 \rightarrow 2) and α -(1 \rightarrow 4) linked branches; (ii) mutans contain more than 50% α -(1 \rightarrow 3) glucosidic linkages

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in the linear backbone; (iii) alternans alternate α -(1→3) and α -(1→6) glucosidic linkages with some degree of α -(1→3) branching; (iv) reuterans consist of a linear backbone of α -(1→4)-linked glucose residues with some α -(1→6) branches; (v) β -D-glucans composed of β -(1→3)-linked glucose molecules with β -(1→2) branches; (vi) levans contain fructose units linked by β -(2→6) glycosidic bonds; (vii) inulins are chains of fructose linked by β -(2→1) glycosidic bonds and (viii) others, like polygalactans, composed of structurally identical repeating units with different glycosidic linkages [19], [20]. On the contrary, HePS are formed by multiple copies of an oligosaccharide, which can contain between three and seven different types of residues. Glucose, galactose and rhamnose usually are the main sugars implicated in the synthesis of HePS, and occasionally, amino-sugars as well as polyol (glycerol), glucuronic acid and phosphate might be also present [81].

HoPS are mainly produced by LAB, since until now none has been reported for bifidobacteria. HoPS such as dextrans or levans are synthesised in g per L quantities by highly specific, secreted or membrane anchored, glycosyltransferases belonging to the glycosyltransferases (GTF) class (levansucrases, mutansucrases, alternansucrases or dextransucrases), which transfer a D-glucose moiety from sucrose to an acceptor monosaccharide or oligosaccharide [19], [23].

HePS are produced by LAB and bifidobacteria in mg per L quantities and their synthesis is more complex because it involves the participation of several enzymes and/or proteins (Figure 4). This synthesis seems to follow the same mechanisms in LAB and in bifidobacteria, and two groups of enzymes are implicated: (i) non-EPS-specific enzymes, called 'housekeeping enzymes', which permit the phosphorylation, internalisation and activation of the substrate and are used by other cell pathways, and (ii) EPS-specific enzymes, encoded by operons, which also contain genes encoding transcriptional regulators. The biosynthetic pathway can be divided into four separate steps: (i) sugar transport into the cytoplasm, (ii) synthesis of sugar-1-phosphates, (iii) activation and coupling of sugars and (iv) export of the EPS [22], [81], [82]. The most frequent mechanism for sugar transport is the bacterial phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS), which contains a group of proteins responsible for binding,

transmembrane transport and phosphorylation of a variety of sugar substrates. Once in the cytoplasm, sugar-6-phosphates are consumed in metabolic pathways, whereas sugar-1-phosphates can participate in EPS synthesis. As PEP-PTS systems generate mainly sugar-6-phosphates, the role of phosphoglucomutases is very important for EPS synthesis. Then, sugar-1-phosphates are converted into sugar nucleotides by different enzymes, and finally, the EPS biosynthesis, using EPS-specific enzymes, takes place (Figure 4).

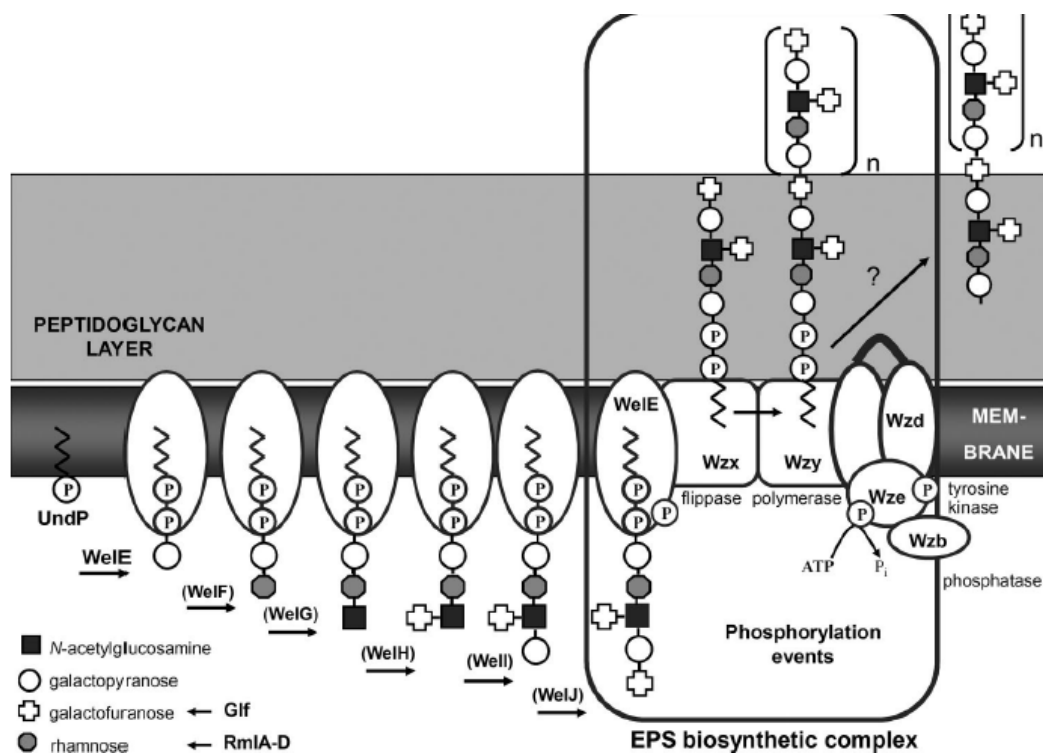


Figure 4. Schematic representation of the putative steps in HePS biosynthesis by *L. rhamnosus* GG. Image reproduced from Lebeer *et al.* (2009).

Genes coding for these EPS-specific enzymes are organised in the *eps* cluster, which differs between LAB and bifidobacteria in the absence for the latter of gene regulators. However, it is been suggested that this role could be played by proteins with unknown function within the cluster or others outside it [83]. The *eps* cluster consists of four separate domains. There is a central core coding for the glycosyltransferases, which is flanked at the ends by genes coding for proteins with strong homology with enzymes used for polymerisation and export. Finally, a

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regulatory domain is present at the start of the gene cluster, when applicable. The sugar nucleotides are assembled on an isoprenoid glycosyl lipid carrier that is attached to the cytoplasmic membrane. The first sugar-nucleotide is donated with a phosphate group to the lipid by the 'priming-glycosyltransferase' (priming-GTF or p-GTF). This enzyme, unlike other GTF, is able to recognise the lipid carrier and does not catalyse a glycosidic linkage, but a phosphoanhydride bond. Then, the repeating-unit is obtained by sequential transfer of nucleotide sugar residues onto the growing chain by other glycosyltransferases. Finally, a flippase or translocase moves the lipid-bound material to the periplasmic face of the membrane, where a polymerase catalyses the polymerisation of the blocks and another protein controls the polymer chain length [19], [22], [24], [82]–[84](Figure 4).

Nevertheless, the ability to produce EPS is often an unstable characteristic genetically, as well as the instability of the ropy texture itself, which is a serious problem for industrial applications. In mesophilic LAB this characteristic seems to be encoded by genes located in plasmids, thus the loss of ropiness would be attributed to the loss of these plasmids. However, in bacteria that carry genes responsible for EPS synthesis in the chromosome, the genetic instability could be due to mobile genetic elements or deletions and rearrangements in the DNA [19], [23].

As commented above, the purification of the isolated EPS and the determination of their structure are very important for their applications [85]. After lyophilisation of the samples, the EPS are often further purified using a chromatographic technique, attending to their charge, solubility and molecular weight. Size-exclusion chromatography (SEC) is one of the most widely used methods for purification of large molecules, as polysaccharides, that separates components of a sample according to their molecular size (not molecular weight). As polysaccharides are polydisperse, only their apparent average molecular weight (M_w) can be determined. To do so, a calibration curve should first be performed by fractionation of polysaccharide standards in the same analytical conditions as the samples. To detect the eluted polysaccharides, a post-column colorimetric method is generally used [86]. As an alternative, high-performance size-exclusion

chromatography (HPLC-SEC) equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors can also be used to determine the M_w and z-average radius of gyration (R_z) of the EPS.

The monosaccharide composition of the EPS is determined by liquid or gas chromatography after acid hydrolysis. In the last case, the monosaccharides should be converted into volatile derivatives prior to analysis. The types of bonds between the monosaccharide residues in the EPS and their percentages is usually ascertained by methylation analysis and gas chromatography/mass spectroscopy (GC-MS) [87]. This is a complex methodology with multiple steps. First, the free hydroxyl groups of the polysaccharide (not involved in any kind of linkage) are chemically methylated [88]. The permethylated EPS is then hydrolysed with acid, giving a mixture of partially methylated monosaccharides that have again free hydroxyl groups in the ring positions that were involved in linkages in the native EPS. The mixture is treated with sodium borodeuteride, producing a double effect: the reduction of the aldehyde groups to alcohols, and their exclusive labelling with a deuterium atom, which will make easier the subsequent MS analysis. In the last step, all the free -OH groups are acetylated. Chemically, the resulting derivatives, denominated 'partially methylated alditol acetates', are then analysed by GC-MS, and each peak of the chromatogram is identified by the retention time and mass spectra parameters. The quantification is associated to the peaks area.

To resolve the structure of an EPS molecule, both the ring size (pyranose/furanose) of the monosaccharide residues and the anomeric configuration of the glycosidic linkages should also be determined. Nuclear magnetic resonance (NMR) is the technique of choice to study the conformation of the polysaccharides and allows elucidation of the type of glycosidic linkages and the structure of the repeating units that constitute the EPS molecules. Before NMR analysis, the purified EPS are dissolved in D_2O so that exchangeable protons are replaced by deuterium (deuteration). A proton nuclear magnetic resonance (1H NMR) spectrum of the EPS gives information about the number of monosaccharides present in the repeating unit by counting the resonances in the anomeric region (4.4-5.5 ppm). The common hexoses are detected as well in

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carbon nuclear magnetic resonance (^{13}C NMR) spectra (95-110 ppm). From the splitting of the anomeric peaks in ^1H spectra, the anomeric configuration can be established. Since NMR spectra of most polysaccharides show peak overlap in the ring region, 2D-NMR techniques are often applied, as well.

Finally, to determine the supramolecular structure and conformation of an EPS, atomic force microscopy (AFM) is currently used. It has been successfully applied to visualise a range of polysaccharides including curdlan [89], oat β -glucan [90] and the *L. suebicus* CUPV221 (1 \rightarrow 3)(1 \rightarrow 2)- β -D-glucan [91].

2.2. Production of phytate-degrading enzymes

Nowadays, vegetarian and vegan trendies along with some nutritional problems (cholesterol raise, lactose intolerance, allergies) lead to an increase in the demand of non-dairy milk alternatives [92]. At this point, fermentation of cereals by LAB plays an important role in the appearance of new functional products. Moreover, these cereal-based products could also serve as vectors for assimilating functional components as antioxidants, dietary fibre, minerals, probiotics, vitamins, etc. However, cereals also contribute with negative effects such as natural contaminants, antinutrients as phytates, inadequacy of essential micronutrients, etc. which lead to final products with poor nutritional quality and sensory properties [93], [94]. Fermentation of cereals by LAB will represent a solution by transforming the sensorial qualities of the product, changing it chemically, producing different metabolites (organic acids, oligosaccharides, polyphenolic compounds), enriching the product with micronutrients, lowering serum cholesterol levels or stimulating the immune system [94].

Phytic acid, phytate or *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate, is widely distributed in plant seeds, where it serves as the storage form of a large proportion of phosphate. It is considered an anti-nutrient because of its potent chelation capacity. This property is due to the negative charges provided by the phosphate groups linked to the inositol ring, which form insoluble complexes with proteins, amino acids and divalent cations, decreasing their bioavailability [25].

Therefore, the incapacity of human beings to metabolise phytate present in the diet, leads to nutritional deficiencies of iron and zinc in children of developing countries, whose staples are cereals, which contain phytates. In addition, phytates also have an ecological impact because of the incapacity of some animals to metabolise phytate from the diet. As a result, a considerable amount of phosphates and inorganic P are excreted, causing a negative impact on the environment: eutrophication of surface water, algal blooms, hypoxia, death of fish and other aquatic animals, production of nitrous oxide, etc. [95]. However, its capacity for complexing iron also confers it antioxidant ability by the avoidance of free-radicals formation. Moreover, complexing of calcium with phytic acid diminish calcifications formed in the kidneys or heart vessels and it interacts with proteins making soluble complexes inhibiting some enzyme activities. Finally, it has anticarcinogenic activity, demonstrated in rat models of colon, liver, lung, mammary glands, prostate or skin cancers [25], [96].

Lower forms of *myo*-inositol phosphate (IP₂, IP₃, IP₄), also called inositol phosphates, have been also described to positively affect human health [97]. Therefore, in order to achieve dephytinisation several methods have been used, such as food processing, activation of seed endogenous phytases, addition of exogenous phytases as well as fermentations with phytase-producing microorganisms. Food processing includes milling, soaking and germination, which can contribute to removal of phytic acid. Grain milling is very effective; however, minerals and dietary fibre are lost. By soaking, part of the phytic acid (between 16% and 25%) can be degraded. However, a loss of minerals and proteins soluble in water occurs. Germination is also very effective, but it is a slow process not adequate for industry [25], [95], [96].

Phytases are *myo*inositol-1,2,3,4,5,6-hexakisphosphate phosphohydrolases that dephosphorylate phytates in sequential steps, releasing phosphate, the inositol ring, and the inositol phosphates (IP₅-IP₁) that are also formed in the process. These phytases occur naturally in diverse organisms: plants, animals, bacteria, yeasts, fungi and protozoan. They differ greatly between one another; structure, mechanism of action, temperature, pH, etc. which lets a wide range of choices for

different purposes. Thermoresistant ones are very important for standing products' processing temperatures, or acidic ones are more suitable for feeding. In addition, today some phytases have been cloned [93] in more suitable microorganisms, like LAB, for food fermentation, for instance.

2.3. Production of conjugated linoleic acid (CLA)

CLA is the term used to describe a mixture of positional and geometrical isomers of linoleic acid (LA; *cis*-9, *cis*-12-C18:2) with conjugated double bonds (Figure 5) produced by bacteria present in the rumen of some animals. Different pathways have been reported in which a linoleic acid isomerase (LAI) is the main enzyme responsible for the formation of CLA. The fact that CLA are conjugated implies the presence of a carbon atom, named as methylene carbon, between each carbon harbouring the double bonds. Depending on the spatial configuration of the double bonds, as well as their position within the carbon chain, a wide variety of different isomers can be formed. The most important ones, biologically, are *cis*-9, *trans*-11 (*c9*, *t11* or ruminic acid) or *trans*-10, *cis*-12 (*t10*, *c12*) (Figure 5). They have been reported to have some health benefits: anti-carcinogenic, anti-atherogenic, anti-osteoporosis, anti-inflammatory, anti-oxidant, anti-cholesterolemic, anti-diabetic, lipolysis stimulators, immune system modulators, etc. [26]–[36]. In general, the *c9*, *t11* isomer is the responsible for the majority of these effects and its presence in animal products (milk fat above all) raises to 75-90%. The *t10*, *c12* isomer is more related to anti-obesity and anti-diabetic effects, and it appears in a range of 3-5%. Recently, the *trans*-9, *trans*-11 (*t9*, *t11*) isomer has been described as a potent anticarcinogenic, too [98]. However, the estimated CLA intake that a person consumes per day, depending on the food source and sex (0.2 to 1.5 g [29]) does not reach the minimum required for the production of the health benefits. Some authors believe that this quantity must be around 3-4 g / day /person to take advantage of the beneficial effects [27], [31], [33], thus, the normal daily diet is not enough to reach that amount.

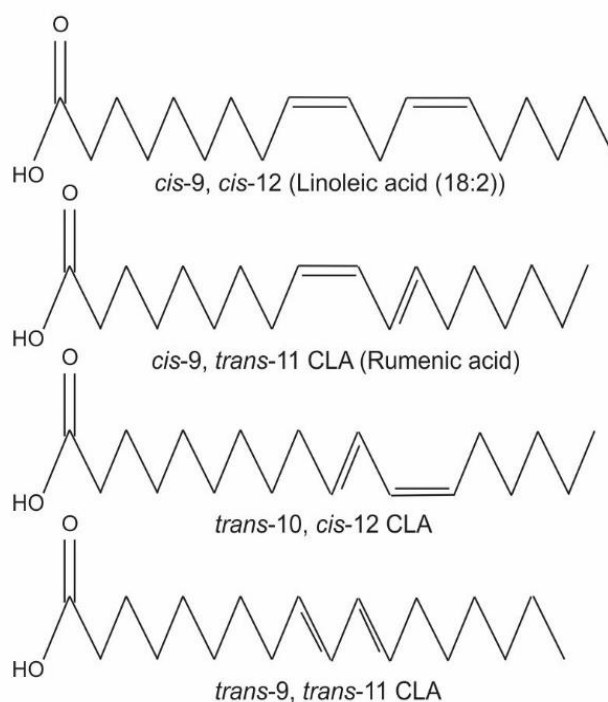


Figure 5. Chemical structures of LA and some of its conjugates.

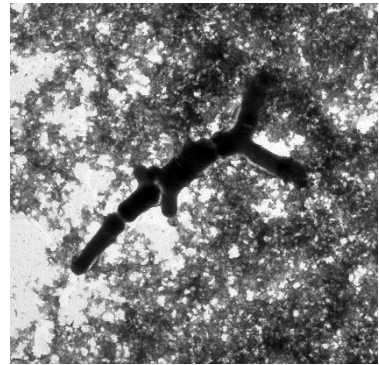
Currently, one solution has been the formulation of CLA supplements or the addition of the isomers to some products, as fermented milks. The CLA incorporated to foods is synthesised chemically by alkaline isomerisation of fatty acids, reduction of acetylenic fatty acids by selective hydrogenation, dehydration of fatty acids containing an alcohol homo-allylic function or stereomutation of conjugated fatty acids in *cis* configuration. Nevertheless, these methods do not allow the production of a specific isomer, thus, the need for additional equipment for the separation and purification of the interesting one is crucial. In addition, some of the methods require very specific substrates, not always available from natural sources [26]. This reason drove to the search for bacteria harbouring enzymes as the LAI for producing CLA isomers from polyunsaturated fatty acids (PUFAs) and LAB have attracted special attention.

OBJECTIVES

The main objectives of this work are summarised below.

1. Evaluation of the technological properties of the bifidobacteria employed in this study.
2. Isolation and chemical characterisation of the EPS synthesised by different bifidobacteria and LAB.
3. Evaluation of the bioactivity of the EPS isolated from bifidobacteria and LAB: effect of the EPS on their adhesion capacity.
4. Assessment of the LAB strains from the UPV/EHU culture collection (CUPV) as producers of phytase activity and/or conjugated linoleic acids.

CHAPTER 1. EPS PRODUCTION BY BIFIDOBACTERIA



1.1. Introduction

Bifidobacteria, Gram-negative, non-spore forming, non-motile, catalase-negative and anaerobe microorganisms, are a predominant bacterial group present in the human GIT. In addition, they have a long history of safe use in food and as probiotics [99]–[101]. These microbes protect the host by acting as a barrier against exogenous food-borne pathogens, promote nutrient supply and shape and maintain normal mucosa immunity. In response, they have access to key nutrients and enjoy of a stable and required environment for their growth. Some of the beneficial health effects (anti-tumour, cholesterol-lowering, immunomodulating, etc) exerted in the host are promoted by the production of EPS. At the same time, these polymers protect them from the stomach acidity and bile salts and improve their adherence to intestinal mucosa [84], [99]. In addition, the type of EPS produced can modify the levels of adhesion [102]. EPS are gaining ground in the food industry because they can improve properties as texture, rheology or body of dairy products above all. Moreover, they open a field for new products as low-fat cheeses or skimmed-milk based dairy products [19].

However, *Bifidobacterium* strains have very stringent growth conditions. Some of them are very sensitive to oxygen, their growth in milk is low or limited compared to that of other LAB and they generally do not survive to some processes used in food industry as freeze-drying, for instance [103]. Thus, the technological properties of the strains studied have to be taken into account when searching for a probiotic.

The aim of the present chapter was to characterise the technological and probiotic properties of two EPS-producing *Bifidobacterium* strains isolated from infant faeces, as well as to perform a physicochemical characterisation of their HePS and to test them as prebiotics.

1.2. Materials and Methods

1.2.1. Bacterial strains and culture conditions

Bifidobacterium longum INIA P132 and *Bifidobacterium infantis* INIA P731, isolated from healthy breast-fed infant faeces [104], were selected to be studied on the basis of theirropy phenotype. The commercial probiotic strain *Bifidobacterium animalis* BB12 (Chr. Hansen A/S, Hørshom, Denmark) was used for comparison. All bifidobacteria were routinely cultured on Reinforced Clostridial Medium (RCM) broth (Becton, Dickinson and Company), incubated at 37 °C for 48 h in an anaerobic atmosphere (anaerobiosis generators, BD GasPak™), and conserved at -80 °C upon addition of 10% glycerol.

1.2.2. Amplification by PCR of priming-glycosyltransferase genes

The p-GTF enzymes are involved in the initiation of the synthesis of HePS and can be encoded by different genes in different strains of *Bifidobacterium* [105]. To detect the p-GTF coding genes of the two *Bifidobacterium* strains, their genomic DNA was isolated using the 'Wizard® Genomic DNA Purification kit' (Promega) following the manufacturer's instructions. Two sets of degenerated primers previously designed [105] were used: *cpsD_F4-cpsD_R6* and *rfbP_F5-rfbP_R5*. PCRs were performed with Taq polymerase (Invitrogen) and conditions were: 3 min at 94 °C, 30 cycles of 45 s at 94 °C, 30 s at 60 °C or 56 °C for *rfbP_F5-rfbP_R5* or *cpsD_F4-cpsD_R6* and 50 s at 72 °C, with a 10 min final step at 72 °C. The DNA sequence of the resulting amplicons was determined at Secugen (Madrid, Spain). Homologies of the DNA sequence of the amplicons and the inferred amino acid sequences, respectively, with the genes and proteins deposited in the data banks were searched with Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/) and Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) programs.

1.2.3. EPS isolation and purification

The bifidobacteria were seeded on RCM agar plates, collecting the polymers from the biomass on the plates' surfaces with ultrapure water (1.5 mL/plate) as

previously described [106] with modifications. Briefly, cell suspensions were mixed with one volume of 2 M NaOH and incubated overnight at room temperature (RT) with shaking at 180 rpm. Then, trichloroacetic acid was added at a final concentration of 20% (v/v) and kept 1-2 h at 4 °C under stirring. Supernatants were collected after centrifugation at $18566 \times g$ at 4 °C (20 min) and pH was adjusted to 6.5 by addition of NaOH. Then, the EPS were precipitated with 3 volumes of cold absolute ethanol and incubation overnight at -20 °C. The precipitates were sedimented by centrifugation at $18566 \times g$ at 4 °C for 10 min and washed 3 times with 80% (v/v) cold ethanol. Finally, EPS-preparations were dialysed in 12-14 kDa molecular weight cut-off (MWCO) membranes (Iberlabo) against deionised water, and freeze-dried.

For further reduction of contaminants (DNA, RNA and proteins), the freeze-dried EPS were dissolved (1 mg/mL) in a solution with 50 mM Tris-HCl, 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.5, and kept at 70 °C overnight. To eliminate non-dissolved material, the preparations were centrifuged at $8609 \times g$ for 15 min at RT. Then, DNase I and RNase A (both from Sigma-Aldrich) were added to the supernatants at a final concentration of 2.5 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$, respectively, and enzymatic digestions were performed at 37 °C for 6 h with shaking. Afterwards, the EPS-preparations were deproteinised by: (i) treatment with proteinase K (Sigma) at 30 $\mu\text{g}/\text{mL}$ for 18 h at 37 °C with moderate stirring and (ii) two phenolisation processes. The latter were performed by addition of 1 mL phenol:chloroform:isoamyl alcohol (25:24:1) to each EPS solution, vortex for 7 min, centrifugation at $8609 \times g$ at RT for 5 min and recovery of the upper aqueous phases containing the EPS. Afterwards, the EPS preparations were treated with one volume of chloroform:isoamyl alcohol (24:1), vortexed for 7 min and fractionated as indicated above. Finally, samples were dialysed and freeze-dried. Lyophilised EPS were dissolved in ultrapure water (0.1 mg/mL) and concentration was estimated from the neutral carbohydrate content, determined by the phenol-sulphuric acid method [86] using glucose as standard. The calibration curve (Appendix 1, 1.1) was done with solutions containing 0, 10, 20, 30, 40, 50, 80, 100, 120, 140, 160, 180 and 200 mg/L, prepared by dilution of a standard stock solution of 1 g/L of glucose. For each solution, three 0.5 mL aliquots were mixed with one volume of phenol (5% v/v) and vortexed. Then, 2.5 mL

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sulphuric acid (95.5% v/v) was added, mixtures were vortexed and incubated for 5 min at 100 °C. Finally, tubes were cooled in an ice bath and absorbance was measured at 490 nm ($A_{490\text{nm}}$). The linear regression equation of the standard curve was $A=0.1010C - 0.0062$, where A refers to $A_{490\text{nm}}$ of glucose and C corresponds to the glucose concentration.

Contaminant DNA, RNA and protein contents were determined in EPS suspensions at 1 mg/mL using specific fluorescent staining kits and the Qubit®2.0 fluorometric detection methods (ThermoFisher Scientific).

1.2.4. Detection of EPS by electron microscopy

To detect EPS by transmission electron microscopy (TEM), the bifidobacteria were grown in RCM broth. Aliquots (100 μL) of the cultures were centrifuged (5 min, $15700 \times g$ at 4 °C) and the sediments were resuspended in 100 μL of deionised water. For visualisation, bacterial suspensions (50 μL) were processed as previously described [107], with some modifications. Briefly, glow-discharged carbon-coated Formvar grids were subjected to ionic discharge for 1 min. Then, they were placed facedown over a droplet of each suspension for 15 s, and each grid was removed, washed, blotted briefly with filter paper, negatively stained with 1% (w/v) uranyl acetate, blotted quickly and air-dried. Finally, samples were examined in the Electron Microscopy Facility at the Biological Research Centre (CIB, Madrid, Spain) using a JEOL 1230 microscope operated at 100 Kv.

1.2.5. EPS characterisation

To determine neutral sugars composition, 1-2 mg of sample were weighted in glass vials and hydrolysed with 1 mL of 3 M TFA for 1 h at 120 °C. Tubes were cooled down and 100 μg of *myo*-inositol were added as internal standard. Then, the reaction mixture was evaporated to dryness in order to eliminate the acid, and the monosaccharides were derivatised to alditol acetates [108]. Alditols are obtained by overnight reduction of the aldehyde groups of monosaccharides with a water solution of sodium borohydride (1 mL, 1.5 mg per mg of EPS). Then, the unreacted

sodium borohydride was destroyed by dropping concentrate HCl in the sample solution until neutral or acidic pH. The solution was evaporated to dryness and washed three times to dryness with 2 mL of methanol to remove borates. Acetylation was carried out by addition of 250 μ L of pyridine and 250 μ L of acetic anhydride and incubation at 100 °C for 1 h. Finally, solutions were evaporated to dryness and the samples were re-dissolved in 200 μ L of chloroform and transferred to gas-chromatography vials. The analysis was done in a 7890A instrument (Agilent) coupled with a 5975C mass detector, using a HP5-MS column and a temperature program. The oven was set at 175 °C for 1 min and then, temperature raised at a rate of 2.5 °C/min until 215 °C. Finally, temperature was increased in a 10 °C/min rate to 225 °C and kept for 1.5 min. One microlitre of sample was injected at 275 °C with a 1:50 split. The carrier gas was helium and passed at 1 mL/min. The sugars were identified by comparison of their retention times (Rt) with those previously obtained for commercial monosaccharides, analysed in identical conditions. For quantification, a calibration curve was constructed (Appendix 1, 1.2) from 1 mg/mL working solutions of eight monosaccharides (arabinose, xylose, rhamnose, galactose, glucose, mannose, glucosamine, and galactosamine) in water. Standard mixtures containing 25, 50, 100 and 200 μ g/mL of each monosaccharide, and a constant amount (100 μ g) of the internal standard (*myo*-inositol) were prepared, and the sugars were converted into alditol acetates and analysed by GC-MS as explained above.

The linkage types were determined by a methylation assay as previously described [109]. Briefly, between 1 and 3 mg of sample was completely dissolved in 1 mL of dimethyl sulfoxide (DMSO) under an ultrasounds bath. Then, NaOH pellets (*ca.* 1 pellet for 5 samples) were mashed, added to each sample and kept for 2 h in the ultrasounds bath. Once cooled down, 1 mL of methyl iodide was added in ice for avoiding evaporation, placing the vials again in the ultrasounds bath for around 2 h. The methylated polysaccharide was cleaned by liquid-liquid extraction using water (2.5 mL) and dichloromethane (1.5 mL) and recovered from the organic phase (at the bottom). The extractions were repeated until samples were clean enough and the dichloromethane was finally evaporated to dryness. The methylated EPS was hydrolysed and derivatised as explained for neutral sugars

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analysis, except that reduction was made with deuterated sodium borohydride. Finally, samples were analysed in the same instrument using a temperature program that started at 170 °C for 1 min and then raised to 220 °C in a 2 °C/min rate. Since not commercial standards of partially methylated alditol acetates are available, the compounds were identified from their mass spectra and retention times and quantified as the relative proportion of each peak using peak areas.

The presence of N-acetyl, carboxyl, phosphate or sulphate groups and the α - or β -anomeric configuration of the monosaccharides was assessed by Fourier-transformed infrared (FT-IR) spectroscopy as previously described [110]. First, KBr pellets of the samples were prepared and the spectra were recorded in the range of 4000-700 cm^{-1} .

Finally, the M_w of the EPS was obtained by HPLC-SEC as previously described [110]. The instrument used was a 1100 Series System (Agilent Technologies) with a RI detector. It was equipped with a guard column (PL-aquagel-OH Guard, 8 μm , Agilent Technologies) and a PL aquagel-OH Mixed-H column (8 μm , Agilent Technologies). The column was eluted with 0.1 M NaNO_3 and 0.02% NaN_3 (pH 7), at 35 °C and at a flow rate of 1 mL/min. The sample solutions were prepared at 0.75 mg/mL and 100 μL were injected. Data processing was performed using the Agilent ChemStation with integrated gel permeation chromatography (GPC) data analysis software. A calibration curve was obtained by using dextran standards (1.82×10^2 , 3.78×10^2 , 5.94×10^2 , 1.08×10^3 , 4.44×10^3 , 9.89×10^3 , 4.35×10^4 , 6.67×10^4 , 1.24×10^5 , 1.96×10^5 , 2.76×10^5 , 4.01×10^5 and 1.22×10^6 Da) from Sigma-Aldrich. Samples and standards were filtered through 0.2 μm filters (Chromafil Xtra, Macherey-Nagel) prior to the injection.

1.2.6. THP-1 cell line culture and immunomodulation assay

The human monocytic cell line THP-1, obtained from the CIB cell bank, was used for the immunomodulation assay. The cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 g/mL) at 37 °C in an atmosphere

containing 5% CO₂, and they were sub-cultured every 2 days. First, human monocytic THP-1 cells (10⁶ cells/mL) were differentiated to a macrophage-like state by treatment with 40 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) for 72 h in 12-well cell culture plates (Falcon-Software), which resulted in their inability to proliferate [111]. Differentiated, plastic-adherent cells were washed twice with the same medium without PMA and incubated for 24 h at 37 °C. Then, PMA-THP-1 cells were co-stimulated with 700 ng/mL of the lipopolysaccharide (LPS, Sigma-Aldrich) of *Escherichia coli* O111:B4 and with the purified EPS (100 µg/mL) for 18 h in the same conditions of incubation [112]. Cell-free culture supernatants were collected and stored at -80 °C to analyse the concentration of the secreted pro-inflammatory TNF-α and the anti-inflammatory IL-10 cytokines using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD OptEIA™; BD biosciences) as indicated by the manufacturers. The THP-1 monocytes (un-differentiated cells) and the supplemented medium were used as controls. Each EPS was tested in triplicate in two independent experiments.

1.2.7. Survival to gastrointestinal conditions

Survival to gastric and intestinal conditions was tested based on Haller *et al.* [113] by consecutive exposal of bacterial suspensions to pH 3 (phosphate-buffered saline (PBS) with hydrochloric acid) and to bile salts (1.5g/L, Oxoid). Each step was performed over 1 h at 37 °C and anaerobic atmosphere. Experiments were performed in duplicate and viable cell population variation was determined by plate counting on RCM agar.

1.2.8. Biofilm formation

Cells grown on RCM agar were resuspended in mTSB (Biolife) supplemented with 20 g/L of bacto proteose-peptone (Oxoid). This suspension was used to inoculate (10%) either mTSB or mTSB supplemented with 0.2% oxgall (Oxoid), and each inoculated broth was loaded into the wells of polystyrene microtiter plates (Nunc 167008) and incubated at 37 °C for 24 h under anaerobic conditions. Biofilm

formation was assessed by the crystal violet method [114]. *B. animalis* BB12 was included for comparison purposes. Control wells with non-inoculated broth were used as blanks and negative controls. Each strain and treatment were tested in at least three independent experiments, each with eight biological replicates.

1.2.9. Caco-2 cell culture and adhesion assays

The Caco-2 human enterocyte cell line, obtained from the cell bank at CIB, were grown in MEM-Alpha Medium (Invitrogen), supplemented with 20% (v/v) heat-inactivated fetal bovine serum. The incubation was carried out at 37 °C in an atmosphere containing 5% CO₂. Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest™, Becton Dickinson) at a final concentration of 1.25×10^4 cells/well (1.25×10^5 cells/mL) and grown as monolayers of differentiated cells for 15 days. The culture medium was changed every 2 days. Cell concentrations were determined before adhesion experiments in a counter chamber (Type Neubauer 0.100 mm Tiefe. 2. Depth Profondeur 0.0025 mm²) using a Nikon Eclipse TS 100 microscope.

For adhesion experiments, Caco-2 cell medium was changed for Dulbecco's Modified Eagle medium (DMEM, Invitrogen). Late exponential-phase cultures of the bifidobacteria were diluted in a final volume of 1 mL of DMEM (Invitrogen), to give 1.25×10^5 colony forming units (cfu)/mL and added to Caco-2 cells (ratio 1:1) in a final volume of 0.1 mL per well. After incubation for 1 h at 37 °C and 5% CO₂, un-adhered bacteria were removed by three washes with saline solution. The cell-associated bacteria were detached from the plastic surface by incubating for 10 min at 37 °C with 0.1 mL of 0.05% trypsin-EDTA per well. The detachment reaction was stopped by adding 0.1 mL of saline solution pH 7.0. To determine the number of cell-associated bacteria appropriate dilutions were plated onto RCM plates. All adhesion assays were conducted in triplicate, with two biological replicates in each.

1.2.10. Technological properties

These experiments along with those of the biofilm formation and the survival to GIT conditions were performed by Ángela Bernardo under the supervision of Eva Rodríguez at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, Madrid, Spain).

Survival as frozen cultures was measured after 21 days of storage at -80 °C. The strains were grown in RCM for 48 h at 37 °C in anaerobic conditions and glycerol was added as cryopreservant to a final concentration of 5% (w/v). Viable cell population was determined by plate counting on RCM agar before and after the process.

For freeze-drying survival assay, cells were grown, collected and resuspended in reconstituted skimmed milk (10% w/v; Central Lechera Asturiana) as protective medium, aliquoted in cryotubes and froze at -80 °C for 24 h. Subsequently, aliquots were lyophilised and stored at 5 °C for 21 days. Freeze-dried cells were reconstituted using peptone water and viability was determined by plate counting in RCM agar.

Growth and survival of the bifidobacteria in milk was tested by inoculating the strains in reconstituted skimmed milk (10% w/v) and incubating in anaerobic conditions and 37 °C for 24 h. Change in bifidobacteria levels was assessed by plate counting in RCM agar.

Survival in milk under refrigerated conditions was performed by collecting the cells grown in RCM agar, resuspending them in skimmed milk, and storing the suspensions at 5 °C. Viable cell population was determined by plate counting on RCM agar and checked at 14 and 28 days.

1.2.11. Statistical analysis

The bacterial adhesion to Caco-2 cells was evaluated by two-way analysis of variance (ANOVA). For the experiments of EPS immunomodulation of THP-1 cells

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the SAS 9.4 software (SAS Institute Inc.) was used, applying the T-Student test to assess the significance of the addition of the LPS from *E. coli* O111:B4 and then, the Dunnett's test to evaluate the significance of the differences between samples and controls. Results of survival to gastrointestinal conditions and biofilm formation were subjected to ANOVA analysis using a general linear model and means' comparison was carried out by Tukey's test, performed using SPSS Statistics 22.0 software (IBM Corp.) at the INIA.

1.3. Results and Discussion

1.3.1. Detection of EPS by electron microscopy

B. longum INIA P132 and *B. infantis* INIA P731 were selected for use in a former work because of their mucous and ropy phenotype. This characteristic has been related to the production of EPS [115], [116]. Accordingly, analysis of bifidobacterial cultures by TEM revealed cells with EPS attached to them as well as un-attached EPS (Figure 6A and 6B). Bifidobacteria are pleomorphic, thus, their shape varies depending on the strain and the growth medium when exposed to stress conditions, adopting conventional rod or bifurcated ‘Y’/‘V’ morphologies [53], [58], [117]. As observed in Figures 6C and 6D, the two bifidobacteria analysed can adopt the ‘Y’ morphology.

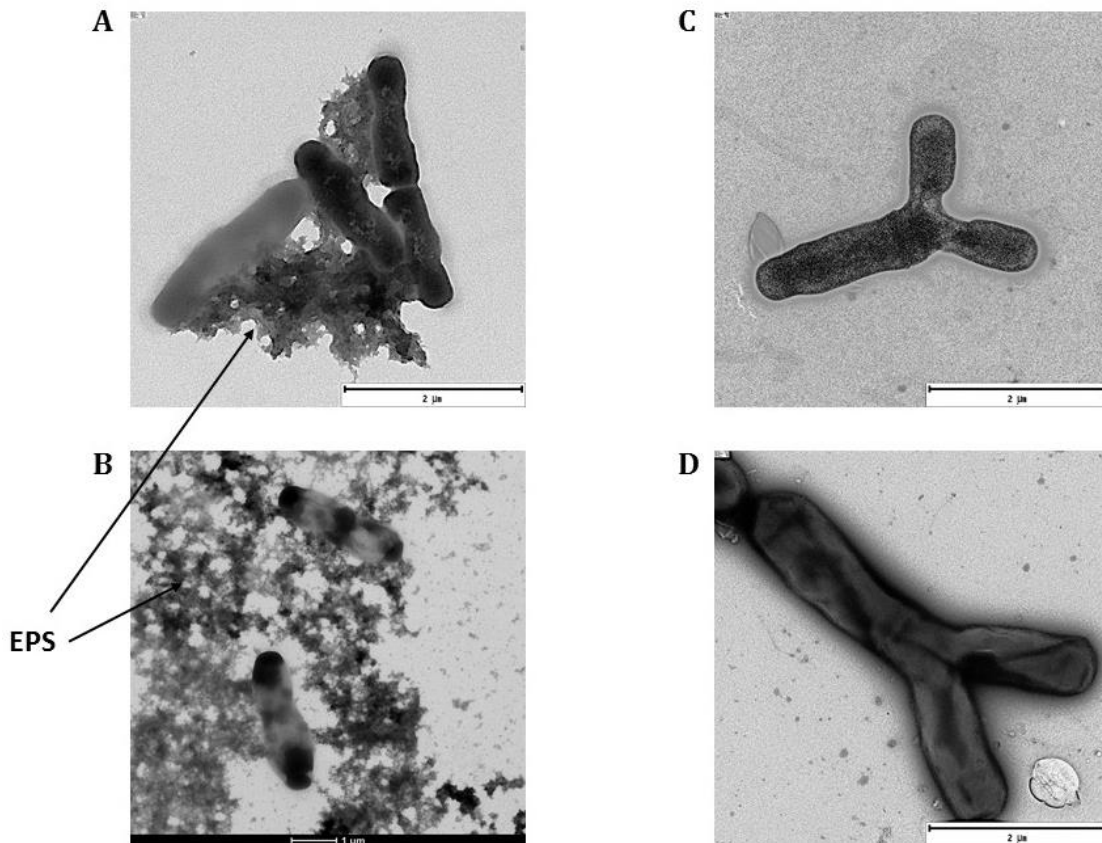


Figure 6. Visualisation of bifidobacteria and their EPS by TEM. *B. infantis* INIA P731 (A) and *B. longum* INIA P132 (B) with their EPS. (C) and (D) show the ‘Y’ morphology of INIA P731 and INIA P132 strains, respectively.

1.3.2. Detection of genes encoding the p-GTF

It has been reported that a p-GTF enzyme catalyses the first step of the synthesis of the *Bifidobacterium* HePS, by transferring a sugar-1-phosphate to a lipophilic carrier molecule embedded in the bacterial membrane [118]. Moreover, two genes included in *eps* clusters, *cpsD* and *rfbP*, which encode putative p-GTF, have been detected in bifidobacteria and are annotated in the data banks as 'galactosyl-transferase' and 'undecaprenyl-phosphate sugar phosphotransferase', respectively. The two proteins only have homology at their C-terminal region, which includes the catalytic domain, and are highly conserved in bifidobacteria, which harbour one or both coding genes depending on the strain. Thus, the differences between the amino acid sequences of the p-GTF could be due to a domain responsible for the sugar specificity of each enzyme, located at their N-terminal regions [53].

The PCR amplification of the 3'-region of the p-GTF coding genes with two different pairs of oligonucleotides produced only high yield of one of the expected amplicons for each strain: the 373 bp DNA fragment of *rfbP* for *B. infantis* INIA P731 and the 301 bp DNA fragment of *cpsD* for *B. longum* INIA P132 (Figure 7A). The determination of the DNA sequence of the specific amplicons and the homology search with the BLAST program confirmed that *B. infantis* INIA P731 harbours a gene with 99%-90% identity to the *rfbP* of several *Bifidobacterium breve* and *B. longum* strains, as well as *B. longum* INIA P132 carries a gene with 100%-96% identity to the *cpsD* of other *B. longum* strains. Moreover, the sequenced region of the genes from *B. infantis* INIA P731 and *B. longum* INIA P132 showed between them an identity of 70.1% indicating that the DNA region encoding the catalytic domain of the p-GTF protein has evolved from a common ancestral gene. Finally, the alignment of the predicted amino acid sequence of the two gene products (Figure 7B) revealed a fragment of the C-terminal region of the proteins highly-conserved in p-GTF (73.9% identity) [100], [105], with a glutamate described as a probable catalytic residue in the p-GTF of *Lactococcus lactis*, and a tyrosine specific for galactosyltransferases [100], [119]. Moreover, a tyrosine in the position 200 has been reported as being a phosphorylation site and necessary for the functionality of the p-GTF [120]. However, the sequences obtained were not long enough to find out whether the Y²⁰⁰ was present or not. These data, together

with the observation of mucoid colonies with ropy phenotype on RCM-agar plates and the visualisation of EPS by TEM, encouraged us to isolate the EPS produced by the two strains.

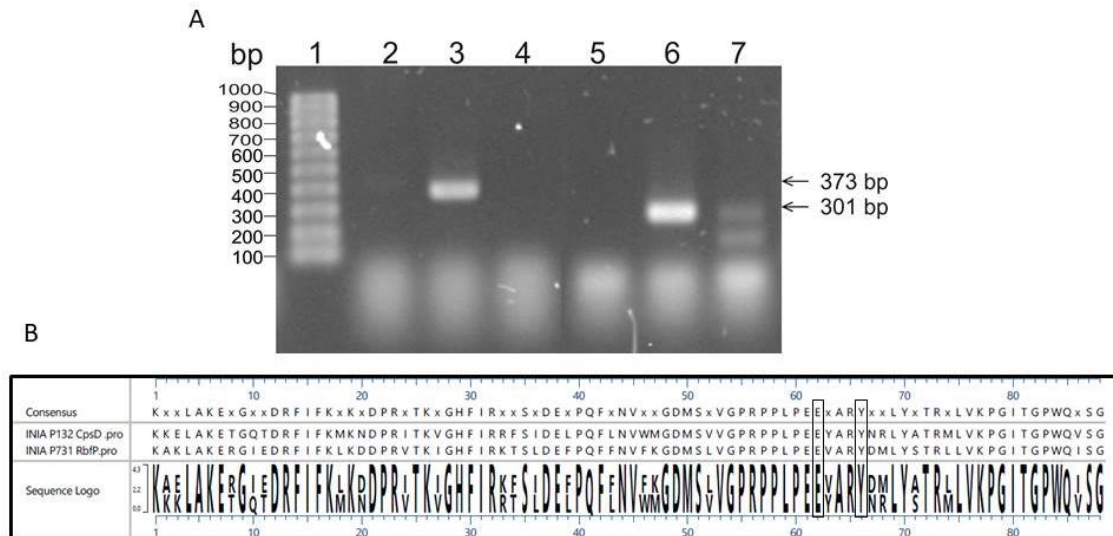


Figure 7. Genetic analysis of EPS production by *Bifidobacterium* strains.

(A) Detection by PCR of *cpsD* and *rfbP* in 1% agarose gel. Lanes: 1, BIORAD Ez Load 100 bp Molecular Ruler; 2, PCR with only *rfbP* F5-R5 primers (negative control); 3, PCR of INIA P731 DNA with *rfbP* F5-R5 primers; 4, PCR of INIA P132 DNA with *rfbP* F5-R5 primers; 5, PCR with only *cpsD* F4-R6 primers (negative control); 6, PCR of INIA P132 DNA with *cpsD* F4-R6 primers; 7, PCR of INIA P731 with *cpsD* F4-R6 primers. (B) Clustal Omega alignment and consensus sequences of the inferred amino acid sequences of CpsD from INIA P132 and RfbP from INIA P731. The boxes highlight the glutamate (E) and tyrosine (Y) residues conserved among p-GTF at the putative catalytic centre.

1.3.3. Isolation and partial characterisation of the EPS

The EPS were separated from biomass harvested from RCM-agar plates, recovering 0.5-0.8 mg and 0.2-0.3 mg of the polymers per plate for *B. longum* INIA P132 and *B. infantis* INIA P731, respectively. These recoveries are within the ranges (0.78-4.34 mg EPS/plate) previously described for other bifidobacteria [121], but lower than the 3.6-3.8 mg/plate reported for *B. longum* NB667 [122]. The crude EPS precipitates contained residual amounts of DNA (0.01-0.03%), RNA (0.026-0.04%) and protein (1.5-2.5%) and, after enzymatic elimination of nucleic acids and deproteinisation these values were further reduced to <0.01%, <0.02% and <1%. Higher protein levels (1.9-8.9%) were reported for EPS preparations from other intestinal *Bifidobacterium* strains [121], [123].

Results and Discussion

Both EPS preparations were partially characterised to analyse their similarities and/or differences. IR spectra (Figure 8A) showed the typical profile of polysaccharides. Absorption bands in the region of 3400, 1400 and 1060 cm^{-1} , correspond to the hydroxyl stretching vibration [122]. Those around 2925-2930 cm^{-1} are due to C-H stretching [124]–[126], and signals in the 1860-1660 cm^{-1} region result from carbonyl stretching [122], [126]. Absorption between 1200-1000 cm^{-1} is due to the stretching vibration of glycosidic linkage (C-O-C) and C-O or C-O-H groups [124], [125]. The fingerprint region ($<1500 \text{ cm}^{-1}$), characteristic of each molecule [126], presented a band around 1020 cm^{-1} . In the anomeric region, 950-700 cm^{-1} [125], [126], a band at 895 cm^{-1} was observed in the spectrum of the crude *B. infantis* INIA P731 EPS, indicating its predominance of β anomers [124], while no bands were observed in this region for the EPS produced by the other strain studied, highlighting a first difference between them.

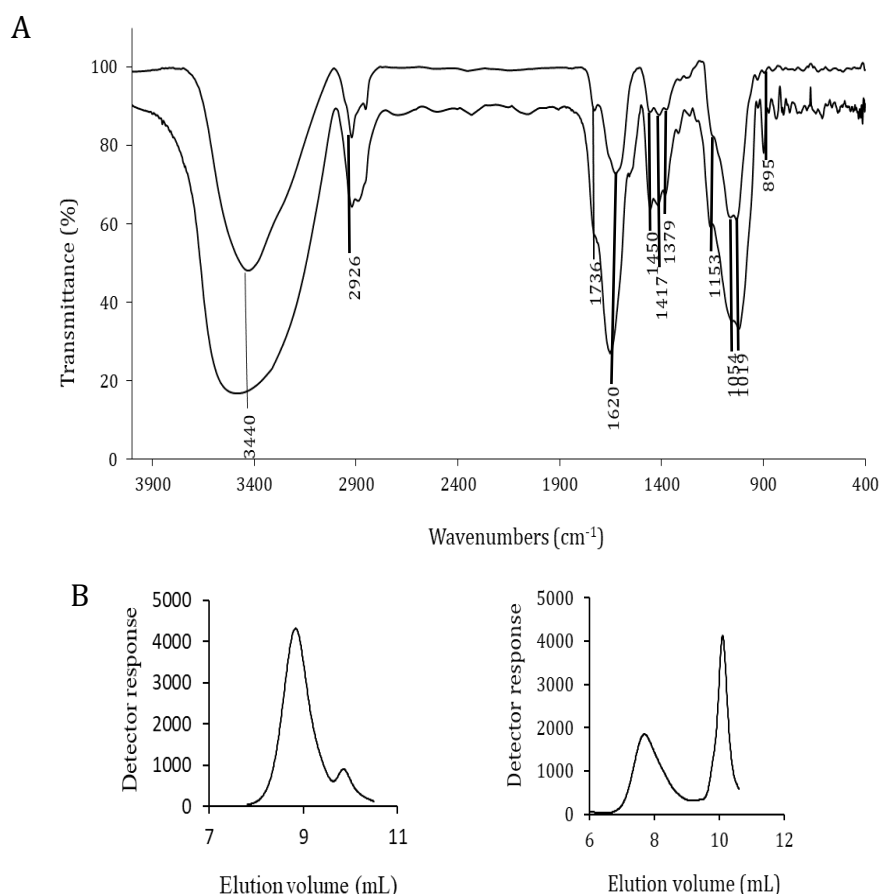


Figure 8. Physicochemical analysis of bifidobacterial EPS preparations. (A) IR spectra. Up, EPS from *B. longum* INIA P132. Down, EPS from *B. infantis* INIA P731. (B) HPLC-SEC elugrams. Left, EPS fractions from *B. infantis* INIA P731. Right, EPS fractions from *B. longum* INIA P132.

Regarding the monosaccharide composition, both strains contained glucose, galactose and rhamnose, although in different proportions: 16:10:1 for *B. longum* INIA P132 and 28:10:8 for *B. infantis* INIA P731. These results correlate with the fact that bifidobacteria synthesise various HePS composed of these three monosaccharides [105], [127]–[129]. As several repeating units have been reported for bifidobacterial HePS, the types of *O*-glycosidic linkages in the two HePS preparations were investigated by methylation analysis. The results, depicted in Table 1, revealed the same seven predominant types of residues in both of them.

Table 1. Linkage types and their proportions (%) in the crude EPS of each strain, deduced from a methylation analysis.

Linkage types	Proportion (%)	
	<i>B. infantis</i> INIA P731	<i>B. longum</i> INIA P132
Rhap-(1-3)	10.3	1.6
Glcp-(1-	14.9	22.1
Galp-(1-4)	0.8	15.2
Glcp-(1-4)	59.3	39.9
Galp-(1-3)	1.6	0.0
Glcp-(1-6)	1.1	2.3
Glcp-(1-4,6)	1.2	3.4
Galp-(1-3,6)	10.0	15.7

The majority of glucose molecules were present as linear-chain units 1,4-linked, and at terminal positions of the side-chains, and also low percentages of 1,6 and (1,4,6)-linked glucopyranose were also detected. The relative proportion of the glucopyranose residues in both samples was similar. On the contrary, as already expected from monosaccharide analysis, the amount of rhamnose and galactose units differed considerably in the two HePS analysed. Rhamnose was exclusively attached to its adjacent residue in the polysaccharide chain by 1,3 linkages, representing almost 20% in the HePS from *B. infantis* INIA P731. Galactose was found in branching points, as a pyranose substituted at *O*-3 and *O*-6, and as linear-

chain units 1,4-linked or 1,5-linked, representing about 30% of the HePS from *B. longum* INIA P132. Unfortunately, this uncertainty could not be resolved with this methodology since both residues are transformed into the same partially methylated alditol acetate: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactose. The presence of either 1,4-Galp and 1,5-Galf in HePS from bifidobacteria has been reported [53]. In addition, the backbone of the extracellular HePS from *B. longum* JLB05 contains α -(1,4)-Galp, and no galactofuranose [127]. Moreover, pyruvic acid was reported in these HePS, and this or another organic acid could be present in the polymers analysed in the current work, since a slight carbonyl band (1736 cm⁻¹), more evident in the sample from *B. longum* INIA P132, was observed in the IR spectra (Figure 8A). Analysis of the HePS preparations from the two bifidobacteria by analytical HPLC-SEC (Figure 8B) indicated that they contained two fractions of different M_w, as reported before by other authors [121], [123], [126]. *B. infantis* INIA P731 contained a major peak of M_w about 1.9 × 10⁵ Da and a smaller one of 1.2 × 10⁴ Da. In *B. longum* INIA P132 the two polysaccharides were in similar amounts, with the peak of high M_w being ca. 1.0 × 10⁶ Da and the other of 1.3 × 10⁵ Da. These M_w are in the range of those of other HePS produced by bifidobacteria [126], [127].

1.3.4. Immunomodulatory activity of HePS from bifidobacteria

The EPS seem to play a role in counteracting the inflammatory effect produced by probiotic strains, thus, preventing them from being attacked by the immune system [130], [131]. Moreover, the EPS' physicochemical differences (negative charges, high or low molecular weight, etc) seem to affect their immunostimulation capacity.

Therefore, we evaluated the immunomodulatory activity of the HePS of the two bifidobacteria *in vitro*. To this end, human PMA-THP-1 macrophages were treated with the HePS preparations and with *E. coli* LPS to induce an inflammatory response. The levels of the TNF- α inflammatory and IL-10 anti-inflammatory cytokines secreted by treated PMA-THP1 were compared to those secreted by

macrophages either untreated or exposed only to LPS, which were used as controls (Figure 9).

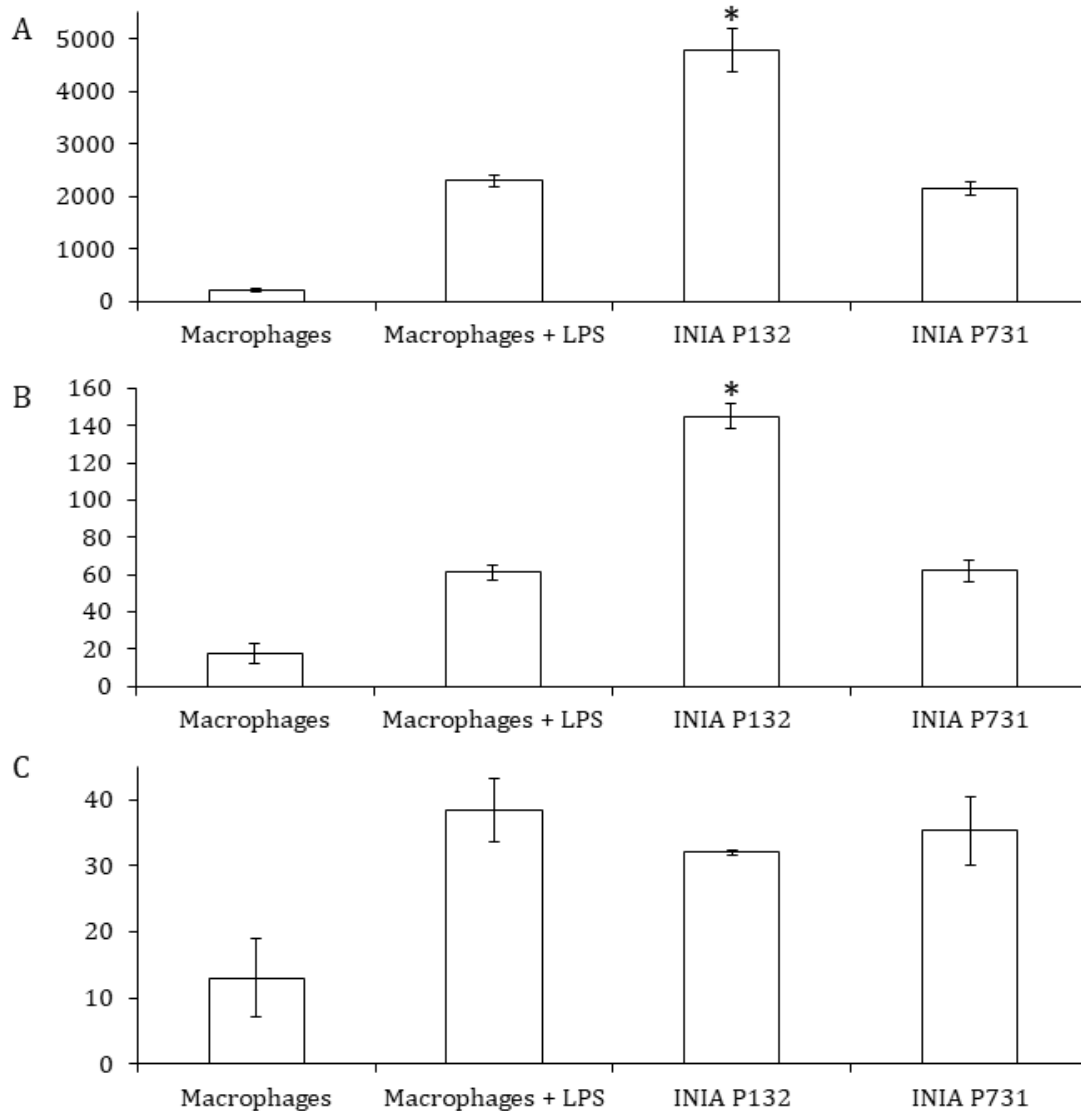


Figure 9. Evaluation of the cytokines production in the supernatants of THP-1-PMA macrophages after the co-treatment with LPS and the HePS produced by bifidobacteria. Levels of TNF- α (A), IL-10 (B) and ratio TNF- α /IL-10 (C) are depicted. Data were analysed by the T-student's t-test. Then, Dunnett's test was employed to assess the significant differences between the samples and the controls. Significances of $p \leq 0.05$ are indicated with *.

All treatments induced the production of both cytokines (Figure 9A and 9B). The levels of the individual cytokines were similar for treatments with LPS alone and in co-treatment with HePS from *B. infantis* INIA P731, but in the presence of the HePS

from *B. longum* INIA P132 a significantly higher production of TNF- α and IL-10 ($p < 0.05$) was observed. The TNF- α /IL-10 ratios calculated for the challenged macrophages were consistent with an inflammatory response (Figure 9C) but, even though the differences among the ratios in the three treatments were not statistically significant, an anti-inflammatory trend of the HePS tested can be observed in this model (Figure 9C). According to Hidalgo-Cantabrana *et al.* [53], high molecular mass EPS show a lower capacity to influence the release of anti- and pro-inflammatory cytokines by macrophages. However, these results could also be explained by an over-stimulation of the eukaryotic cells provoked by the LPS, which can mask the effect of the EPS.

1.3.5. Survival of bifidobacteria to simulated gastrointestinal conditions

The probiotic potential of the two EPS-producing bifidobacteria was first assessed by measuring their survival under *in vitro* GI conditions (Table 2). The two *Bifidobacterium* strains showed good stability to the GI conditions, with reductions around 0.3 log cfu/mL, although their survival was lower than that of *B. animalis* BB12. This tolerance to acidic pH and bile salts exhibited by the two strains suggests their potentiality to survive passage through the GI tract and to reach the intestine at sufficient levels to exert their effects.

1.3.6. Biofilm formation by bifidobacteria

The HePS involved in biofilm formation can affect colonisation and survival of bifidobacteria in the gut [53]. In the present work, *B. infantis* INIA P731, in the absence of oxgall, showed the better result for biofilm formation of the two EPS-producing bifidobacteria (Table 2). Neither of the two strains increased the biofilm formation in the presence of oxgall, as has been described before for certain strains of bifidobacteria and lactobacilli [114], [132] and both new bifidobacterial strains showed lower biofilm formation than *B. animalis* BB12 (Table 2).

1.3.7. Analysis of the adhesion ability of the bifidobacteria

Adherence to human epithelial cells is one of the *in vitro* tests listed in the guideline for the evaluation of probiotics in food [66]. Thus, the adhesion capacity of *B. longum* INIA P132 and *B. infantis* INIA P731 was assessed using the enterocyte-like Caco-2 cell line. Some authors have reported that the presence of EPS in the surface of bacteria has a negative effect in their adhesive properties [106], [133], [134], while others described the production of these polymers as useful for probiotics to interact with eukaryotic cells [12], [42], [135]. In this work, in accordance with the results obtained for the biofilm formation, *B. longum* INIA P132 showed low binding capacity to the enterocytes, whereas adhesion of *B. infantis* INIA P731 to the epithelial intestinal cells was significantly high ($p < 0.05$), exceeding *B. animalis* BB12 levels (Table 2).

Table 2. Probiotic characterisation of bifidobacterial strains.

Strain	¹ Survival to gastrointestinal conditions	² Biofilm formation		³ Adherence to Caco2 cells
		mTSB	mTSB + 0.2% oxgall	
<i>B. longum</i> INIA P132	-0.32 ± 0.27^a	0.02 ± 0.01^{ab}	0.01 ± 0.01^a	1.45 ± 0.29^a
<i>B. infantis</i> INIA P731	-0.31 ± 0.05^a	0.21 ± 0.13^c	0.08 ± 0.06^b	35.91 ± 5.24^b
<i>B. animalis</i> BB12	0.18 ± 0.05^b	0.62 ± 0.14^d	0.71 ± 0.20^e	20.34 ± 5.48^c

¹Survival to gastrointestinal conditions expressed as change in log cfu/mL (mean \pm SD). Values with different superscript differ significantly (Tukey test, $p < 0.05$).

²Biofilm formation expressed as increment of OD_{570nm} (mean \pm SD). Values with different superscript differ significantly (Tukey test, $p < 0.05$).

³Adhesion to Caco-2 cells, expressed as the percentage of cfu (mean \pm standard error (SE)). Values in each column with different superscript differ significantly (Two-way ANOVA, $p \leq 0.05$).

1.3.8. Technological properties of *Bifidobacterium* strains

The development of a functional food containing live probiotic cultures requires that the probiotic strain is able to survive both the manufacturing process and during the shelf life of the product. A preliminary technological characterisation of the two bifidobacterial strains was performed by assessing their survival as frozen or freeze-dried cultures, and their growth and survival in milk under refrigeration conditions. *B. longum* INIA P132 showed better stability as frozen and freeze-dried

Results and Discussion

culture than *B. infantis* INIA P731 (Table 3). Moreover, *B. longum* was able to grow in milk, while the levels of *B. infantis* INIA P731 decreased in these conditions. This strain showed better stability in milk under refrigerated conditions. According to these results, more research is needed to improve the yield and survival of the strains under the manufacturing conditions and to test their behaviour in different food matrices.

Table 3. Technological characteristics of the two *Bifidobacterium* strains.

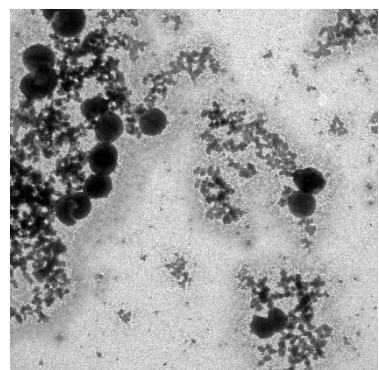
Strain	-80 °C survival (21 days)	Freeze-drying survival (21 days)	Growth in milk (24 h)	4 °C storage (14 days)	4 °C storage (28 days)
<i>B. longum</i> INIA P132	-0.53 ± 0.15	-0.65 ± 0.03	1.52 ± 0.54	-0.92 ± 0.14	-1.70 ± 0.11
<i>B. infantis</i> INIA P731	-1.06 ± 0.15	-1.81 ± 0.26	-0.99 ± 0.21	-0.61 ± 0.10	-1.21 ± 0.05

Data are expressed as change in log cfu/mL-1 (mean ± SD) after each procedure.

1.4. Conclusions

The mucoid aspect and the ropiness of *B. longum* INIA P132 and *B. infantis* INIA P731 colonies suggested them as EPS producers, which was confirmed after obtaining EPS preparations from solid cultures. The polymers were partially characterised as mixtures of HePS with different ratios of rhamnose, galactose and glucose. The same linkage types were detected in both EPS preparations, although their proportions varied. Additionally, the biological activity of these HePS mixtures was studied. Although not conclusive, an anti-inflammatory tendency was observed in experiments performed with PMA-THP-1 macrophages. Alongside to the EPS activities, both strains showed good survival under gastrointestinal conditions, and *B. infantis* INIA P731 displayed higher biofilm formation and adhesion capacity to enterocytes, which is a very remarkable property of probiotic strains. On the other hand, *B. longum* INIA P132 showed better technological suitability. Taken together, these findings suggest that the two strains evaluated, as well as their HePS, might be used as adjuncts in the food industry and give an added value to fermented products.

CHAPTER 2. EPS PRODUCED BY PEDIOCOCCI



2.1. Introduction

Pediococcus is a LAB genus belonging to the family *Lactobacillaceae*. Fifteen different species are included in the genus (Euzeby 'http://www.bacterio.net/pediococcus.html#r', August 2018): *acidilactici*, *argentinus*, *cellicola*, *claussenii*, *damnosus*, *dextrinicus*, *ethanolidurans*, *halophilus*, *inopinatus*, *lolii*, *parvulus*, *pentosaceus*, *siamensis*, *stilesii* and *urinaeequi*. The bacteria included in these species are coccoid cells with spherical, perfectly round and rarely ovoid shape. They appear disposed in pairs, tetrads or clusters, but never in typical chains because their division occurs in two perpendicular directions. The size of their diameters ranges from 0.36 to 1.43 μm depending on the strains or species, but it remains uniform within a culture. They are strong Gram-positives, non-motile, non-spore forming, not encapsulated and catalase negative or possess a pseudocatalase activity, depending on the species. They are facultative anaerobic and homofermenters. Surface colonies are greyish-white, smooth, circular, low convex with entire margins and their optimum temperature and pH for growth are 25-35 °C and around 5.0, respectively [136], [137].

Pediococci usually appear as responsible for the spoil of different beverages because of the production of EPS. The most abundant EPS produced by strains belonging to this genus is a 2-substituted (1,3)- β -D-glucan (β -D-glucan) [138], [139] synthesised by the GTF glycosyltransferase [140], [141]. This HoPS is produced by LAB isolated from alcoholic beverages: *Pediococcus damnosus* or *O. oeni* strains in wines [9], [10], *Lactobacillus* and *Pediococcus* strains in ciders [11], [13], [14], [52], [142] and *Lactobacillus* and *Pediococcus* species in beers [15], [16]. The GTF is encoded by the *gtf* gene, which generally is present in plasmids [40], [41], although a chromosomal location has been detected in *O. oeni* [10], [40].

Generally, for LAB and bifidobacteria, genetic studies and characterisation of the EPS produced by them have shown that these bacteria are able to synthesise more than one EPS. Characterisation of these polymers has revealed that some strains of LAB produce HoPS and HePS [11], [13], [110], [143] or several HePS, as also described for bifidobacteria [122], [144]–[146]. However, simultaneous

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production of HePS and HoPS by a pediococcal strain has not been reported until now.

These polymers often show useful properties, such as improvement of the rheological properties of food and even beneficial effects for health as prebiotics and immunomodulators [145], [147]. For instance, HePS-producing *Streptococcus thermophilus* strains have been widely used in fermentations to obtain dairy products such as cheeses and yoghurts because of the rheological properties their EPS confer to the final products [148]–[151]. The interest in the identification and characterisation of new LAB producing the β -D-glucan is due to the fact that this polymer immunomodulates human macrophages, and its presence increases the adhesion capability to enterocytes of the producing bacteria [12], [42]. Moreover, this EPS has prebiotic activity [152], [153] and confers resistance to both technological and gastrointestinal stresses to lactobacilli upon heterologous expression [154].

Therefore, the characterisation of *Pediococcus ethanolidurans* CUPV141, a novel strain isolated from cider that produces the 2-substituted (1,3)- β -D-glucan and a phosphorylated HePS was performed. As far as we know, this is the first instance of detection of this species in cider and the first characterised example of a *Pediococcus* producing HoPS and HePS. Moreover, the presence of these two EPS seems to play a role for adhesion of the bacteria to biotic surfaces.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth conditions

The EPS-producing *P. ethanolidurans* CUPV141 strain was isolated from ropy cider (containing 6% ethanol and at pH 3.4-3.8) of the Basque Country (Spain) as previously described [14], in Carr-agar medium (3% yeast extract, 2% agar, 1 mL of a 2.2% solution of bromocresol green/L and 2% ethanol) with 50 mg/mL pimarinic acid to avoid the growth of yeasts and moulds. The isogenic, non-ropy strain *P. ethanolidurans* CUPV141NR was generated by chemical mutagenesis with the antibiotic novobiocin (Sigma-Aldrich) at a final concentration of 50 µg/mL. The *Pediococcus parvulus* 2.6 strain [138] was used for comparative purposes. All strains were stored at -80 °C in MRS medium [155] containing 20% glycerol. The experimental assays were performed in MRS medium pH 5.5 at 28 °C and under an atmosphere containing 5% CO₂. For EPS isolation, a semi-defined medium (SMD) was used [138].

2.2.2. Genomic and plasmidic DNA preparations

For genomic DNA extraction, NucleoSpin® Tissue kit (Macherey-Nagel) was used according to the manufacturer's instructions and supplementing the lysis buffer with lysozyme (Sigma-Aldrich) at 30 mg/mL and mutanolysin (Sigma-Aldrich) at 2 U/µL. Once isolated, samples were stored at -20 °C until use.

Total plasmid DNA preparations of *P. ethanolidurans* CUPV141 and CUPV141NR strains were obtained and purified by isopycnic CsCl density gradient to eliminate non-supercoiled forms of the plasmids as previously described [156]. Plasmidic samples were maintained at -80 °C until use.

Fluorescent quantification of the DNA in genomic and plasmidic DNA preparations was determined with a Qubit fluorimeter using the Qubit HS dsDNA Assay Kit (Molecular Probes).

2.2.3. 16S rRNA amplification by PCR

The strain was identified in the laboratory of the UPV/EHU by Ana Isabel Puertas González.

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The template for PCR amplification was genomic DNA from *P. ethanolidurans* CUPV141. The flanking primers 616V and 630R [157] and the internal primer 699R [158] were used to obtain two amplicons (1466 pb and 1009 pb) of the 16S rRNA gene. The PCR products were purified with a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. Subsequent sequencing reactions were performed at Secugen (Madrid, Spain). The DNA sequences obtained were used as templates for the identification of the strain in the public database EZBioCloud [159].

2.2.4. Quantification of the 2-substituted (1,3)- β -D-glucan produced by *P. ethanolidurans* strains

A competition ELISA was performed for the specific detection of the EPS synthesised by strains of *P. ethanolidurans*, based on *Streptococcus pneumoniae* serotype 37 antibody, as previously described [141]. The EPS of *P. parvulus* 2.6 was isolated according to Notararigo *et al.* [109] and immobilised in each well of a 96-Well Nunc-Immuno MicroWell MaxiSorp plate (Thermo Fisher Scientific). EPS quantification was performed using a standard curve of serial dilutions of the purified *P. parvulus* 2.6 EPS dissolved in PBS, generated by the competition for the primary antibody.

To quantify the amount of EPS released to the growth medium or attached to the bacteria, *P. ethanolidurans* strains were grown in MRS medium (1 mL) to a final concentration of 1×10^8 cfu/mL in 1.5 mL Eppendorf tubes. Then, the cultures were centrifuged ($9300 \times g$, 4 °C, 10 min), the supernatants were transferred to another Eppendorf tube and the bacteria were resuspended in 1 mL of PBS pH 7.2. Dilutions of the culture supernatants and of the bacterial suspension in PBS were used for quantification, measuring the optical density (OD) at 415 nm in a microtiter plate reader model 680 (Bio-Rad). The determinations were performed in triplicate.

2.2.5. Characterisation of the *gtf* and *p-gtf* genes

Plasmid DNA from *P. ethanolidurans* CUPV141 strain was used to determine the 1,704 bp nucleotide sequence of the *gtf* gene by the dideoxy method at Secugen as previously described for the *gtf* gene of *P. parvulus* strains [12].

For detection of the priming-GTF coding gene (*p-gtf*), degenerate primers [24] and genomic DNA from the two *P. ethanolidurans* strains were used for DNA amplification. The 20µL reaction mixtures for each sample contained: 1 U of Taq DNA polymerase (Sigma), 1X PCR Buffer (Sigma), 2.5 mM MgCl₂ (Sigma), 0.1 mM dNTP mixture (TaKaRa), 6.25 mM of each primer and 200 ng of total template DNA. Conditions for the PCR were slightly modified from Provencher *et al.* [24] as follows. First, an incubation at 94 °C for 9 min. Then, 5 cycles at 94 °C for 30 s, 62 °C for 31 s and 72 °C for 32 s. Finally, 40 cycles at 94 °C for 30 s, 52 °C for 31 s, and 72 °C for 32 s.

After fractionation of the amplicons in 2.5% agarose gels, they were purified using the 'NucleoSpin® Gel and PCR Clean-up' kit, according to the manufacturer's instructions, and its nucleotide sequence determined at Secugen.

2.2.6. Informatics analysis of genes and inferred protein sequences

The amino acid sequence of the GTF glycosyltransferase was inferred from the nucleotide sequence of the *gtf* gene with EditSeq 15 software (DNASTAR® Lasergene 15). The sequences of the protein, the *gtf* gene and the DNA fragment of *p-gtf* were compared with those from other bacteria, deposited at the National Centre for Biotechnology Information (NCBI) database, using the BLAST. Multiple sequence alignments (MSA) of the sequences obtained in the search were performed with MegAlign Pro 15 software (DNASTAR® Lasergene 15) using the Clustal Omega algorithm. In addition, phylogenetic trees were obtained using Tamura-Nei [160] or Kimura [161] metrics for DNA and protein sequences, respectively.

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Finally, mutations in the amino acid sequence of the GTF glycosyltransferase of each bacterium were gathered in a secondary structure model of the *P. parvulus* 2.6 enzyme, previously inferred using the SOSUI program [40].

2.2.7. Detection of the *gtf* gene by Southern blot hybridisation

Genetic localisation of the *gtf* gene was performed following the protocol previously described [156]. Briefly, samples of plasmid DNA preparations from *Pediococcus* strains and from *E. coli* V517 [162] were fractionated by electrophoresis in a 0.7% agarose gel and DNA molecules were revealed by staining with ethidium bromide at 0.5 µg/mL. The gels' images were obtained with Gel Doc 200 (BioRad). Plasmids from *E. coli* V517 were used to generate a standard curve in which their relative migration in the gel was represented *versus* their known size [162], which was used to determine the molecular mass of the pediococcal plasmids. The plasmidic DNA bands were transferred to a nylon membrane prior to hybridisation. The internal regions of the *gtf* gene used as probes were generated by PCR amplification, in a reaction catalysed by Phusion High Fidelity Polymerase (BioLabs), using as substrate total plasmid DNA preparation of *P. parvulus* 2.6 and the previously described primers GTFSF and GTFSR [40]. Then, the amplicon was labelled with digoxigenin-dUTP by using the DIG high prime DNA labelling and detection starter kit II (Roche). Each DIG-labelled DNA probe (25 ng/mL) was used for hybridisation at 40 °C following the specifications of the kit's supplier. The hybridisation bands were revealed with the chemiluminescent substrate CSPD, detecting the signals with the LAS-3000 imaging system (Fujifilm).

2.2.8. Agglutination immunological analysis

Agglutination tests were performed with *S. pneumoniae* type 37-specific antisera according to the protocol previously described [40]. Briefly, cultures in late-exponential phase (1×10^9 cfu/mL) from the two *Pediococcus* strains were centrifuged at $8609 \times g$ for 47 min at 4 °C and after removal of the supernatants, the sedimented bacteria were concentrated 100-fold by resuspension in PBS pH 7.2 with vigorous vortex. Then, 10 µL of each bacterial suspension were mixed

with 1 μL of anti-type 37 antibody and incubated for 2 h at 4 °C. Afterwards, each sample (4 μL) was observed by phase contrast microscopy using a Leica DM 1000 microscope.

2.2.9. Isolation of EPS

P. ethanolidurans CUPV141 and CUPV141NR were grown in MRS broth for 24 h. Then, the volume of bacteria corresponding to a 2% inoculum was sedimented by centrifugation (18500 $\times g$, 10 min, 4 °C), resuspended in fresh MRS pH 5.5 medium and incubated at 28 °C in a 5% CO₂ atmosphere for 24 h. Finally, a 2% inoculum was sedimented again, in the same conditions, to inoculate the final fermentation in SMD pH 5.5 medium [138]. When the cultures reached the late-exponential phase of growth, bacteria were sedimented by centrifugation of the cultures (18500 $\times g$, 20 min, 4 °C), and the EPS were precipitated from the supernatants, dialysed and freeze-dried as explained in section 1.2.3 in Chapter 1. Lyophilised EPS were dissolved in ultrapure water (0.1 mg/mL) and concentration was estimated from the neutral carbohydrate content, determined by the phenol-sulphuric acid method [86] using glucose as standard, as explained in section 1.2.3, as well.

2.2.10. Partial characterisation of the crude EPS

P. ethanolidurans CUPV141 and CUPV141NR were incubated in MRS broth for 21 h. Then, the volume corresponding to one mL of an OD_{600nm} of 1.0 was centrifuged (9600 $\times g$, 10 min, 4 °C). Supernatants were discarded, and the sediments were resuspended in 0.5 mL of PBS pH 7.2. The similarities or differences in the EPS production between the two *P. ethanolidurans* strains, were visualised by TEM according to Zarour *et al.* [107], as detailed in section 1.2.4 of the chapter 1, with two modifications. The glow discharge process for the hydrophilisation of the grids lasted for *ca.* 30 sec instead of 1 min, and the uranyl acetate water solution was 0.5% (w/v) instead of 1%. Samples were observed at the Microscopy Service of the UPV/EHU.

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Monosaccharide composition of the polymers, as well as methylation analysis for the elucidation of the *O*-glycosidic linkages involved in the structure of the EPS were developed following the protocols described in section 1.2.4. The calibration curves for the monosaccharide type elucidation were performed as explained in section 1.2.5 and are depicted in the Appendix 1, section 1.2.

Finally, ¹H NMR analysis of the EPS produced by the ropy strain was performed as previously described [138] at the UPV/EHU NMR Service (SGIker).

2.2.11. Adhesion properties

2.2.11.1. Self-aggregation assay

P. ethanolidurans CUPV141 and CUPV141NR strains were grown in MRS medium pH 5.5 (1% inoculum) for 15 h. Then, the volume corresponding to 1×10^8 cfu/mL was centrifuged ($12000 \times g$, 10 min, 4 °C) and after removal of the supernatant, 1 mL of fresh MRS pH 5.5 medium was added. Two tubes of each strain were incubated at 28 °C. Samples were recovered at 5 h and 24 h (one tube of each strain for each time) as follows: the tubes were gently centrifuged ($5000 \times g$, 3 min, 4 °C), and after removal of the supernatants the bacteria were carefully resuspended in 50 µL PBS pH 7.2. Aliquots of 5 µL of this suspension were visualised by phase contrast microscopy using a Leica DM 1000 microscope.

2.2.11.2. Bacterial adhesion to Caco-2 cell

The experiments were performed following the protocol detailed in section 1.2.9 in Chapter 1. The sole modification was that the bacteria to Caco-2 cells ratio was 10:1.

2.3. Results and Discussion

2.3.1. *P. ethanolidurans* CUPV141 produces a HoPS

The mucosal (ropy) phenotype of some bacteria is related to the production of EPS [116], and among others, we have previously isolated the 2-substituted (1,3)- β -D-glucan-producing *P. parvulus* 2.6 strain from cider due to its ropy phenotype [142]. In the search for novel β -D-glucan-producing bacteria, the CUPV141 strain was isolated from a ropy cider and selected by its mucosal phenotype upon growth in a medium containing glucose. Determination of the nucleotide sequence of the 16S rRNA coding gene (accession number in GenBank: MH298647, Appendix 2, 2.1) identified this strain as *P. ethanolidurans* and, as far as we know, this is the first instance of isolation of this species from a ropy cider. A specific ELISA method developed in our group [141] was used to test and quantify the production of the 2-substituted (1,3)- β -D-glucan by *P. ethanolidurans* CUPV141 cultures ($OD_{600nm}=1.0$), which amounted to 59.8 ± 4.8 mg/L. Afterwards, we proceeded to characterise the genetic determinant responsible for the β -D-glucan synthesis, encoding the GTF enzyme. Previously designed oligonucleotides [40] were used to amplify the *gtf* gene of CUPV141, and to determine its nucleotide sequence (accession number in GenBank: MH028492, Appendix 2, 2.2), which was compared to those of the *gtf* genes from GenBank at the NCBI site. The BLASTn analysis revealed a 99% identity of the CUPV141 *gtf* with its homologues from *Lactobacillus suebicus* CUPV221, *Lactobacillus diolivorans* G77 (CUPV218) and *P. parvulus* CUPV22, CUPV1 and 2.6 strains isolated from Spanish cider and from *P. damnosus* IOEB8801 isolated from French wine, showing that these genes have evolved from a common ancestor. Thirteen *gtf* nucleotide sequences encoded by several LAB, belonging to the genera *Pediococcus*, *Lactobacillus* and *Oenococcus*, were compared to that of CUPV141 strain to obtain the MSA depicted in Appendix 3, section 3.1, and the phylogenetic unrooted tree shown in Figure 10A. According to the phylogenetic tree, the *gtf* gene is highly conserved among the species and genera studied, although the MSA showed some nucleotide changes. Also, a high identity was observed between the *gtf* genes of *P. ethanolidurans* CUPV141 and *P. parvulus* 2.6, the reference bacterium for this gene. Only four changes, located at positions 86, 217, 1291 and 1524, differentiate these strains. The last was a silent mutation and the other three resulted in changes of the GTF of *P. parvulus* 2.6 (Trp29Leu,

Results and Discussion

Leu73Phe and His43Tyr). Thus, two divisions can be made according to the origin from which the species were isolated. The bacteria isolated from cider and *P. parvulus* IOEB8801 (from wine) are grouped together. All of them contain a plasmid harbouring the *gtf* gene, except *L. suebicus* CUPV221, for which the location of the gene has not been established. The second division mainly grouped isolates from beer, besides two *O. oeni* isolated from champagne and cider, both described as having the *gtf* gene at a chromosomal localisation. Taking into consideration this classification, there are also some mutations to emphasise. For instance, those having changes at positions 272, 1524 or 1548, where the majority of *gtf* genes of bacteria isolated from cider harbour two cytosines and one adenine, while the genes of beer isolates carry two thymines and one guanine, respectively. Moreover, the mutation in position 272 (a change Ala91Val) only occurred in the GTF of the isolates from cider. The other two were silent mutations. Although the physiological role of the 2-substituted (1,3)- β -D-glucan production is unknown, the high conservation of the *gtf* gene could be due to a bacterial adaptation to the alcoholic environment of the different beverages. The plasmidic localisation of the gene in the *Pediococcus* and *Lactobacillus* strains suggests a horizontal transfer of the gene, which might have conferred an evolutionary advantage.

The *P. ethanolidurans* CUPV141 *gtf* gene was translated using EditSeq 15 software and used as template for a BLASTp search. Most of the high identity hits coincided with those detected for the GTF enzymes, but the search also revealed identities, not detected at the nucleotide level, such as the GTF from *Propionibacterium freundenreichii* (33%) or the Tts glycosyltransferase of *S. pneumoniae* (36%). These results indicate a convergent functional evolution to yield glycosyltransferases encoded from unrelated genes. Similar results were previously reported from comparison of the amino acid sequence of *S. pneumoniae* Tts with those of the enzymes from *P. freundenreichii* (52% identity and 72% similarity) and *P. parvulus* 2.6, *P. damnosus* IOEB8801 and *O. oeni* I4 (32% identity and 51% similarity among all of them) [10], [40], [163].

Moreover, the amino acid sequences of CUPV141 and thirteen glycosyltransferases, encoded by related genes (Figure 10A) were aligned

(Appendix 3, 3.2). A phylogenetic tree was also generated (Figure 10B) disclosing a high degree of conservation, with small evolutionary distances among the GTF of different LAB species. Again, the species clustered according to the source from which they were isolated, with a clear grouping of the isolates from cider and beer, and high similarities in the active centre of all the enzymes compared.

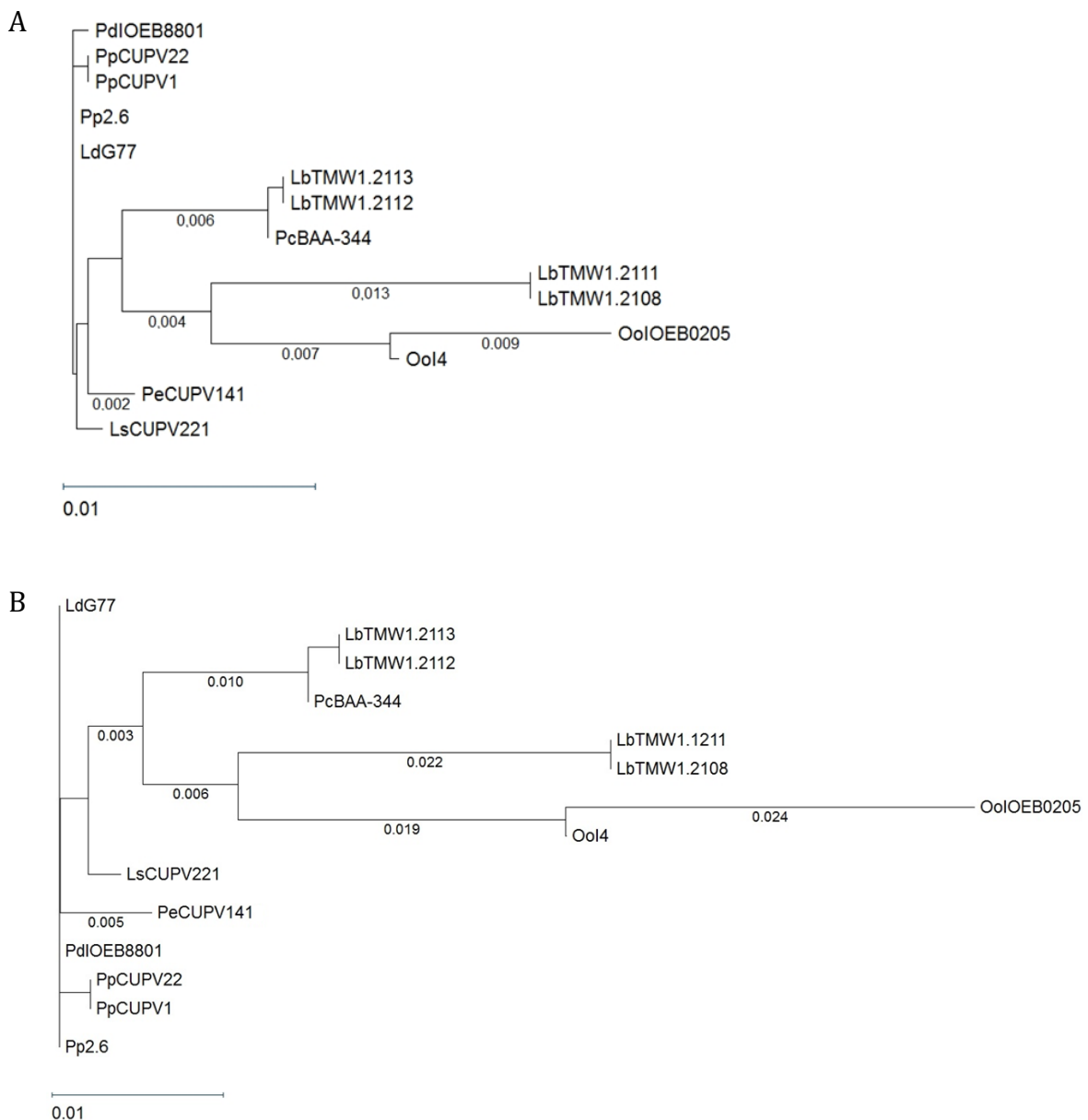


Figure 10. Phylogenetic trees pertaining to (A) the *gtf* genes and (B) the GTF protein of different lactic acid bacteria.

The trees were obtained with the Tamura-Nei (1993) and Kimura metrics, for DNA and proteins, respectively, from an MSA generated with the Clustal Omega algorithm. Complete names and details of the strains are depicted in Table 4.

Table 4. Bacterial strains used for the *gtf* MSA and their codes.

Code	Strain	<i>gtf</i> gene location	Source of isolation	Reference
<i>Pediococcus</i>				
PeCUPV141	<i>P. ethanolidurans</i> CUPV141	Plasmid pPE3		This work
Pp2.6	<i>P. parvulus</i> 2.6	Plasmid pPP2	Cider	[40]
PpCUPV1	<i>P. parvulus</i> CUPV1	Plasmid		[12]
PpCUPV22	<i>P. parvulus</i> CUPV22	Plasmid		
PdIOEB8801	<i>P. damnosus</i> IOEB8801	Plasmid pF8801	Red wine	[164]
PcBAA-344	<i>P. clausenii</i> ATCC BAA-344	Plasmid	Beer	[165]
<i>Oenococcus</i>				
OoI4	<i>O. oeni</i> I4	Chromosome	Cider	[40]
OoIOEB0205	<i>O. oeni</i> IOEB205	Chromosome	Champagne	[10]
<i>Lactobacillus</i>				
LsCUPV221	<i>L. suebicus</i> CUPV221	Unknown	Cider	[12]
LdG77	<i>L. diolivorans</i> G77 (CUPV218)	Plasmid pLD1		[40]
LbTMV1.2108	<i>L. brevis</i> TMV1.2108	Plasmid pl12108-6	Beer	[15]
LbTMV1.2111	<i>L. brevis</i> TMV1.2111	Plasmid pl12111-5		
LbTMV1.2112	<i>L. brevis</i> TMV1.2112	Plasmid pl12112-4		
LbTMV1.2113	<i>L. brevis</i> TMV1.2113	Plasmid pl12113-4		

In addition, the differences in amino acids of the fourteen GTF glycosyltransferases were assembled (Figure 11) in a previous topological prediction of the GTF

glycosyltransferase of *P. parvulus* 2.6 and *L. diolivorans* G77 (CUPV218) [40]. According to this model, the translated polypeptide seems to have four transmembrane regions at the C-terminal domain and two more at its N-terminus flanking the catalytic domain. This suggests that the enzyme synthesises the EPS in the cytosol and that the active protein could be an oligomer of the polypeptide, which could form a membrane pore through which the polymer could be secreted to the environment.

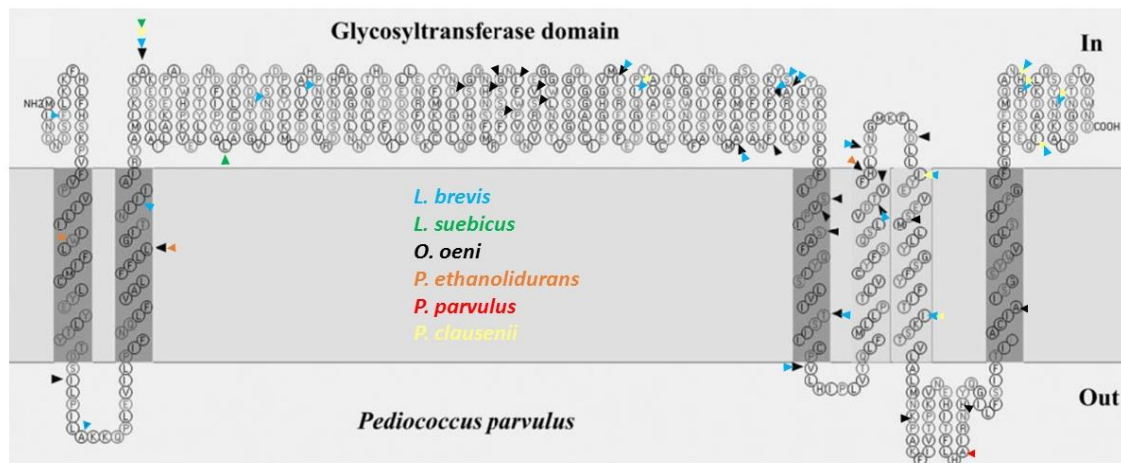


Figure 11. Mutations of different LAB in the protein sequence of the GTF, pertaining to the secondary structure predicted for *P. parvulus* 2.6 and *L. diolivorans* G77 (CUPV218). The prediction was performed using the SOSUI program (<http://harrier.nagahama-bio.ac.jp/sosui/>). Each coloured arrowhead represents a bacterial species: black for *O. oeni*, blue for *L. brevis*, green for *L. suebicus*, yellow for *Pedococcus clausenii*, orange for *P. ethanolidurans* and red for *P. parvulus*.

The alignment (Appendix 3, 3.2) and superimposition (Figure 11) of the amino acid sequences revealed that the main differences are located at the transmembrane regions, which could be explained as an adaptation for optimal insertion into the membrane of each particular bacterium. Some variations (N219D, N233H, A241S, A244G, V246I, N256S, A257V, M288I and T337A) were also observed in the sequence of the active centre of the proteins, especially in *O. oeni* IOEB0205, but none of them affected the aspartic acid residues (Asp143, Asp198 and Asp200 or Asp295) that seem to constitute the essential catalytic tetrad [85]. Among the mutations detected in the enzymes of the *L. brevis* strains some of them are located at the catalytic domain (K136N, V159I, M288I and

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M299I), but none of them should affect the activity of the enzyme. The protein from *L. suebicus* CUPV221 presents one change in the amino acid sequence, I126L, at the beginning of the predicted active centre. Also, as reported before [12], *P. parvulus* CUPV1 and CUPV22 strains only differ from the protein of *P. parvulus* 2.6 in one amino acid (T489A), which is located at the loop between the fifth and sixth predicted transmembrane regions. Finally, the four nucleotide mutations present in CUPV141 *gft* gene, which resulted in three changes in the encoded polypeptide, are highlighted in orange in Figure 11 and were located at the first, second and fourth predicted transmembrane regions, respectively.

2.3.2. Isolation and partial characterisation of the EPS from *P. ethanolidurans* CUPV141

Culture media for routine growth often contain components that interfere with the quantification of the EPS released to the medium by bacteria, i.e. MRS medium [155]. For this reason, SMD medium was used for the isolation of the EPS produced by CUPV141. As reported before [166], the production of EPS can be affected by the growth conditions, as well as by the growth medium. Therefore, the bacterial strain was cultivated for 48 h at two different pHs in SMD medium, giving a slightly higher production of EPS when the medium was adjusted to pH 5.5 (58.9 ± 2.2 mg/L) rather than pH 4.8 (53.5 ± 1.2 mg/L). Thus, for the subsequent isolation of EPS from the supernatants, both *Pediococcus* strains were grown in SMD medium at pH 5.5 for 48 h at 28 °C in an atmosphere containing 5% CO₂.

The yield of EPS recovered from the supernatant of CUPV141 was 69 mg per litre of culture. Among the cider isolates, *P. parvulus* CUPV1 and *L. suebicus* CUPV221 produced similar amounts of EPS, while *P. parvulus* 2.6 or *P. parvulus* CUPV22 have been reported to produce 193 and 243 mg per litre, respectively [52].

In order to elucidate the partial chemical structure of the EPS, several analyses were carried out. The polymer released to the medium by CUPV141 contained glucose as the major monosaccharide, and small amounts of galactose and galactosamine were also detected (less than 5%). In addition, a peak was identified

as glycerol-3-phosphate using the NIST library, which is an uncommon component of EPS from *Pediococcus* strains.

Methylation analysis gave evidence of three main units of partially methylated alditol acetates corresponding to terminal, 3-*O*-substituted and 2,3-di-*O*-substituted glucopyranose in relative proportions 1:1:1. In addition, the ^1H NMR spectrum of the EPS (Figure 12) showed the signals reported for the 2-substituted (1,3)- β -D-glucan of *P. parvulus* 2.6 [138], which confirmed that *P. ethanolidurans* CUPV141 releases this β -D-glucan as the major extracellular polysaccharide. However, other minor components were identified in this EPS: (1,2)-galactopyranose, (1,6) and (1,3,6)-glucopyranose, and (1,4)-glucosamine, suggesting that this isolate produced also a HePS. To the best of our knowledge, the production of both a HoPS and a HePS by *Pediococcus* strains has not been previously reported.

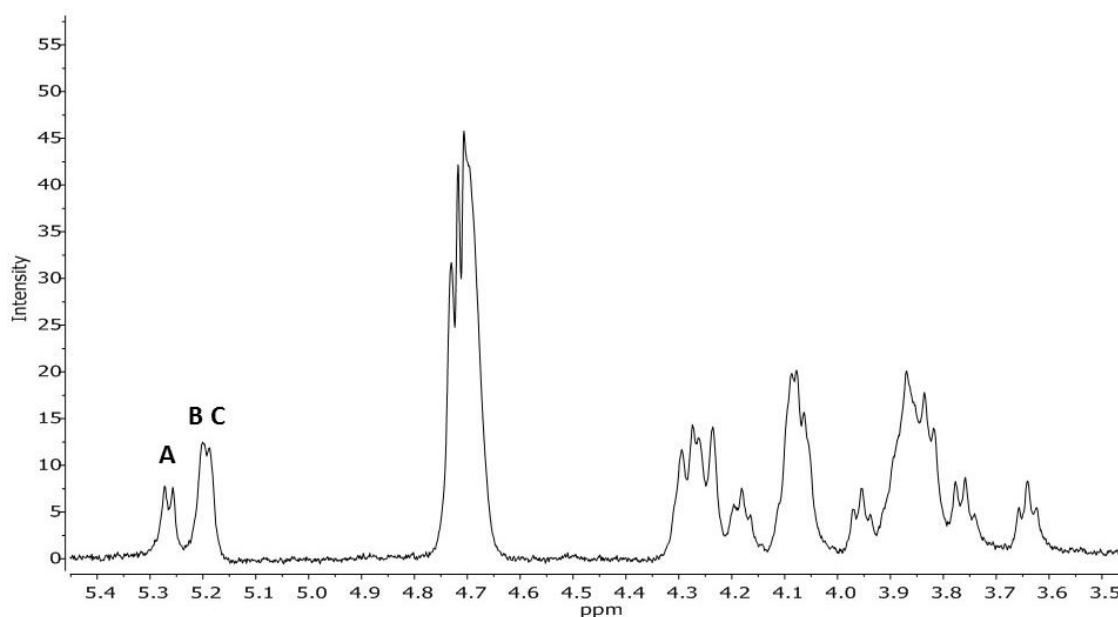


Figure 12. ^1H RMN spectra of CUPV141 EPS recovered from the supernatant.

A, B and C signals correspond to the anomeric protons of terminal β -D-glucopyranose unit, 3-linked- β -D-glucopyranose residue and 2,3-linked- β -D-glucopyranose unit, respectively, as described in Dueñas *et al.* (1997) for the 2-substituted (1,3)- β -D-glucan produced by *P. parvulus* 2.6.

2.3.3. The *P. ethanolidurans* CUPV141NR mutant strain

Chemical mutagenesis of *P. parvulus* 2.6 resulted in the generation of the 2.6NR isogenic strain that did not produce β -D-glucan [142]. Therefore, with the aim of abolishing the production of the HoPS for further studies on the biological activity of the EPS, and for a better characterisation of the HePS synthesised, *P. ethanolidurans* CUPV141 was subjected to chemical mutagenesis and the isogenic non-ropy CUPV141NR strain was generated. The Tts glycosyltransferase of *S. pneumoniae* serotype 37, which is homologous to the GTF of *P. parvulus* 2.6 and *P. ethanolidurans* CUPV141, synthesises a capsular HoPS [167] very similar to the β -D-glucan produced by the pediococcal enzyme. Thus, anti-serotype 37 antibodies are able to agglutinate 2-substituted (1,3)- β -D-glucan-producing bacteria [40], [167]. Therefore, an evaluation of the HoPS production of the mutant and the parental strain by an agglutination immunoassay with anti-serotype 37 antibodies was performed. The results revealed a clear difference between the aggregation capabilities of the two strains, showing, after a 24 h-incubation period, that *P. ethanolidurans* CUPV141 formed huge aggregates in the presence of the antibodies, while CUPV141NR strain produced small aggregates (Figure 13).

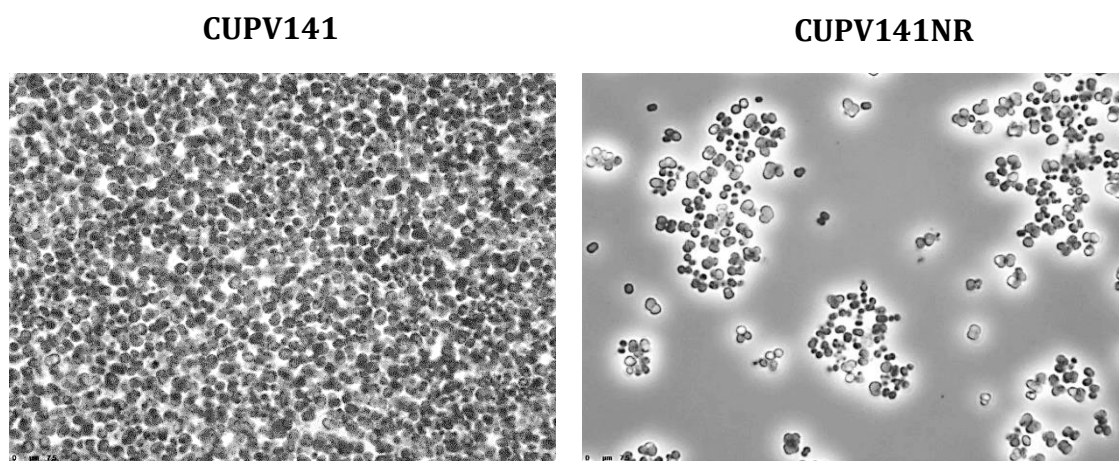


Figure 13. Immunoagglutination in the presence of anti-serotype 37 antibody of the (1,3)(1,2)- β -D-glucan producing *P. ethanolidurans* CUPV141 and CUPV141NR.

These results suggested that the mutant strain still produced the β -D-glucan, although at very low levels. Specific quantification of the 2-substituted (1,3)- β -D-

glucan with the ELISA immunoassay using anti-serotype 37 antibodies confirmed that, at an $OD_{600nm}=1.0$, the non-ropy CUPV141NR strain released 0.096 ± 0.002 mg/L of β -glucan to the supernatant, whereas the wild-type CUPV141 strain produced 42.40 ± 0.05 mg/L.

The recovery of EPS from the supernatant of CUPV141NR yielded 29 mg per litre of culture, a quantity much lower than that produced by the ropy strain. Acid hydrolysis of the EPS released glucose, galactose and glucosamine in a molar ratio 2.4:1:0.9, and the GC-MS peak of glycerol-3-phosphate was observed again. The main linkage types in the EPS, deduced from a methylation assay, revealed a polymer structurally different from the major β -glucan produced by the ropy strain, but with the same components detected in minor amounts in that preparation, namely: *O*-2 substituted galactopyranose, terminal glucopyranose, *O*-6, *O*-2,6 and *O*-3,6 substituted glucose, and *O*-4 substituted glucosamine. The proportion of terminal residues was far lower than that expected from the amount of branching points, which suggests that the glycerol 3-phosphate units detected in the hydrolysates may occupy terminal positions in the side chains of this branched polymer. Further analyses are needed to determine the structure of this polymer, but the current data confirm that *P. ethanolidurans* CUPV141 synthesises and secretes at least two polysaccharides: the 2-substituted (1,3)- β -D-glucan and a HePS with glycerol-3-phosphate. This is the first instance of a *Pediococcus* strain producing both a HoPS and a HePS, and the first report of a phosphorylated EPS in this genus, although other HePS with phosphorylated glycerol have been reported in *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL 1073R-1 [168], *Lactobacillus plantarum* EP56 [169] and *Lactobacillus johnsonii* FI9785 [170].

2.3.4. Detection of the plasmidic location of *P. ethanolidurans* *gtf* gene

The *gtf* gene of *P. parvulus* 2.6 is located in the pPP2 plasmid, which is not present in the 2.6NR strain [40], [142], [156]. Thus, by homology, the *gtf* gene in *P. ethanolidurans* CUPV141 could be located in a plasmid, and the reason for the non-ropy phenotype of the isogenic CUPV141NR strain could be the loss of this plasmid. To confirm this hypothesis, total plasmid DNA preparations from *P.*

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ethanolidurans CUPV141, CUPV141NR and *P. parvulus* 2.6 were purified by fractionation in a CsCl gradient to eliminate open circles and linear forms of the plasmids. Then, the purified plasmid DNA preparations were fractionated in an agarose gel (Figure 14A). As expected, three bands were detected in the preparation of *P. parvulus* 2.6 corresponding to the previously identified pPP1, pPP2 and pPP3 plasmids with molecular weights of 39.1 kbp, 24.5 kbp and 12.7 kbp, respectively ([156] and M_w inferred with the standard curve in Appendix 1, 1.3). A different plasmidic pattern was detected in the *P. ethanolidurans* CUPV141 DNA preparation, including four bands, which should correspond to plasmids named pPE1, pPE2, pPE3 and pPE4 with an inferred molecular weight of 45.6 kbp, 40.2 kbp, 34.4 kbp and 33.4 kbp, respectively (Appendix 1, 1.3). Only three bands were observed in the preparations of the *P. ethanolidurans* CUPV141NR strain, which lacked the pPE3 plasmid. Southern blot hybridisation gave evidence of the presence of the *gtf* gene in *P. parvulus* 2.6 and in the *P. ethanolidurans* wild-type strain, and not in the mutant (Figure 14A). Moreover, the hybridisation bands revealed that pPE3 harbours the *gtf* gene in CUPV141, whose molecular weight differs from that of the pPP2 *gtf*-carrier plasmid in *P. parvulus* 2.6. The non-detection of *gtf* in the CUPV141NR strain correlated with its non-ropy phenotype; however, as stated above, the immunodetection and specific quantification of the (1,3)- β -D-glucan indicated that this bacterium produces low levels of the HoPS. This could be due to the presence of pPE3 in CUPV141NR with a low copy number, undetectable by Southern blot hybridisation. Thus, plasmidic DNA from both *Pediococcus* strains was used for the detection of the *gtf* gene by the more sensitive PCR amplification method. The reaction products were analysed in an agarose gel (Figure 14B), revealing that the expected amplicon of 1.7 kbp had been generated with both plasmidic DNA preparations. However, the intensity of the band was very weak in the CUPV141NR DNA preparation even though a 5-fold higher reaction volume, compared to that of CUPV141 DNA, was loaded in the gel (Figure 14B). Consequently, the overall results support a drastic decrease of the copy number of pPE3 as a consequence of the novobiocin treatment of CUPV141 to generate the CUPV141NR strain.

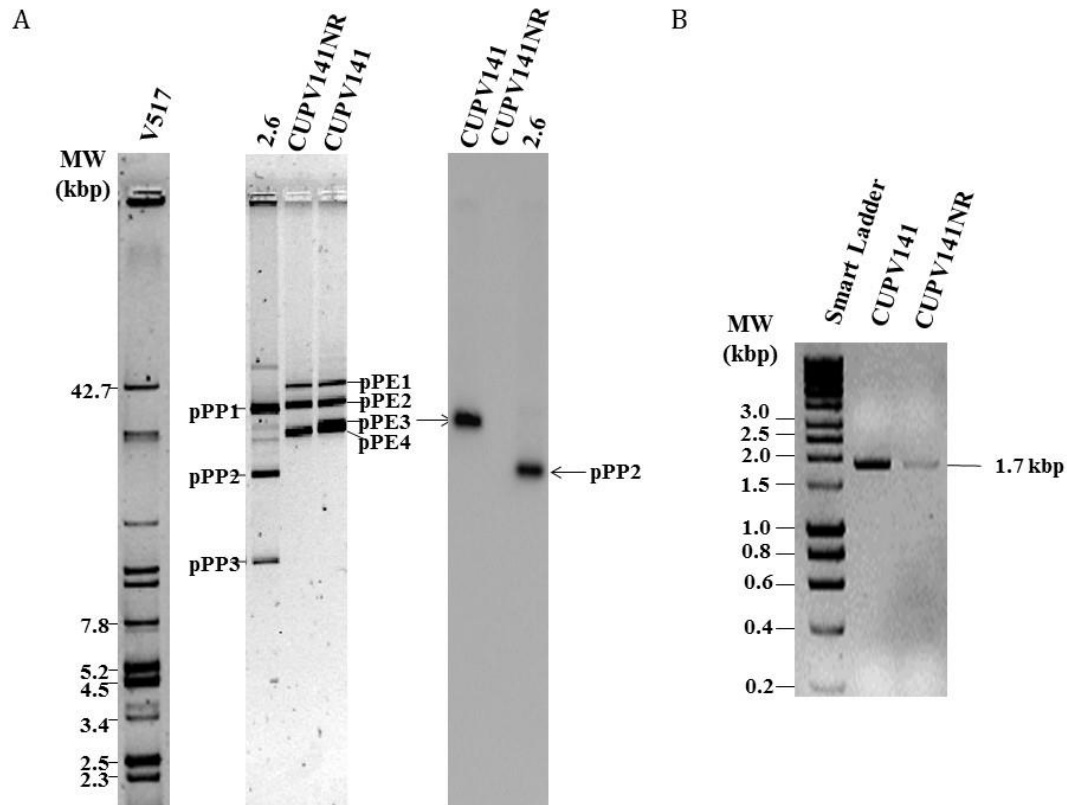


Figure 14. Detection of plasmids of *P. ethanolidurans* CUPV141 and CUPV141NR and of *P. parvulus* 2.6.

(A) Detection of the *gtf* gene by Southern blot hybridisation. Left, analysis in a 0.7% agarose gel of plasmids preparations of LAB strains and of *E. coli* V517. Right, hybridised membrane of samples transferred from the agarose gel. (B) Analysis in 0.7% agarose gel of *gtf* PCR amplicons obtained with genomic DNA from CUPV141 and CUPV141NR strains. Smart Ladder, molecular weight standard.

2.3.5. Genetic determinant of the initiation of HePS synthesis in *Pediococcus* strains

HePS are synthesised by a more complex molecular mechanism than HoPS, which requires the action of several proteins. It is known that the first enzyme in the process is the p-GTF, which transfers the first phospho-sugar residue from an activated nucleotide sugar to the undecaprenyl phosphate-lipid carrier embedded in the membrane [171]. The p-GTF from *O. oeni* has been recently characterised [172], but no studies in *P. ethanolidurans* have been performed until now. Thus, the detection of the p-GTF coding gene was approached by PCR amplification of the highly conserved C-terminal sugar transferase domain of the enzyme, using degenerate primers previously described [24]. The nucleotide sequence of the

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obtained fragments (Appendix 2, 2.3) was the same for the ropy and non-ropy strains. Furthermore, BLASTn analysis revealed 96%, 85% and 74% identities with regions of genes encoding putative proteins annotated as a sugar transferase of *Lactobacillus sanfranciscensis* TMV1.1304, a glycosylphosphotransferase of *Vagococcus lutrae* MIS7, and a p-GTF of *L. plantarum* 26.1, respectively. These nucleotide sequences were aligned using the Clustal Omega algorithm (Appendix 3, 3.3) and a phylogenetic tree was also obtained (Figure 15). In addition, the MSA included a DNA sequence of a gene encoding a putative undecaprenyl-phosphate galactose phosphotransferase from the genome of *P. parvulus* 2.6 [173], and the partial DNA sequences of the *p-gtf* genes from different bacteria isolated from cider in a previous work [11].

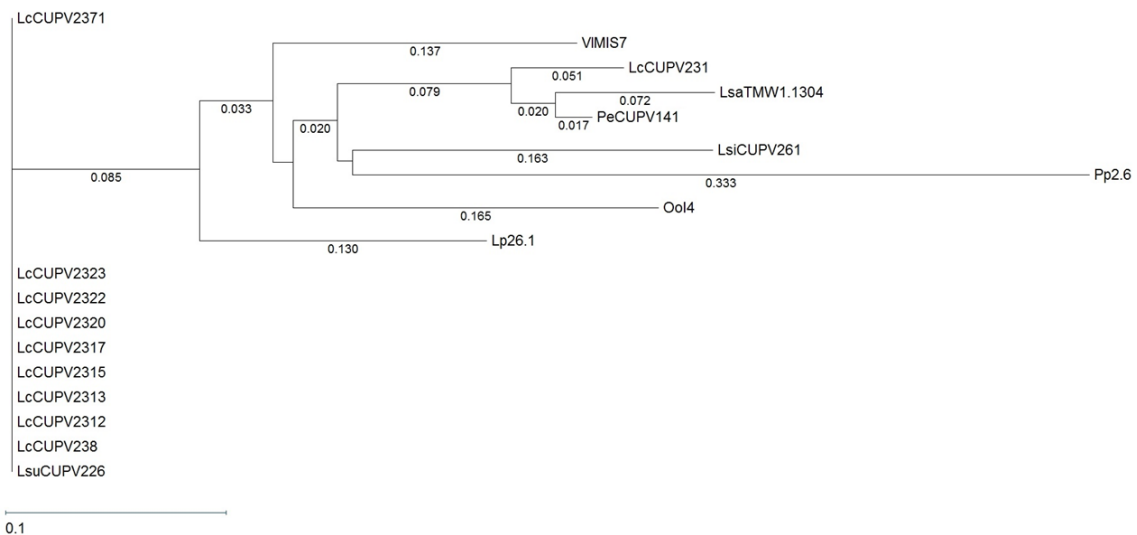


Figure 15. Phylogenetic tree pertaining to the *p-gtf* gene nucleotide sequences of different LAB.

The tree was obtained with the Tamura-Nei (1993) metric from an MSA generated with the Clustal Omega algorithm. Complete names and details of the strains are depicted in Table 5.

The MSA showed a very poor conservation of the gene between different species. For instance, *P. parvulus* 2.6 showed three triplets more than the rest of the bacteria at position 125. However, the homology was very high among different strains of *Lactobacillus collinoides*, as is also observed in the phylogenetic tree, except for the CUPV231 strain, which has an identity of 63.4% (104 out of 164

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Table 5. Bacterial strains used for the *p-gtf* MSA and their codes.

Code	Strain	Source of isolation	Reference
PeCUPV141	<i>P. ethanolidurans</i> CUPV141	Cider	This work
Pp2.6	<i>P. parvulus</i> 2.6		[173]
Lp26.1	<i>L. plantarum</i> 26.1	Dairy and cereals	[175]
LsaTMV1.1304	<i>L. sanfranciscensis</i> TMV1.1304	Sourdough	[176]
LsiCUPV261	<i>L. sicerae</i> CUPV261	Cider	[11]
LsuCUPV226	<i>L. suebicus</i> CUPV226		
LcCUPV238	<i>L. collinoides</i> CUPV238		
LcCUPV2312	<i>L. collinoides</i> CUPV2312		
LcCUPV2313	<i>L. collinoides</i> CUPV2313		
LcCUPV2315	<i>L. collinoides</i> CUPV2315		
LcCUPV2317	<i>L. collinoides</i> CUPV2317	Cider	[11]
LcCUPV2320	<i>L. collinoides</i> CUPV2320		
LcCUPV2322	<i>L. collinoides</i> CUPV2322		
LcCUPV2323	<i>L. collinoides</i> CUPV2323		
LcCUPV2371	<i>L. collinoides</i> CUPV2371		
LcCUPV231	<i>L. collinoides</i> CUPV231		
OoI4	<i>O. oeni</i> I4	Cider	[11]
VIMIS7	<i>V. lutrae</i> MIS7	Fermented food	Unpublished, GenBank: AGM39429.1

2.3.6. Analysis of the adhesion ability of the *P. ethanolidurans* CUPV141 and CUPV141NR strains

To carry out this test, bacteria have to be sedimented and thus the supernatant containing part of the EPS is removed. Therefore, the concentration of the 2-substituted (1,3)- β -D-glucan bound to the bacteria after sedimentation and resuspension was determined by the specific ELISA immunoassay method prior to the analysis. As expected, the results revealed differences between the two strains, since CUPV141 and CUPV141NR carry 80.2 ± 6.0 ng/mL and $<0.30 \pm 0.06$ ng/mL of HoPS, respectively. Consequently, a comparative analysis of the two strains analysed should provide information on the contribution of the HoPS to adhesion. First, the ability of the bacteria for self-aggregation was investigated (Figure 17).

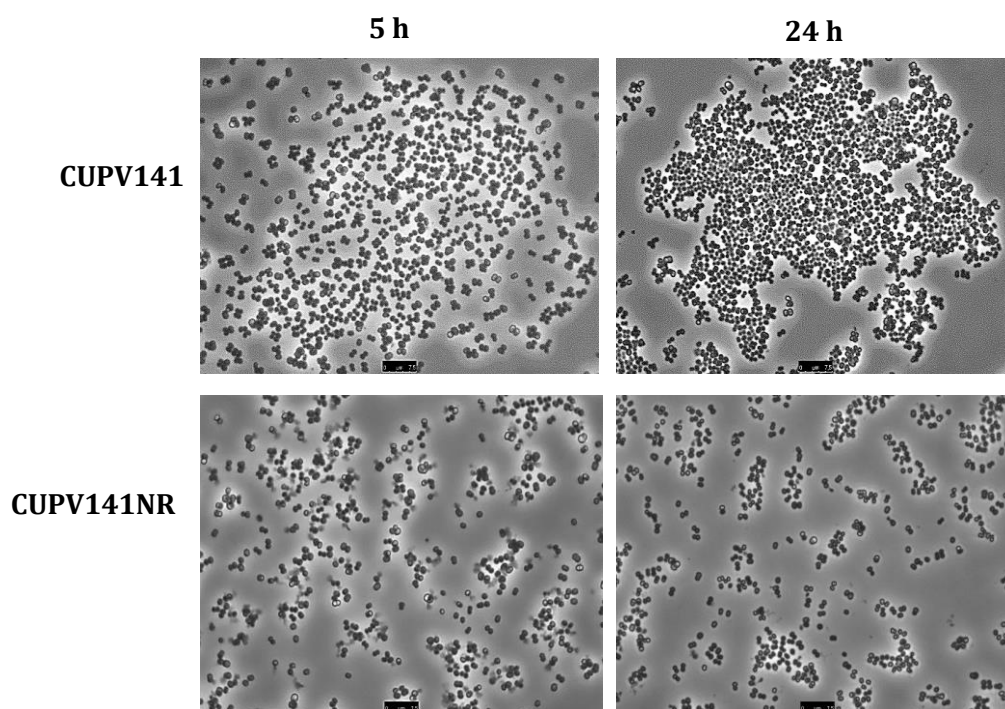


Figure 17. Aggregation of *P. ethanolidurans* CUPV141 and CUPV141NR strains, incubated for 5 h and 24 h.

After 5 h of incubation, the culture of *P. ethanolidurans* CUPV141 showed some aggregates, whereas fewer complexes appeared in the culture of CUPV141NR. However, the differences at this incubation time were not very appreciable. By contrast, after 24 h of incubation the aggregates formed by the CUPV141 strain

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were bigger than those in the 5-hours culture. An increase of aggregation of the CUPV141NR strain was not observed for the 5 h to 24 h incubation period. Thus, these results indicated that bacterial cell-to-cell interactions are mediated or potentiated by the 2-substituted (1,3)- β -D-glucan.

Secondly, the capacity of *P. ethanolidurans* CUPV141 and CUPV141NR to interact with human epithelial cells was also assessed using the enterocyte-like Caco-2 cell line (Figure 18).

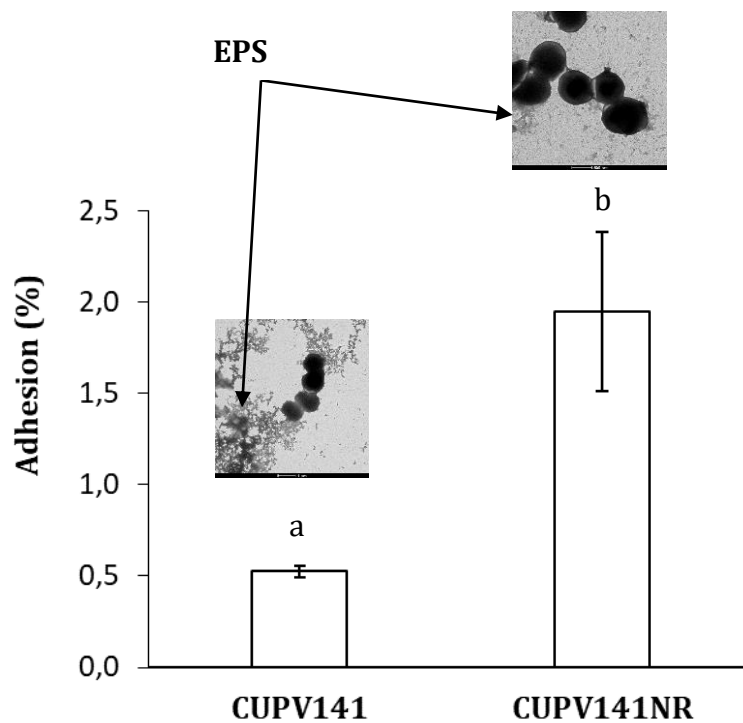


Figure 18. Adhesion of *P. ethanolidurans* CUPV141 and CUPV141NR strains to Caco-2 cells.

Values are expressed as the percentage of cfu added to the assay. The results are the mean of three independent experiments. The insets show electron micrographs of the bacteria. The arrows mark the β -glucan (EPS). Different letters represent statistical significances with $p \leq 0.05$.

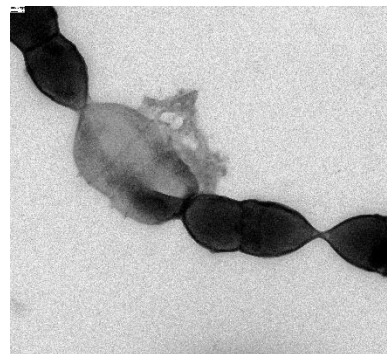
EPS production, which in CUPV141 formed a kind of net in the medium, while in CUPV141NR appeared as small aggregates. The ability of both strains to bind the enterocytes was significantly lower than that reported by Fernández de Palencia *et al.* [177] for the commercial probiotic *Lactobacillus acidophilus* La-5 (7%).

However, comparing the two strains described in this work, the non-ropy bacterium showed higher adhesion ($1.95 \pm 0.44\%$) to the eukaryotic cells than the ropy strain ($0.52 \pm 0.03\%$), which can be attributed to the presence of the phosphorylated HePS. Phosphate groups increase the net charge of the EPS and can be very important to mediate the interactions between bacteria and their hosts, as reported for several *Lactobacillus* strains [168]–[170]. Similarly, chemical phosphorylation of a dextran produced by *Leuconostoc mesenteroides* enhanced its immunostimulatory capacity [178]. For neutral EPS, some authors have reported that their presence in the surface of bacteria has a negative effect in their adhesive properties [106], [133], [134], while others described the production of these polymers as useful for probiotics to interact with eukaryotic cells [12], [42], [135]. The positive effect of the HoPS on binding to intestinal cells was demonstrated for the β -glucan-producing *P. parvulus* 2.6 and CUPV22 strains, which showed adhesion capacities to Caco-2 cells of 6.1% and 10.5%, respectively, that were reduced when the EPS was removed by washing prior to the binding assay [12], [42]. Thus, the different behaviour of the CUPV141 ropy strain could be due to its lower production of 2-substituted (1,3)- β -D-glucan. The overall results obtained for *P. ethanolidurans* illustrate the different roles of the two polysaccharides produced by this species: there is an involvement of the 2-substituted (1,3)- β -D-glucan in cell-to-cell adhesion, while the HePS would lead these bacteria to interact with eukaryotic cells, for colonisation of new environments. However, further research would be necessary for the elucidation of the mechanism through which these kinds of adhesions take place.

2.4. Conclusions

In the search for novel bacterial producers of the extracellular 2-substituted-(1,3)- β -D-glucan, a *P. ethanolidurans* (CUPV141) strain was isolated for the first time from a ropy cider of the Basque Country. We have demonstrated that this isolate secretes not only that β -glucan, but also a HePS composed of glucose, galactose, glucosamine and glycerol-3-phosphate, being the first *Pediococcus* strain described to produce this kind of polymer. However, our *in silico* analysis of priming-glycosyltransferase coding genes involved in HePS synthesis suggests that this is a general characteristic shared by different pediococci. Southern blot hybridisation allowed localising the GTF glycosyltransferases-coding gene responsible for the synthesis of the β -D-glucan in a 34.4 kbp-pPE3 plasmid of this strain. The role of the HoPS in bacterial self-aggregation, as well as the most relevant role of the HePS in bacteria-eukaryotic cells interactions were inferred from interactomic experiments using the ropy and the non-ropy strains. Nevertheless, the molecular mechanisms by which *P. ethanolidurans* CUPV141 polysaccharides perform biotic interactions, the detailed structure of the HePS produced by this strain, and its existence in other pediococci remain unknown and deserve further work.

**CHAPTER 3. EPS PRODUCED BY *LEUCONOSTOC* AND
*LACTOBACILLUS***



3.1. Introduction

Dextrans are HoPS synthesised by dextransucrases, a type of glucansucrases mostly belonging to the glycoside hydrolase family 70 [179], using sucrose as substrate. Depending on the linkage specificity of the dextransucrase, dextrans have different types of glucosidic linkages and different proportion of branching. Thus, depending on each bacterium and its specific dextransucrase, dextrans can be different one from another. Still nowadays, the most used dextran in industry is that produced by *Lc. mesenteroides* NRRL B-512F, with a 95% of α -(1→6) glucosidic linkages [180]. In industry, dextrans can improve the texture, rheology and palatability of some beverages, and they serve as cryoprotectants or moisture-increasers [181]. In addition, they can be produced *in situ* in fermented dairy food, developing their prebiotic role, or during sourdough fermentation to improve texture and storage life of bread [182], [183]. Moreover, dextrans are applied in other fields than food industry. They have been reported as having antiviral activity in salmonids, to serve as plasma substitutes or as coating for chromatographic columns [152], [181], [184], [185]. In addition, they are utilised in the chromatographic purification of whey proteins [186] or because of the glycation they perform with whey to affect the immunoglobulin-E binding capacity with blood sera in patients with cow milk protein allergy [187]. Furthermore, dextrans modified at their 3-position ring carbon are able to reduce/modify the activity of glucosyltransferase enzymes produced by oral strains of *Streptococcus mutans*, reducing the formation of dental plaque [188]. However, dextrans' production by LAB is not always desirable for the food and beverage industries due to the fact that sometimes they have a deleterious effect on the final products. For instance, they can form a slime film that spoils meat products, and also, they have been characterised as being produced by beverages-spoiling strains as *O. oeni* in wine [39], [143] or *Lactobacillus diolivorans* in ciders [38].

In 1878, Van Tiehem described the first microorganism responsible for dextran production and named it *Leuconostoc mesenteroides* [179]. Since then, a lot of different bacteria have been isolated as dextran-producers, from other *Leuconostoc* to *Lactobacillus*, *Weissella*, *Streptococcus* or *Oenococcus* species [38], [39], [107], [182], [185], [189].

Introduction

The multiple applications of dextrans, as well as the increasing demand on free-additive products, make necessary the search for new dextran-producers. Thus, the aim of this chapter was the isolation of dextran-producing bacteria from different food origins and the characterisation of the dextrans they produce.

3.2. Materials and Methods

3.2.1. Bacterial strains and growth conditions

The isolation of both strains used in this chapter as well as the identification of one of them (*Lactobacillus mali* CUPV271) was performed by Ana Isabel Puertas at UPV/EHU.

Two LAB strains were isolated, respectively, from the ropy slime of the surface of a vacuum-packed sliced cooked ham and from an apple must from a Spanish cider producer (Basque Country) as follows. Serial dilutions in Ringer's solution of the slime and the apple must were cultivated in MRS [155] agar plates (pH 6.0), containing 2 μ L/mL pimaricin and 5% sucrose, at 28 °C under a microaerophilic atmosphere (CampyGen™, ThermoScientific) for 24 h. These bacteria were identified as *Leuconostoc carnosum* CUPV411 and *L. mali* CUPV271 by sequencing a fragment of their 16S rRNA coding genes at Secugen (Madrid, Spain). The data were deposited in GenBank with accession numbers MH628089 and MH628046, respectively (Appendix 2, 2.4 and 2.5). LAB were grown at 30 °C without shaking in MRS medium containing either 2% glucose (MRSG) or 2% sucrose (MRSS). The media were buffered at pH 6.8 or 5.5 for growth of either *Lc. carnosum* or *L. mali*. The strains were stored in MRSG containing 20% (v/v) glycerol at -80 °C. For EPS production, a semi-defined (SMD) medium containing 2% sucrose (SMDS) and no glucose [138] was used with the aim of avoiding the contamination with polysaccharides present in the MRS medium.

3.2.2. Production, purification and quantification of EPS from LAB

The protocol used for the isolation of the EPS produced by *L. mali* and *Lc. carnosum* was that described in section 2.2.9 in Chapter 2. MRSG and SMD media were replaced by MRSS and SMDS at pHs 5.5 and 6.8 for *Lactobacillus* and *Leuconostoc*, respectively. Finally, EPS was recovered by addition of one volume of cold ethanol instead of three volumes.

3.2.3. Monosaccharide composition, methylation and FT-IR analyses

With the aim of elucidating the type of EPS isolated from the two strains, neutral sugar composition, linkage types and the IR spectra were recorded as previously described in section 1.2.5 in Chapter 1.

3.2.4. NMR spectroscopy analysis

Samples were weighted (*ca.* 1 mg) and dissolved 1:1 (w/v) in D₂O and their spectra were recorded at 333 K on a Bruker Avance NEO spectrometer operating at 500.13 MHz (¹H) and 125.75 MHz (¹³C), BBOF probe with z-gradients. Chemical shifts are given in ppm, using the acetone signal (2.16 ppm in ¹H, 30.7 and 215.7 ppm in ¹³C) as reference. To record the 1D spectra, solvent suppression (WATERGATE) was used. The homonuclear correlation spectroscopy (COSY) spectra were recorded using a presaturation to remove the residual signal of solvent (3K x 512 increments) with 8 scans. The heteronuclear single quantum coherence spectroscopy (HSQC) with solvent suppression was performed (2K x 256 increments) with 128 scans. The heteronuclear multiple bond correlation (HMBC) experiment was performed (4K x 256 increments) with 128 scans. To improve the sensitivity, a BBI with z-gradient was used to record the HSQC and HMBC spectra.

3.2.5. Detection of dextrans' production at the cellular level

For phenotypic determination at the cellular level, LAB cultures were grown in MRSG liquid medium to $A_{600\text{nm}} = 1.0$. Then, 100 μL of appropriate dilutions were streaked on MRSS- and MRSG-agar plates and incubated for 11 days. The detection of EPS in the LAB colonies was performed by TEM. Three or four colonies of each strain from MRSS- and MRSG-agar plates were carefully suspended in 50 μL of sterile distilled water to form a turbid suspension, which was subjected to negative staining with uranyl acetate, prior TEM analysis as previously described in section 2.2.10 in Chapter 2.

3.2.6. Caco-2 cell culture and LAB adhesion assays

The maintenance and differentiation of Caco-2 cell line as well as the bacterial binding assays were performed as detailed in section 2.2.11.2 in Chapter 2. In addition, to evaluate the influence of dextran in the adhesion capability of LAB, they were exposed to conditions to support or impair production of dextran. To this end, LAB were grown either in MRSG or MRSS to the middle of the exponential phase, and cultures were diluted to a final volume of 1 mL of DMEM (Invitrogen) supplemented respectively with either 0.5% of glucose or 0.5% sucrose, to give 1.25×10^6 cfu/mL. The rest of the protocol remained unchanged.

3.2.7. Physicochemical characterisation of the isolated dextrans

3.2.7.1. Determination of the molar mass distribution by SEC-MALLS

The molar mass distribution of the purified dextrans was analysed by means of size exclusion chromatography coupled with multiangle laser light scattering detection (SEC-MALLS) as previously described [190]. In short, each lyophilised sample was resuspended in 0.1 M NaNO₃ at a concentration of 5 mg/mL, kept overnight under gentle stirring and centrifuged ($10,000 \times g$, 10 min) before analysis. The HPLC system (Waters, Milford, MA) consisted of a separation module Alliance 2695 connected with two detectors: a refractive index (RI 2414, Waters) to determine the amount of dextran using calibration curves obtained from standards of dextran (Fluka-Sigma), ranging from 5×10^3 to 4.9×10^6 Da [129] and the MALLS Dawn Heleos II (Wyatt Europe GmbH, Dembach). The quantification of dextrans was achieved with the Empower software (Waters) and the molar mass distribution analysis with the Astra 3.5 software (Wyatt Europe GmbH). Two SEC columns placed in series were used: TSK-Gel G3000 PW_{XL}+TSK-Gel G5000 PW_{XL} protected with a TSK-Gel guard column (Supelco-Sigma) and the separation was carried out at 40 °C with a flow rate of 0.45 mL/min using 0.1 M of NaNO₃ as mobile phase.

3.2.7.2. Thermal analysis

Thermogravimetric analysis (TGA) was carried out using a thermogravimeter Q-500 (TA Instruments), under dynamic nitrogen and air atmospheres (90 mL/min) at a heating rate of 10 °C/min, within the temperature interval from RT to 580 °C. Samples were weighted (between 8 and 14 mg) in a platinum pan.

3.2.7.3. Analysis by differential scanning calorimetry (DSC)

A differential scanning calorimeter DSC 3+ (Mettler Toledo) was used to obtain DSC curves under a nitrogen atmosphere flowing at 3 mL/min. The samples (9-12 mg) were deposited in aluminium crucibles maintained at -30 °C during 3 min. Then, two heating scans separated by a cooling stage at 10 °C/min were done. The first heating scan was performed to erase the thermal history of the materials, as reported previously [191], [192], and was raised from -30 °C to 210 °C, maintaining the final temperature for 5 min. In the cooling stage, the temperature fell to -30 °C and was maintained for 3 min. Finally, in the second scan 300 °C were reached.

3.2.7.4. X-ray diffraction (XDR) analysis

The identification of the crystalline and/or amorphous structure of the purified dextrans was analysed. The X-ray powder diffraction patterns were collected by using a PHILIPS X'PERT PRO automatic diffractometer operating at 40 kV and 40 mA, in theta-theta configuration, secondary monochromator with Cu-K α radiation ($\lambda = 1.5418 \text{ \AA}$) and a PIXcel solid-state detector (active length in 2θ 3.347°). Data were collected from 5 to 60° 2θ (step size = 0.026 and time per step = 90 s) at RT, 0.04 rad soller slit and variable divergence slit giving a constant 5 mm area of sample illumination.

3.2.7.5. Atomic force microscopy (AFM) analysis

Aqueous solutions of the two dextrans were prepared at 1 mg/mL with filtered (0.45 μm) deionised water and kept *ca.* 16 h to assure solubilisation. Then, serial

dilutions were made to obtain a final concentration of 1 $\mu\text{g}/\text{mL}$. About 5 μL of this solution were dropped onto a cleaved mica substrate and allowed to dry at RT for 24 h in a desiccator. AFM images were obtained with a NanoScope V microscope (Digital Instruments) operating in tapping mode, with 512 x 512 pixels, and TESP 0.01-0.025 ohm-cm Antimony (n) doped Si tips ($T=3,8 \mu\text{m}$, $f_0=320 \text{ kHz}$) (Bruker). As a contrast enhancement technique [193], phase imaging extension was used. Scan rates ranged from 1 to 2 Hz.

3.2.8. Rheological analysis

The rheological behaviour of the two dextrans was determined as previously described [107]. Briefly, the lyophilised EPS were dissolved in ultrapure water at different concentrations, stirring at RT and then allowing them to settle overnight before each analysis. The viscoelastometer used was a Thermo-Haake Rheostress I (ThermoFisher Scientific), equipped with a cone-plate (60 mm diameter, 2° cone angle) geometry. Each solution (2 mL) was measured in two steps: the first one consisted on 3 min of resting without shear to maintain the temperature at 20 °C. Then, an interval shear-rate range was applied for 3 min between 1 and 500 s^{-1} . Each experiment was repeated at least three times. A Haake Rheowin Data Manager was used for analysing the continuous steady-state flow from the apparent viscosity and shear rate relationship. Viscosity at shear near zero (η_0) was extrapolated and regressed using the Cross model [194].

3.2.9. Statistical analysis

Adhesion data were analysed by two-way ANOVA to determine the significant differences between the variables at $p \leq 0.05$. The analysis was performed using the SAS 9.4 software (SAS Institute Inc).

3.3. Results and Discussion

3.3.1. Elucidation of the type of EPS produced by *L. mali* CUPV271 and *Lc. carnosum* CUPV411

The EPS produced by the two LAB were purified from the supernatants of 48 h-cultures and analysed to determine their composition and structure. Both EPS contained glucose as the sole monosaccharide, indicating that they produced a glucan-type HoPS.

Their FT-IR spectra were very similar (Figure 19) and showed typical bands of polysaccharides in the regions of 3400, 1400 and 1060 cm^{-1} [122].

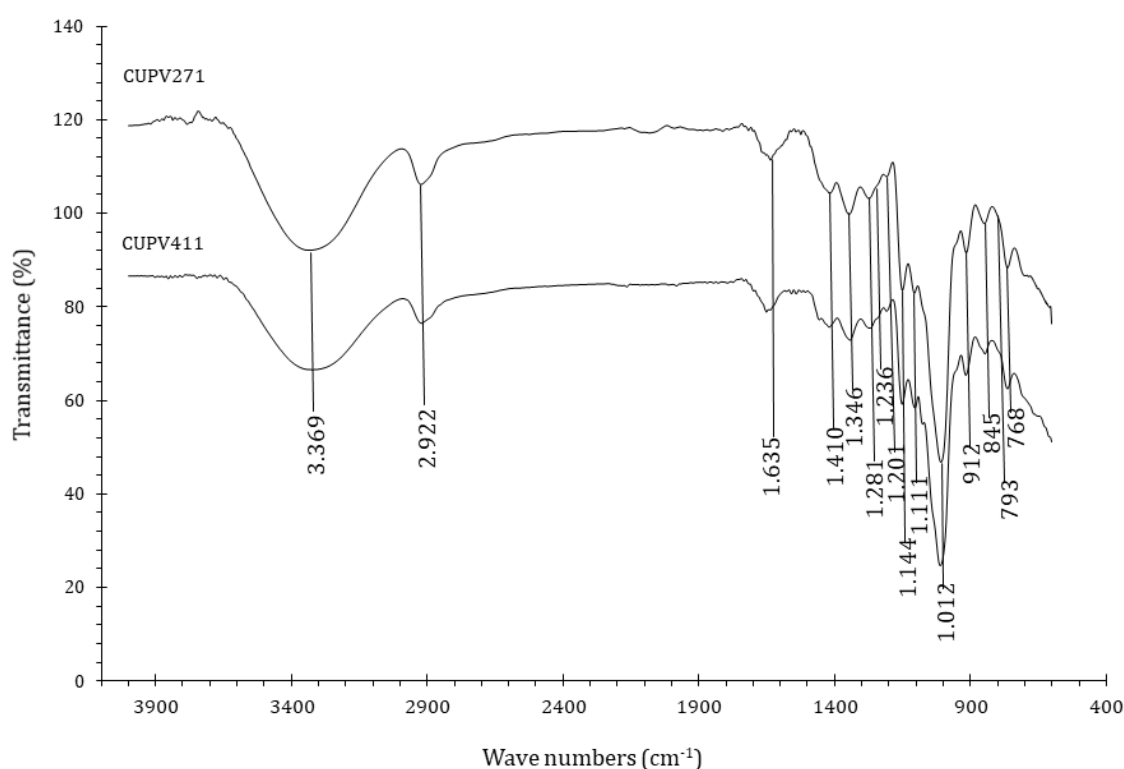


Figure 19. FT-IR spectra of isolated dextrans.

Up, spectrum of the HoPS from *L. mali* CUPV271 and down, the spectrum of the dextran isolated from *Lc. carnosum* CUPV411.

The region between 950-700 cm^{-1} , usually known as the anomeric region [126], [195], showed three bands (912, 845 y 768 cm^{-1}) of α -anomers in both spectra [196], and no bands characteristic of β -linkages were observed, indicating that

they are α -glucans. The broad signal at 3369 cm^{-1} corresponds to the hydroxyl groups stretching vibration [196], very intense in polysaccharides because of their high amount of $-\text{OH}$. The band in the region of $2925\text{-}2930\text{ cm}^{-1}$, represents the stretching vibration of C-H group and that around 1635 cm^{-1} comes from the water bound to dextrans [125], [197], [198]. The following five bands in the range of $1480\text{-}1180\text{ cm}^{-1}$ (1410 , 1346 , 1281 , 1236 y 1201 cm^{-1}) were described to be associated with bending vibrations of alcoholic groups and CH and CH_2 groups [196]. The stretching vibration of glycosidic linkage (C-O-C) and C-O or C—O-H groups is responsible for the signals appearing between 1200 and 1000 cm^{-1} [124]–[126], [196], [199], [200]. Finally, the results from this analysis confirmed the absence of non-glucidic components (phosphates, sulphates, protein) in the polysaccharides analysed.

Methylation analysis (Table 6) showed the predominance of linear residues of (1,6)-glucopyranose in the structure of both polymers as well as the presence of 3,6-di-*O*-substituted glucopyranose (branching points) and terminal units of glucopyranose that amounted to 3.6% in the polysaccharide from *L. mali* and to 6.8% in that of *Lc. carnosum*.

Table 6. Linkage types and their percentages deduced from methylation analysis of dextrans synthesised by *L. mali* CUPV271 and *Lc. carnosum* CUPV411.

Linkage type	Rt (min)	%	
		<i>L. mali</i> CUPV271	<i>Lc. carnosum</i> CUPV411
Glc _p -(1→	6.9	3.6	6.8
→3)-Glc _p -(1→	8.9	2.1	0.7
→6)-Glc _p -(1→	9.9	84.8	81.1
→4,6)-Glc _p -(1→	12.1	1.6	1.5
→3,6)-Glc _p -(1→	12.4	7.9	9.9

These data, together with the α -configuration of the linkages deduced from FT-IR, suggest that these polysaccharides are dextrans. Commercial dextrans have branching degrees around 5%, and the side chains are mainly composed of single α -D-glucopyranosyl units (about 40%), or are two units long (about 45%), while

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only 15% of them contain more than 2 units [201]. Therefore, the small percentage of linear units of glucose (1,3)-linked detected in the methylation analysis may belong to the short side-chains in the dextran structure. Around 1.5% of substitutions at positions *O*-4 were also detected in the two polysaccharides, which is more unusual, as this type of dextrans, branched at positions other than *O*-3, have been reported in fewer cases [202].

For further confirmation of the methylation data, both polysaccharide samples were analysed by NMR spectroscopy, revealing that their ^1H NMR and ^{13}C NMR spectra were very similar. The anomeric proton resonances for the ^1H NMR spectra (Figure 20) of the polymers coincide with those reported for 1,3-branched dextrans as the B-1351 dextran [203].

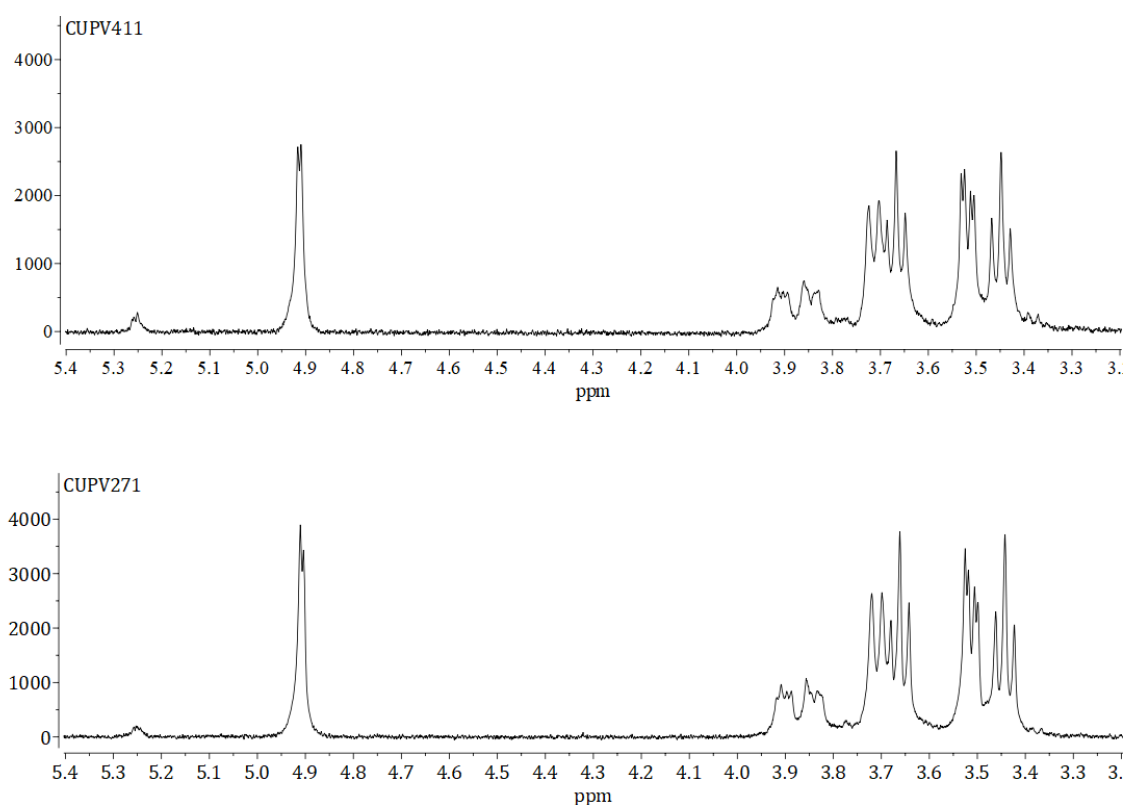


Figure 20. ^1H NMR spectra of dextran produced by *Lc. carnosum* CUPV411 (up) and *L. mali* CUPV271 (down).

A main anomeric signal at 4.91 ppm (coupling constant $J= 3.7$ Hz) attributable to the α -(1 \rightarrow 6)-glucopyranose linkages of the dextran backbone, and a small anomeric signal at 5.25 ppm, were observed in the anomeric region. Integration of the area of both peaks gave a ratio of 5.6/94.4, which also supports the data deduced from methylation analysis. The signals between 3.2-4.4 ppm correspond to the protons of the monosaccharides' backbone [204]. The ^{13}C NMR spectra of the polysaccharides (Figure 21) showed a single anomeric signal from the α -(1 \rightarrow 6)-glucopyranose backbone at 98.3 ppm [205], confirming that the EPS contained α -anomeric carbons instead of β -anomeric carbon atoms with resonances downfield from 102 ppm [206], as we deduced from the FT-IR analysis. However, the signals of the anomeric carbon of the α -glucose branches were not observed in the ^{13}C spectrum.

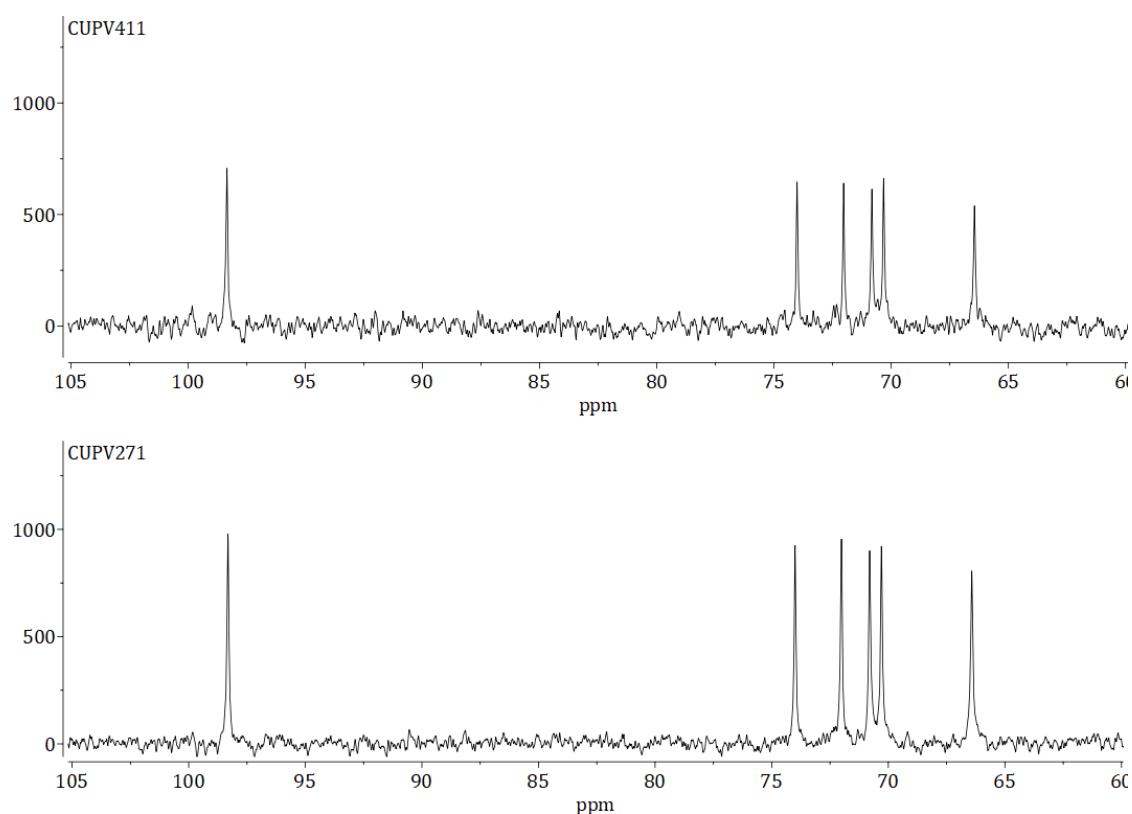


Figure 21. ^{13}C NMR spectra of dextran produced by *Lc. carnosum* CUPV411 (up) and *L. mali* UPV271 (down).

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The assignment of ^1H and ^{13}C resonances of the main monosaccharide (Table 7) was performed on the basis of homonuclear COSY (Figure 22A) and heteronuclear HSQC (Figure 22B) two-dimensional correlation NMR experiments. The system with ^1H anomeric signal at 5.25 ppm could not be assigned, although analysis of various dextrans has shown that this signal is characteristic of α -(1 \rightarrow 3) branched dextrans, [203], [205], [207], [208], which also coincide with our methylation results.

Table 7. Chemical shifts assignments (ppm) for the main monosaccharide α -(1 \rightarrow 6)-glucopyranose.

	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
^1H	4.91	3.51	3.61	3.44	3.85	3.90	3.72
^{13}C	98.3	72.0	74.0	70.3	70.8	66.5	66.5

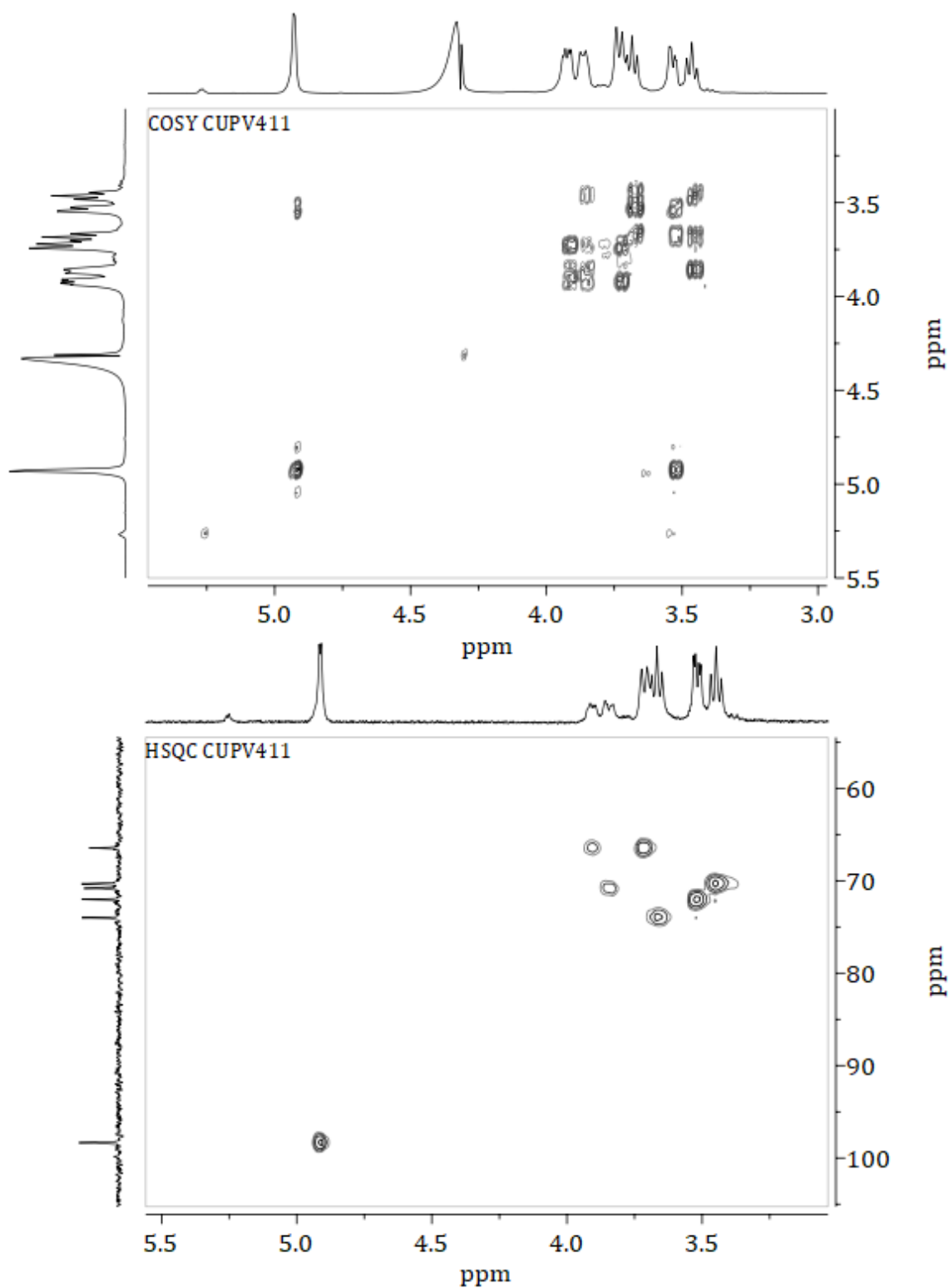


Figure 22. 2D-NMR analysis of the dextran produced by *Lc. carnosum* CUPV411. (A) ^1H - ^1H COSY spectrum and (B) ^1H - ^{13}C HSQC spectrum.

The HMBC spectrum (Figure 23) confirmed the α -(1,6) linkage through the correlation between H6 and H6' signals (3.90 ppm and 3.72 ppm, respectively) and

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C1 signal (98.3 ppm). In addition, the overlap of the HSQC and HMBC spectra (data not shown) only gave information on the (1→6) linkage through the correlation of H1-C1 signals (4.91-98.3 ppm) with the two protons of C6 (H6: 3.90 ppm and H6': 3.72), but signals from the minor components of the polysaccharide were not observable in any of the spectra analysed. Thus, the presence of the α -glucopyranose side chains could not be confirmed with the experiments performed, although the ^1H NMR spectra (Figure 20) showed a signal that, according to the literature, could be interpreted as corresponding to the O-3 branches.

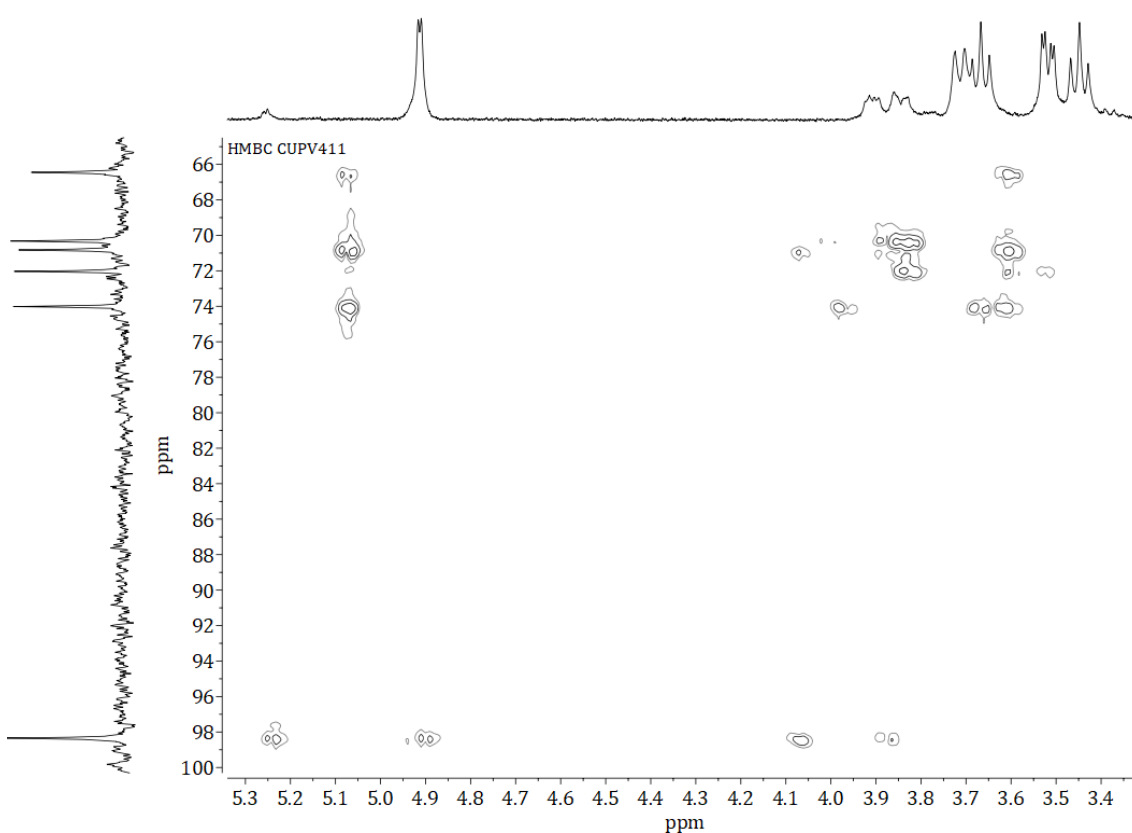


Figure 23. HMBC spectrum of the dextran synthesised by *Lc. carnosum* CUPV411.

The main linkage types in the *Lc. carnosum* CUPV411 dextran are represented in Figure 24.

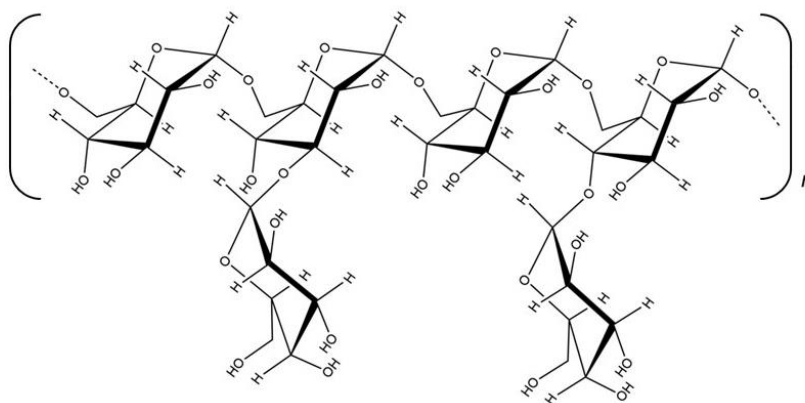


Figure 24. Representation of the main linkage types of the *Lc. carnosum* CUPV411 dextran.

The total number of α -(1 \rightarrow 6)-glucopyranose units in the main backbone is represented by n , and according to the quantitative data from NMR, this value was 94.4%. Side chains (5.6%), mostly of a single α -glucopyranose unit, partially substitute the α -(1 \rightarrow 6) backbone at O -3 and O -4.

3.3.2. Detection of dextran production by *L. mali* CUPV271 and *Lc. carnosum* CUPV411

Dextran is synthesised by dextransucrases using sucrose as substrate [183]. Thus, for a macroscopic detection of the dextrans synthesised by the two LAB, the bacteria were grown in MRSS-agar plates. In addition, plates containing MRSG-agar were also inoculated as negative controls. As expected, after growth for 48 h in the presence of sucrose, the colonies of both strains were mucoid whereas the colonies generated in MRSG medium did not show this phenotype. Moreover, both LAB developed colonies with a larger size upon growth in MRSS medium (Figure 25).

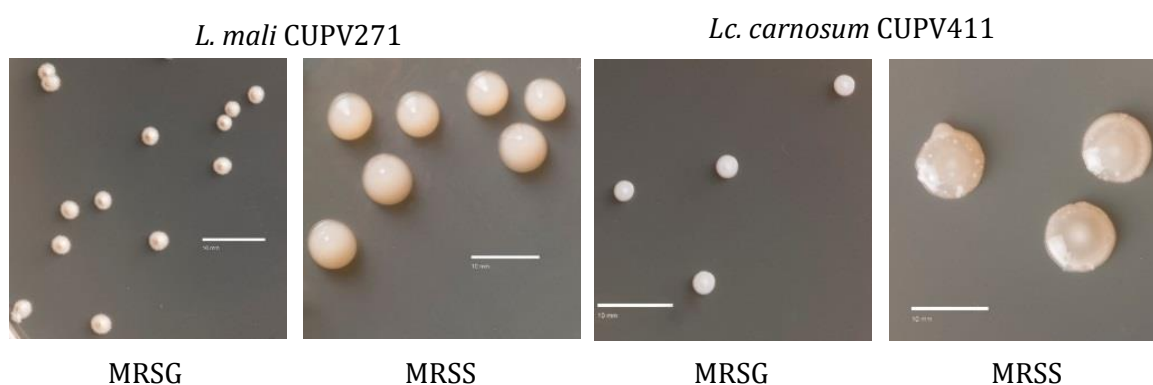


Figure 25. Detection of EPS production by LAB on solid media. Bacterial colonies in MRSG and MRSS after 240 h of incubation.

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Finally, comparing the colonies of both LAB grown in the presence of sucrose, some differences were observed. The CUPV271 strain presented convex colonies firmly adhered to the agar even after 264 h of incubation. On the contrary, CUPV411 colonies were flatter and with less adherence to the agar. This difference in colonies' morphology has also been described for other dextran-producing LAB [107].

Analysis of the two LAB by TEM (inset in Figure 26) confirmed the presence of the EPS attached or surrounding the bacteria in cultures grown in MRSS and not MRSG.

3.3.3. Capacity of *L. mali* CUPV271 and *Lc. carnosum* CUPV411 to adhere to Caco-2 cells

As we have previously observed in some dextran-producing bacteria, the differences in the colonies morphology correlate with different capacities to bind enterocytes in the presence or absence of EPS [107], [134]. Therefore, we tested the adhesion capacity of the LAB grown in media with or without sucrose (MRSS or MRSG) in an *in vitro* assay, measuring the binding of the bacteria to human epithelial Caco-2 cells (Figure 26). The adhesion capacity of *Lc. carnosum* CUPV411 ($2.73 \pm 0.15\%$) did not change regardless of the presence or absence of dextran in the medium, which coincides with the results reported for several *Leuconostoc* strains [107], [134]. On the other hand, the adherence to eukaryotic cells of *L. mali* CUPV271 was significantly reduced from $2.85\% \pm 0.14\%$ to $0.86 \pm 0.07\%$ in conditions allowing dextran synthesis. These results are in accordance with those reported for the dextran-producing *L. sakei* MN1 [134]. However, it should be stated that sometimes the HoPS produced by LAB also enhance the bacterial binding capacity, as previously reported for the β -glucan produced by *P. parvulus* strains [12], [42].

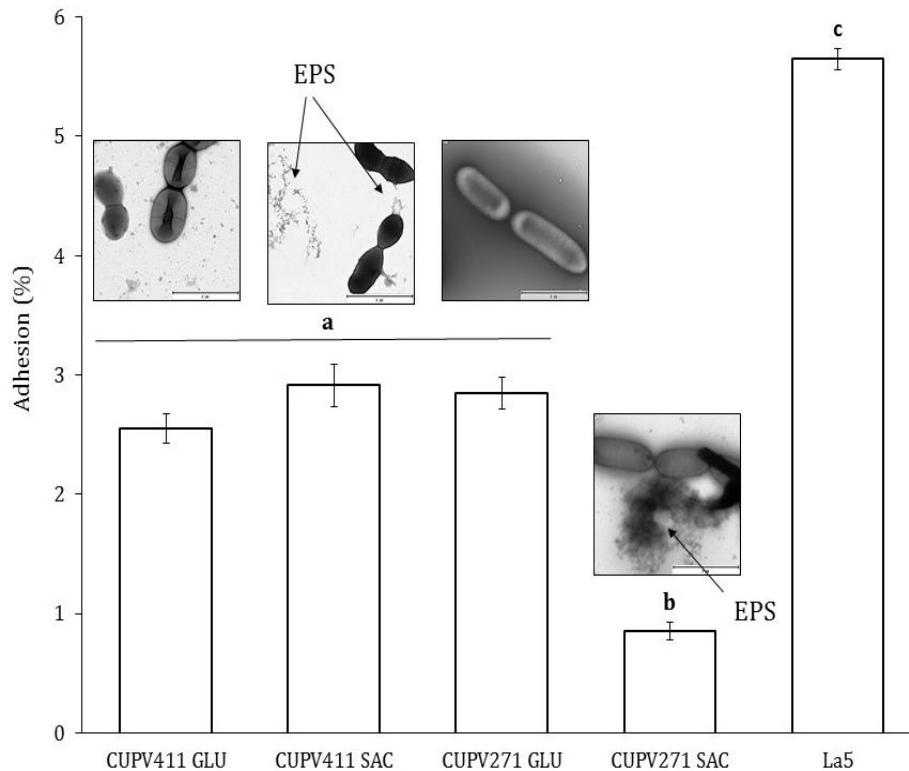


Figure 26. Adhesion of LAB strains to Caco-2 cells. Adhesion levels are expressed as the percentage of cfu. Data were analysed by ANOVA. Differences (a-c) were significant with $p \leq 0.05$.

3.3.4. Physicochemical characterisation of the dextrans

The results presented in sections 3.3.2 and 3.3.3 indicated differences between the dextrans synthesised by *L. mali* CUPV271 and *Lc. carnosum* CUPV411. However, the chemical analyses described in section 3.3.1 showed that both dextrans had similar primary structure and branching degrees. Therefore, with the aim of elucidating whether some significant differences existed between both dextrans, they were subjected to a deeper physicochemical characterisation.

3.3.4.1. Molecular weight distribution

The M_w , weight average radius of gyration (R_w), coefficient v ($\log R_w / \log M_w$) and polydispersity index (PDI, M_w / M_n) of the two isolated EPS were determined by SEC-MALLS. Chromatograms are depicted in Figure 27 and parameters are summarised in Table 8.

Results and Discussion

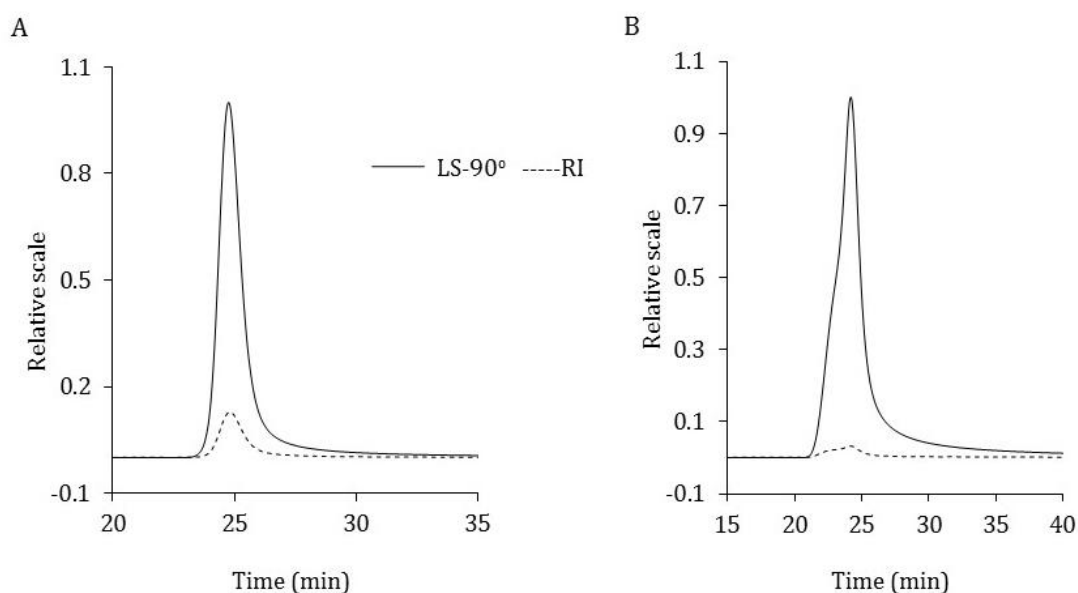


Figure 27. SEC analysis of the EPS synthesised by the studied strains. (A) EPS produced by *Lc. carnosum* CUPV411 and (B) EPS isolated from *L. mali* CUPV271. The figure shows two chromatograms where the continuous lines correspond to the MALLS detector, set at an angle of 90°, and the dashed lines correspond to the RI detector.

Table 8. Physicochemical characteristics of the dextrans produced by *Lc. carnosum* CUPV411 and *L. mali* CUPV271.

	Mean \pm SD (n=2)	
	CUPV271	CUPV411
Elution time (min)	25.6 \pm 0.04	24.9 \pm 0.04
Amount (mg/50 μ L)	223.4 \pm 24.4	106.9 \pm 6.7
M_w (g/mol)	1.23E+08 \pm 2.6E+06	3.58E+08 \pm 1.61E+07
Polydispersity (M_w/M_n)	1.05 \pm 0.08	1.25 \pm 0.02
ν ($\log R_w/\log M_w$)	0.22 \pm 2.62E-04	0.26 \pm 6.55E-05

M_w , weight average molar mass; R_w , weight average radius of gyration.

The chromatogram of the dextran produced by *L. mali* CUPV271 (Fig. 27A) showed a M_w of 1.23×10^8 g/mol, corresponding to a low polydispersity (1.05), very close to monodispersity. The M_w of the EPS of *Lc. carnosum* CUPV411 (Fig. 27B) was in the same log order, 3.58×10^8 g/mol, and showed a moderate polydispersity (1.25). This peak is being considered as a sole distribution of M_w . However, the presence of another slightly small distribution could have been assigned by the deformation at the left side of the peak. Moreover, it could be interpreted as a

shoulder, which might be due to the mixture of aggregates and single molecular structures co-eluting under the same peak, as reported before [209]. Nevertheless, this would not be clearly stated unless a column with more resolution at higher M_w levels is used.

3.3.4.2. Thermal degradation

The patterns of thermal degradation of the two dextrans in aerobic and anoxic atmospheres were analysed by TGA. Table 9 shows data of the thermograms obtained in nitrogen and air atmospheres: thermal decomposition temperatures for 5% and 50% weight loss ($T_{5\%}$ and $T_{50\%}$), temperature of maximum loss rate (T_{max}) and fraction of solid residue at 580 °C.

Table 9. TGA data for the dextrans isolated from *Lc. carnosum* CUPV411 and *L. mali* CUPV271.

Sample	T_5 (°C)		T_{50} (°C)		T_{max} (°C)		Residue (%)		
	In N ₂	In O ₂	In N ₂	In O ₂	In N ₂	In O ₂	In N ₂	In O ₂	
CUPV411	80	83	301	305	287	288 ^a	497 ^b	21.1	1.1
CUPV271	90	85	311	310	305	304 ^a	489 ^b	14.1	0.6

^aSecond or third stage for CUPV271 or CUPV411, respectively.

^bThird or fourth stage for CUPV271 or CUPV411, respectively.

The TGA curves presented in Figure 28 indicate that degradation in anoxic conditions of the polymers of *Lc. carnosum* CUPV411 (Figure 28A, black graph) and *L. mali* CUPV271 (Figure 28B, black graph) took place in three and two steps, respectively. In the first stage, weight loss was observed at a similar temperature range for both dextrans (between 25 and 115 °C) and amounted to *ca.* 5%. This loss might be due to the evaporation of the water embedded in the polymers, which are very hygroscopic. However, it could also be due to the evaporation of the ethanol used for the precipitation of the dextrans, as previously reported [210]. The second degradation stage corresponded to the decomposition of the polymeric chain, accompanied by the rupture of C-C and C-O bonds, generating CO, CO₂ and

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water, as reported before [211], [212]. It was characterised by severe weight losses for both dextrans: 55% (from 226 to 333 °C) for CUPV411, and 78% (from 229 to 441 °C) for CUPV271. A third stage of degradation was observed for the dextran of CUPV411, which consisted of a 14% weight loss (from 333 to 465 °C). In the end, a char residue from both dextrans was formed, corresponding to a carbonaceous or polynuclear aromatic structure.

The thermal degradation of both dextrans in aerobic conditions (Figure 28A and B, grey graphs) was carried out in three and four steps for CUPV271 and CUPV411, respectively. The first stage was again characterised by a weight loss of 5% due to the evaporation of embedded water and remaining ethanol (below 105 °C). A second stage where the majority of the depolymerisation was carried out was outlined by dramatic weight losses of 51% (from 219 to 322 °C) for CUPV411 and 72% (from 221 to 401 °C) for CUPV271. At the end of this degradation phase, a little shoulder was observed in the curve corresponding to the dextran from *L. mali*, which was not considered as a whole stage itself, and which implied a weight loss of around 8% (341-401 °C). On the contrary, *Lc. carnosum* dextran experimented a third phase of thermal degradation in which a 14% weight loss occurred (between 322 and 389 °C). Finally, the last phase of degradation for both dextrans, only present in the aerobic atmosphere, was due to the oxidative degradation of the carbonaceous structure formed in the previous stages. It caused 28% and 21% weight losses for CUPV411 and CUPV271 dextrans, respectively.

Therefore, the thermal degradation of the two dextrans was slightly different, since an additional step was observed for that of *Lc. carnosum* CUPV411 in both atmospheres which, according to the methylation data (Table 6), has more branching points in α -(1,3). However, this is not sufficient to explain the results obtained *in vivo* with the producing LAB. Nevertheless, the high degradation temperatures obtained for both dextrans, either in anoxia or in the presence of oxygen, would mean an advantage for application in the food industry.

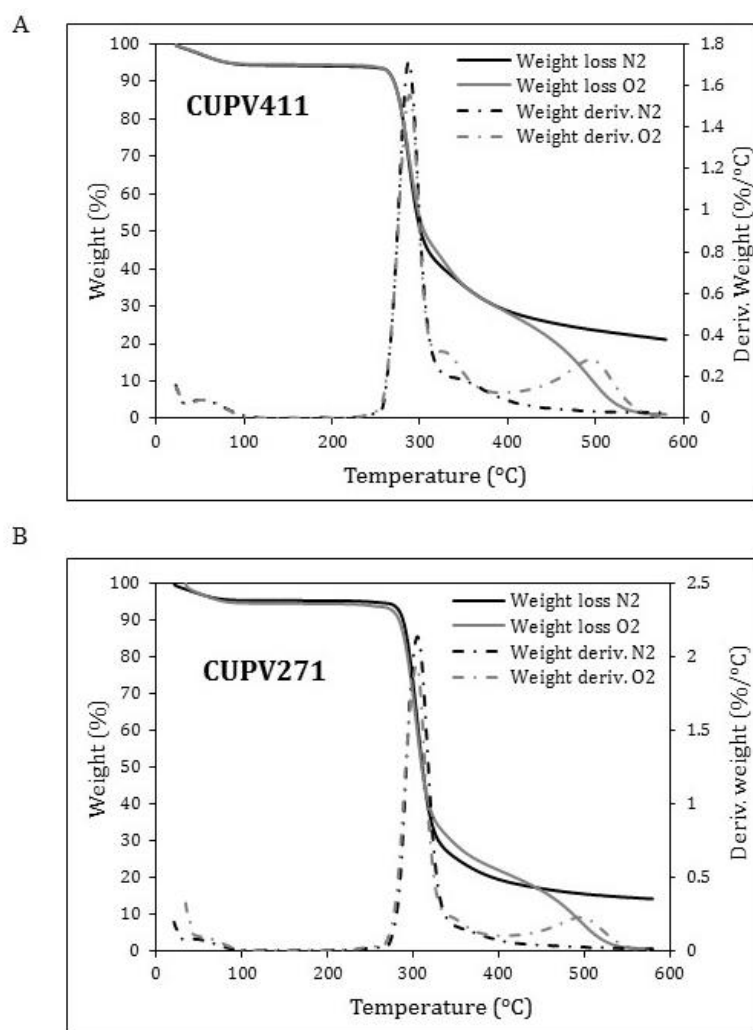


Figure 28. TGA curves. Thermal degradation of the EPS of (A) *Lc. carnosum* CUPV411 and (B) *L. mali* CUPV721.

Black lines correspond to nitrogen atmosphere and grey lines correspond to oxidative degradation. Continuous lines refer to the weight loss expressed in percentage, and dashed lines represent the weight derivative, expressed in %/°C.

3.3.4.3. Thermal properties

The degree of crystallinity of the dextrans was evaluated by DSC. Figure 29 depicts two heating scans of the samples. The first one (Figure 29A), shows a broad endothermic peak around 115 °C for both dextrans, which is due to evaporation of the water embedded in the polymers, as described by other authors [213]. The glass transition temperature (T_g), measured in the last scan (Figure 29B), was taken as the inflection point of the heat capacity change [214]. The T_g values of the polysaccharides were 225.7 °C (CUPV411) and 226.8 °C (CUPV271), which are

close to those reported for dextrans [215]. These high T_g are attributed to the presence of strong hydrogen bonds in these polymers [213], [216].

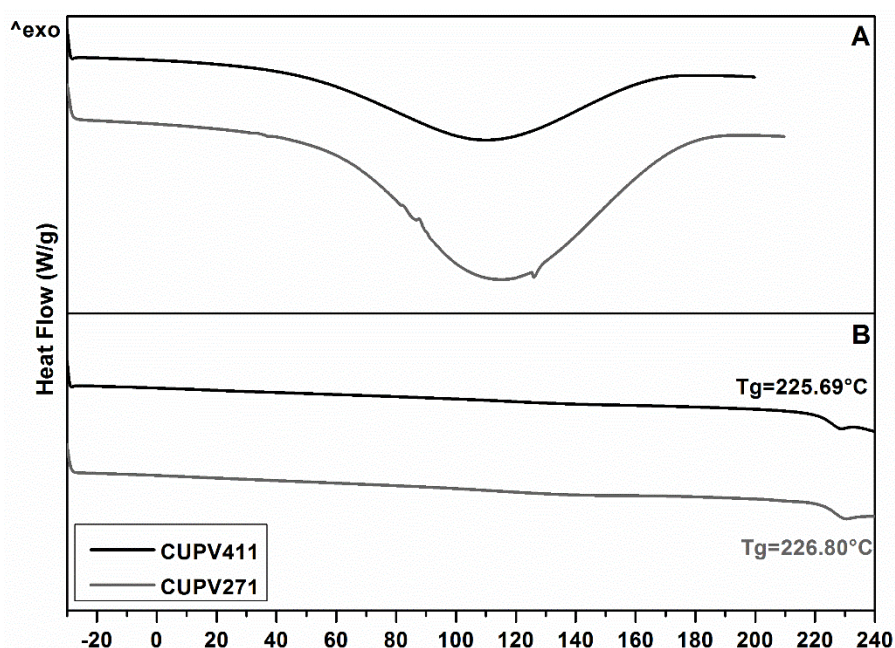


Figure 29. DSC curves. Black lines for *Lc. carnosum* CUPV411 dextran and grey ones for that of *L. mali* CUPV271. (A) First heating scan from -30 °C to 210 °C. (B) Second heating scan in which the T_g of each EPS is observed.

No exothermic peaks have been observed with these dextrans, thus no melting temperature was obtained. In addition, no crystallisation peaks were seen in the cooling stage. Therefore, these results suggest an amorphous behaviour for the EPS studied, as previously reported for other dextrans [213], [216].

3.3.4.4. X-ray diffraction of dextrans

Despite the fact that in the DSC analysis no exothermic peaks were observed, there is a remote possibility for dextrans to form crystals in their structure. The degradation temperature under nitrogen atmosphere was *ca.* 220 °C for both dextrans, thus, if the melting temperature was higher than the degradation temperature, the exothermic peak indicating the melting of the crystals would not be observed by DSC. X-ray diffraction was carried out to check this possibility.

Figure 30 shows the diffraction profiles of dextrans produced by *L. mali* CUPV271 and *Lc. carnosum* CUPV411, confirming the amorphous structure of the polymers. However, a blunt peak appears in the profile of both dextrans in the 2θ range of $15\text{-}25^\circ$, indicating a small amount of the sample with some level of crystallinity, as previously reported [217]. Thus, no differences were found in the formation of crystals between the two dextrans under study.

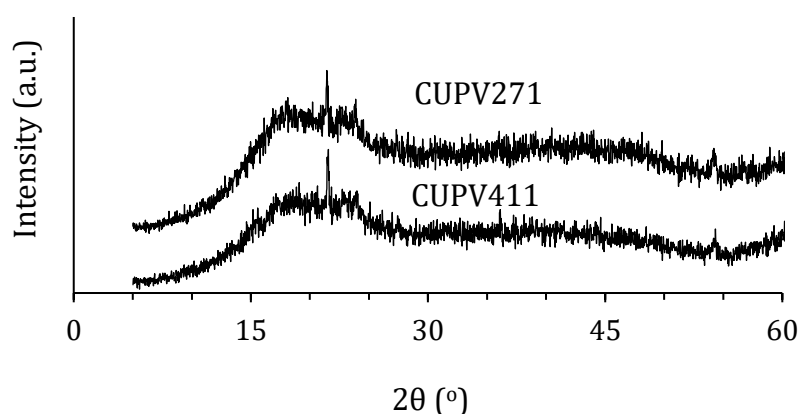


Figure 30. X-ray diffraction diagrams of dextran produced by *L. mali* CUPV271 (up) and by *Lc. carnosum* CUPV411 (down).

3.3.4.5. Atomic force micrographs of dextrans

Finally, atomic force was used to see if differences in the spatial conformation of the dextrans existed. As also reported for the EPS produced by other *Lactobacillus* species [200], [218], the AFM images (height and phase) of the dextran produced by *L. mali* (Figure 31A) revealed a mixture of irregular rounded lumps with few random linear chains, which become visible when saturating the image to the maximum. These fibrillar structures were more evident in the phase micrograph, suggesting that they were thinner than the aggregates and composed of less material. The lumps were very different in shape and size, ranging from 0.13 to $0.54\ \mu\text{m}$, whereas the stretched material formed clusters of chains yielding $0.033 \pm 0.004\ \mu\text{m}$ of width. On the contrary, irregular big aggregates ranging from 0.35 to $0.67\ \mu\text{m}$, and spherical small lumps with a diameter $<0.1\ \mu\text{m}$, were observed for the dextran isolated from *Lc. carnosum* (Figure 31B), as previously described for the dextran produced by *Leuconostoc lactis* KC117496 [219]. Moreover, the

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molecules from both polymers seemed to be tightly packed, suggesting a pseudoplastic behaviour with strong affinity for water molecules [200], [218], which was further confirmed with rheology assays.

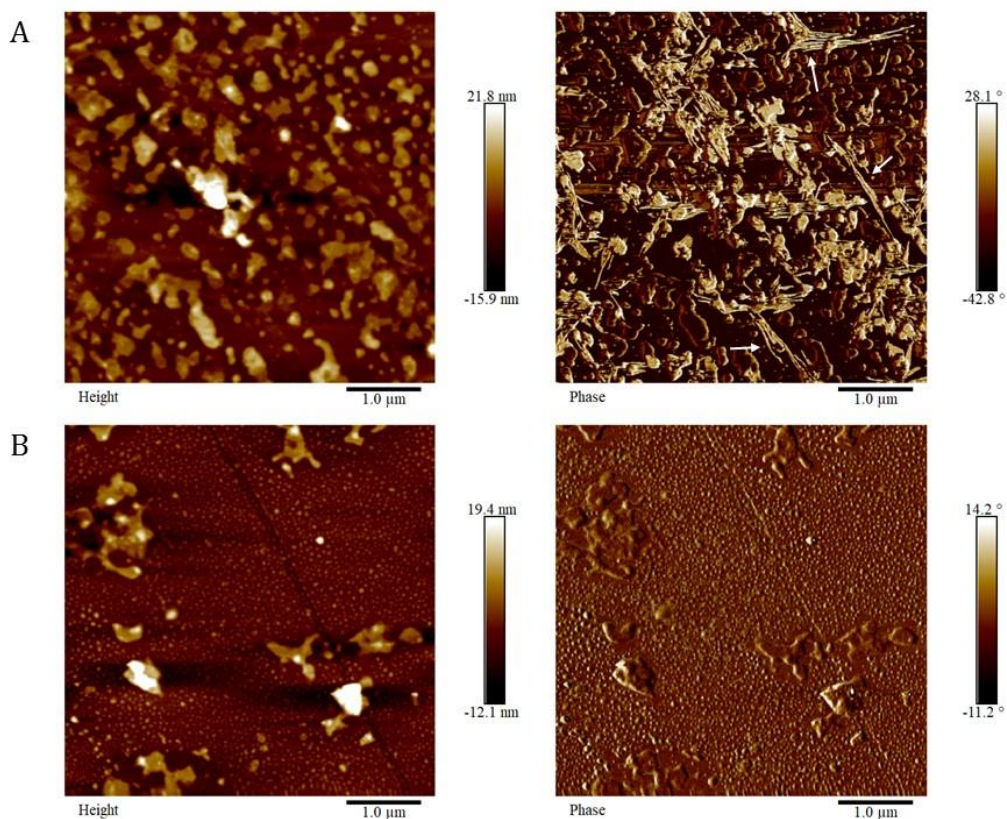


Figure 31. Height (left) and phase (right) AFM planar images of dextrans synthesised by *L. mali* CUPV271 (A) and *Lc. carnosum* CUPV411 (B). White arrows in the phase micrograph of the dextran produced by CUPV271 indicates the fibrillary morphology.

Taken together the results obtained in the different characterisation assays, both dextrans present slight differences in their size or percentage of ramifications, although atomic force micrographs showed some differences at the supramolecular level. Thus, the different spatial distribution of the dextrans might be on the basis of the different behaviours observed *in vivo*.

3.3.5. Rheological properties of dextrans produced by LAB

Since atomic force microscopy suggested the possible pseudoplastic behaviour for the dextrans studied, we evaluated their flow behaviour in solution, under shear at a constant temperature. The performance of the two dextrans was similar, and coincided with that previously described for this type of polymers [107], [189]. Then, only the viscosity curves for the dextran of *Lc. carnosum* CUPV411 will be represented. Ideally viscous or Newtonian flow behaviour was observed at low concentrations (up to a 0.5%), in which the viscosity remained constant over the entire shear rate range (Figure 32). This viscosity was the same as in resting conditions (zero-shear viscosity, η_0). However, at higher concentrations, shear-thinning or pseudoplastic flow behaviour was observed, in which the viscosity decreased with increasing share rates. The hydrodynamic forces generated during the shear could have led to the breakdown of the structural units and the physical networks between the dextrans' chains, as reported before [107], [220].

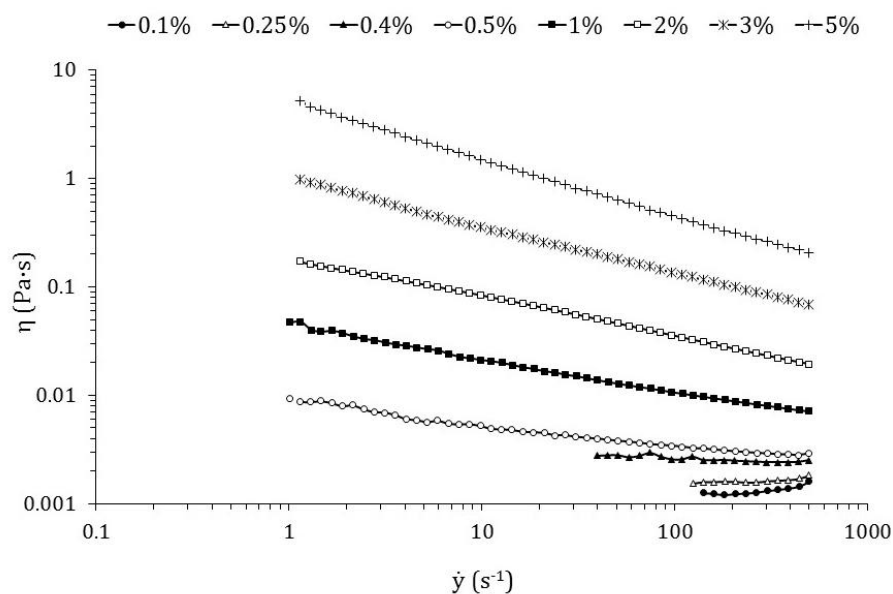


Figure 32. Double logarithmic plot of viscosity curves of dextrans isolated from LAB. Viscosity curves for dextran isolated from *Lc. carnosum* CUPV411 obtained by measuring aqueous solutions at different concentrations in a viscoelastometer.

Pseudoplastic characteristics of a polymer are not uniform in the whole range of shear rates. This behaviour is characterised by showing a plateau value of the zero-

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shear viscosity η_0 at low shear rates below 1 s^{-1} . In this case, measures in the range of $0\text{-}1 \text{ s}^{-1}$ were limited by the characteristics of the instrument used. Therefore, an extrapolation by the Cross model [194] was applied in order to obtain the η_0 value with the aim of comparing the viscosity of the two dextrans in a status near to the rest. At a given concentration of 5%, a higher plateau value of zero-shear viscosity was observed for dextran produced by *Lc. carnosum* CUPV411 (19.56 Pa·s) than that for the one produced by *L. mali* CUPV271 (0.09 Pa·s), indicating a higher M_w for the former dextran, as confirmed by SEC-MALLS.

The critical concentration (C^*), defined as the concentration of a polymer at which the equivalent sphere of a given polymer molecule just touches the equivalent spheres of all of its nearest neighbour molecules [221], was calculated for both dextrans (Figure 33). The data recorded suggested an increase in the Newtonian viscosity with the polymer's concentration. The C^* for the dextran from *L. mali* was 3.8%, while for that from *Lc. carnosum* it was 0.4%. These values are very related to the entanglements occurring both inside a single molecule and between different molecules. Therefore, the less-branched dextran produced by *L. mali* CUPV271 (Table 6) would require more molecules to reach the same number of entanglements than the polymer from *Lc. carnosum* CUPV411, which justifies its higher critical concentration.

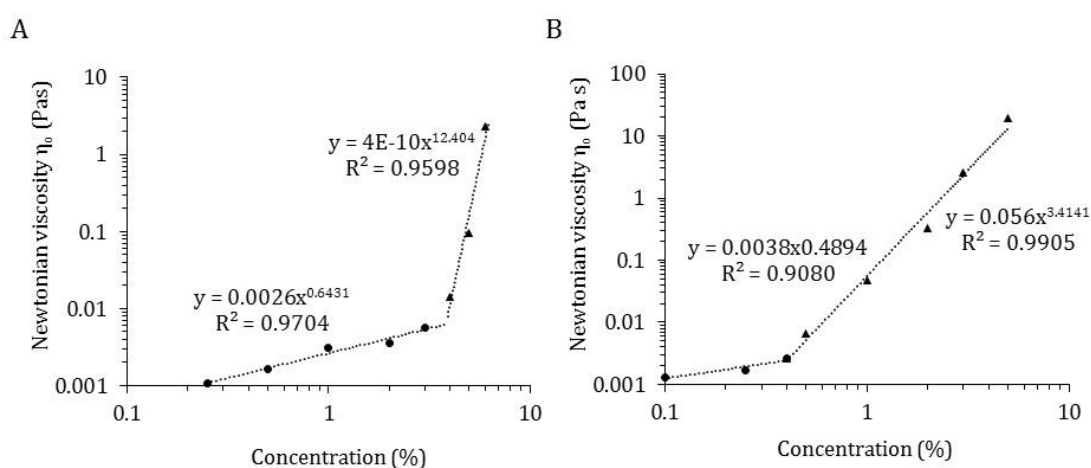


Figure 33. Calculation of the critical concentration (C^*) of the dextrans studied. Representation in a double logarithmic plot of the Newtonian viscosity *versus* the concentration expressed in percentage of the dextrans of (A) *L. mali* CUPV271 and (B) *Lc. carnosum* CUPV411

The shear-thinning data presented above suggest that the dextrans studied would be very suitable to improve the texture or palatability of new fermented products

3.3.6. Dextran yield

As far as our knowledge is concerned, it is the first time that EPS are isolated from *L. mali* and *Lc. carnosum* species. The isolation of the dextrans from the culture supernatants was only possible when sucrose and not glucose was present in the media, as it has been reported by other authors for dextran-producing LAB [107], [134]. The recovery of the EPS after 48 h of incubation in SMDS (2% sucrose), measured by the phenol-sulphuric acid method, was 3.65 ± 0.21 g/L for *Lc. carnosum* CUPV411, similar to the 1.25 ± 0.04 g/L described for *Lc. mesenteroides* RTF10 grown for 13 h in CDMS (0.8% sucrose) [134]. For *L. mali* CUPV271 the recovery was higher than that of CUPV411 in the same conditions, yielding 11.65 ± 1.15 g/L. This concentration exceeded the 2.20 ± 0.09 g/L produced by *L. sakei* MN1 after 13 h of incubation in CDMS [134]. Some species from *Oenococcus* have also been reported to produce EPS. In particular, the strain *O. oeni* S11, isolated from alcoholic beverages (sparkling white wine) in France, produced 3.87 ± 0.02 g/L of dextran in SMDS (1% sucrose) after a two-week incubation period [39]. Thus, the overall results indicate a potential industrial use of the dextrans studied here, although their production levels are well below the 154 g/L reported for *Lc. mesenteroides* NRRL B-512F at optimised conditions for batch fermentation [222]. Considering that neither the medium nor the culture conditions have been optimised in the current work, the perspective for production's improvement is high, supporting the potential use of the *L. mali* CUPV271 dextran for industrial purposes.

3.4. Conclusions

As far as we know, the EPS produced by *Lc. carnosum* and *L. mali* strains have been purified and characterised for the first time. Chemical and spectroscopic analyses revealed that both polymers are *O*-3- and *O*-4-branched dextrans, whose presence affected differentially to the adhesion capacity of the producing LAB. This differential pattern could be due to differences in their supramolecular structures, as deduced from AFM. In addition, these dextrans are amorphous and presented a pseudoplastic behaviour. This shear-thinning property may confer them an advantage in the food industry for improving sensory properties as mouth feel and flavour release. In addition, they would be very suitable for mixing, pouring or pumping, very common processes in the industry. Moreover, *L. mali* CUPV271 produces a considerably higher amount of polymer than *Lc. carnosum* CUPV411, thus, it might be a strong candidate for optimisation aimed to the future development of food, producing the dextran *in vivo*, or being included in the product as an additive.

CHAPTER 4. FUNCTIONAL METABOLITES PRODUCED BY LAB

4.1. Introduction

LAB produce many different metabolites that exert beneficial effects on human health. Among these, are some compounds generated by phytate-degrading enzymes and conjugated linoleic acid (CLA) isomers.

As already mentioned, phytases are hydrolases that act on *myo*-inositol-1,2,3,4,5,6-hexakisphosphate releasing phosphate, the inositol ring, and the lower forms of inositol phosphates (IP5-IP1). Some authors classify phytases as endogenous, exogenous or cell-bound, depending on if they are completely or partially released to the medium or not [25], [223]. In general, phytases from plants are active in a range of 45-55 °C, while their microbial counterparts can tolerate temperatures of 60 °C [224]. Moreover, the optimal temperature for catalysis is another factor that has to be considered.

There exist various classifications of phytases depending on the factors taken into consideration:

- 1) Most authors have described two groups based on the position of the first phosphate hydrolysed: 3-phytases (EC 3.1.3.8) and 6-phytases (3.1.3.26) [95], [96], [225]. Members of the first group are the most frequent and usually have a microbial origin, while the second group has been predominantly observed in plants. However, there also exist some exceptions [96]. Bohn *et al.* [25] also described a third group, 5-phytases (E 3.1.3.72), reported only in lily pollen. These three classes of phytases are accepted by the IUPAC-IUBMB.

- 2) Based on their optimum pH for activity there are two groups: the acidic and the alkaline phytases [95], [96], [225], [226], although sometimes neutral phytases are also added to the classification [25]. In general, phytases of plant seeds act in a range of pH between 4.0 and 5.6; fungal phytases have their optimum activity at pH ranging from 4.5 to 5.5, and finally, bacterial phytases develop their activity at pH between 6.5 and 7.5 [95].

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- 3) According to their active site motifs there exist 4 groups of phytases:
- i) The β -propeller phytases (BPP): these depend on Ca^{2+} , hence they have 3-low affinity and 3-high affinity calcium binding sites. A high number of 3-phytases belong to this group and they have been described predominately in Gram-positive bacteria.
 - ii) The histidine acid phosphatases (HAP): they are the most studied group, to which the 3- and the 6-phytases belong. They are sub-grouped in phytases with low- or high-specific activity for phytic acid hydrolysis. All of them have two conserved motifs: RHGXRXP in the active centre and a catalytic C-terminal HD dipeptide. They also have some amino acids that constitute a specific substrate site called SSS [25], [95], [226], [227].
 - iii) The purple-acid phytases (PAP): these phytases have different conserved motifs: DXG, GDXXY, GNH[E/D], VXXH and GHXH [25], [226].
 - iv) The cysteine-tyrosine phytases (CP): these enzymes form a P loop containing a conserved cysteine (Cys 241) near to its active site. This type of phytase has been described in *Selenomonas ruminantium* [226].

Nowadays, LAB and bifidobacteria are being widely used for the production of bioactive compounds by fermentation. To this end, a lot of research in phytase-producing LAB and bifidobacteria has been performed. Many bifidobacteria are good phytase producers, whereas finding a good phytase-producing LAB is more difficult. Instead, non-specific acid phosphatases are being found in these bacteria, which have high hydrolysis rates of monophosphorylated compounds but a low level of activity against phytate. However, this group of enzymes could also contribute to the hydrolysis of *myo*-inositol with a lower number of phosphate groups [44], [45], [228]. Moreover, some research groups have cloned

Bifidobacterium phytases into LAB with good results in phytate-content reduction in fermented drinks [229]–[231].

The remainder of this chapter will focus predominately on CLA.

Ruminants consume products containing essential PUFAs such as LA (ω -6), which is a toxic compound for some microorganisms present in the rumen. Anaerobic bacteria, such as *Butyrivibrio fibrisolvens*, which is present in the rumen, perform a detoxifying pathway which consists of the biohydrogenation of dietary LA to form saturated fatty acids, such as the less toxic stearic acid [34], [232]. In this metabolic pathway, CLA act as intermediates and the transformation takes place in two steps: first, the isomerisation of the LA carried out by the bacterial linoleate isomerase (LAI) leads to the appearance of CLA. Then, the hydrogenation of a double bond yields the *trans*-vaccenic acid. Finally, *t*-vaccenic acid is reduced to stearic acid (Figure 34). In addition, the presence of an endogenous enzyme in the mammary gland, Δ 9 desaturase, can also form CLA by the conversion of the *t*-vaccenic acid [34], [233], [234].

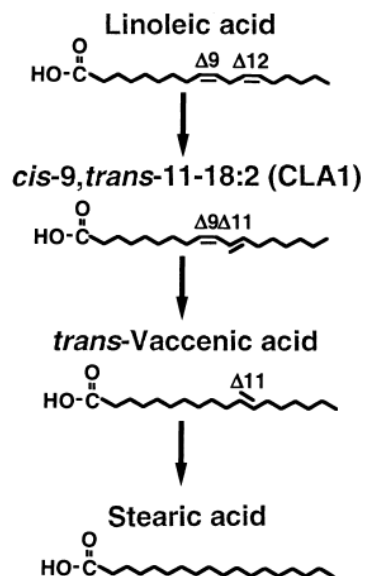


Figure 34. Proposed pathway of linoleic acid hydrogenation to stearic acid by *Butyrivibrio fibrisolvens*.

Image reproduced from Ogawa *et al.* (2005).

Introduction

However, LAB follow different pathways to synthesise CLA. The simplest one, reported for *L. acidophilus* AKY1137 [235], involves a first hydration step in which hydroxyl fatty acids are formed, followed by a dehydrating isomerisation which leads to CLA isomers formation [233], [236] (Figure 35).

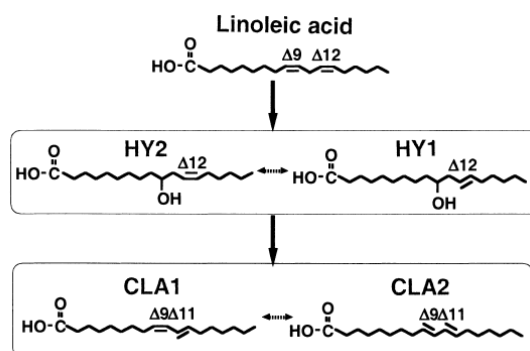


Figure 35. Proposed pathway for linoleic acid isomerisation to CLA by *L. acidophilus*. Image reproduced from Ogawa *et al.* (2005).

LAI from a *L. plantarum* strain was described as a multi-component system consisting of four enzymes, and the first step of the metabolic pathway is carried out by a 10-hydratase called CLA-HY (encoded by the *cla-hy* gene), which acts specifically on the double bond of C9 (Figure 36). In addition, this pathway includes the synthesis of different intermediates such as hydroxyl fatty acids, oxo-fatty acids or CLA isomers [237], [238]. These compounds have gained much attention lately because they have been described to have antifungal, immunomodulatory and lipogenesis stimulatory activities [51], [239]–[241]. In addition, 13-hydratases, which act on the other double bond present in LA (C12), have also been described [242], [243]. Moreover, this hydration has also been reported to be carried out by the myosin cross-reactive antigen (MCRA) as a complementary function in some LAB [236], [244]–[246].

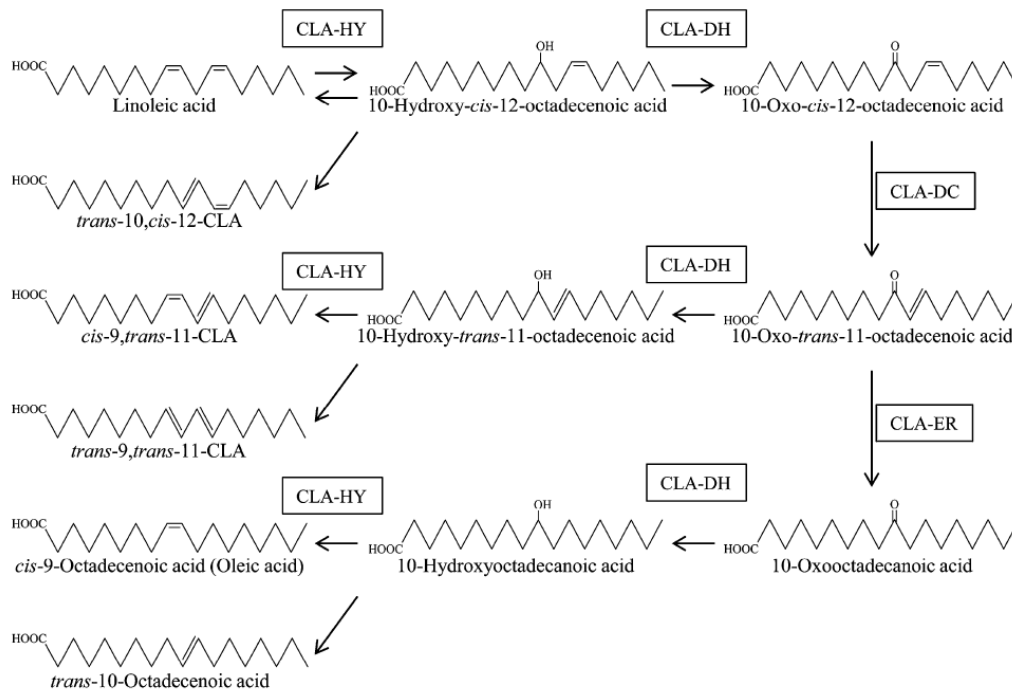


Figure 36. Polyunsaturated fatty acid-metabolism pathway in *L. plantarum*.

Image reproduced from Kishino *et al.* (2013). CLA-HY, responsible for hydration/dehydration; CLA-DH, responsible for oxidation of hydroxyl groups/reduction of oxo groups; CLA-DC, responsible for the migration of carbon-carbon double bonds; CLA-ER, responsible for the saturation of carbon-carbon double bonds.

LAB have attracted special attention as a source of bacterial LAI enzymes. They are considered as GRAS microorganisms by the FDA. Moreover, they are able to ferment dairy products, so they would be very suitable for the development of new functional beverages. In addition, they have been reported to have the ability to synthesise solely a CLA isomer, or a great majority of one of them when producing a mixture [247]–[250]. However, there are some technological difficulties in the production of the isomers due to several factors such as toxicity of high concentrations of LA to the bacteria, the growth temperature, the presence of oxygen in the process, etc. Fortunately, many current studies are being carried out to overcome these drawbacks.

Therefore, the aim of the research in this chapter was to screen a large number of LAB from the CUPV, isolated from ropy natural cider, for their ability to produce phytate-degrading enzymes and CLA.

4.2. Materials and Methods

4.2.1. Strains and culture conditions

The two screenings performed for the search of phytate-degrading activity and production of CLA were performed in LAB (lactobacilli and pediococci) that belong to the CUPV. All LAB analysed were recovered between 1992 and 2009 from ropy ciders, from different factories in the Basque Country (Spain). The lactobacilli and pediococci included in this study were selected on the basis of their ropy phenotype by using a method previously described [251]; their colonies forming long filaments when touched with a loop, and also exhibiting a ropy appearance in MRS broth. Thirty-nine isolates belonged to the genus *Pediococcus*, and were mainly screened for phytate-degrading activity, since two strains of *Pediococcus pentosaceus* had been previously reported to show a specific phytate-degrading activity [252]. Eleven isolates belonging to the genus *Lactobacillus* were mainly screened for CLA production. Table 10 gathers the species names and the active compound for which they were screened.

Table 10. LAB strains used in the screening of bioactive compounds.

Strains	Reference	Screening
<i>P. parvulus</i> CUPV1	[12]	Phytase and CLA
<i>P. parvulus</i> CUPV22		CLA
<i>P. parvulus</i> P2	[52]	Phytase
<i>P. parvulus</i> P3		
<i>P. parvulus</i> P4		
<i>P. parvulus</i> P5		
<i>P. parvulus</i> P6		
<i>P. parvulus</i> P7		
<i>P. parvulus</i> P8		
<i>P. parvulus</i> P9		
<i>P. parvulus</i> P10		
<i>P. parvulus</i> P11		
<i>P. parvulus</i> P12		

<i>P. parvulus</i> P13		
<i>P. parvulus</i> P14		
<i>P. parvulus</i> P16		
<i>P. parvulus</i> P18	[52]	
<i>P. parvulus</i> P19		
<i>P. parvulus</i> P20		
<i>P. parvulus</i> P21		
<i>P. parvulus</i> P22		
<i>P. parvulus</i> P23		
<i>P. parvulus</i> P24		
<i>P. parvulus</i> P25		
<i>P. parvulus</i> P26		Phytase
<i>P. parvulus</i> P27		
<i>P. parvulus</i> P28		
<i>P. parvulus</i> P29		
<i>P. parvulus</i> P30	CUPV	
<i>P. parvulus</i> P31		
<i>P. parvulus</i> P32		
<i>P. parvulus</i> P33		
<i>P. parvulus</i> P34		
<i>P. parvulus</i> P35		
<i>P. parvulus</i> P36		
<i>P. parvulus</i> P37		
<i>P. parvulus</i> P38		
<i>P. parvulus</i> 2.6	[138], [142]	Phytase and CLA
<i>P. ethanolidurans</i> CUPV141	This work	Phytase
<i>L. collinoides</i> CUPV231		CLA
<i>L. collinoides</i> CUPV2322	CUPV	Phytase
<i>L. collinoides</i> CUPV237	[253]	
<i>L. diolivorans</i> CUPV2110	CUPV	Phytase and CLA
<i>L. diolivorans</i> CUPV218	[38]	CLA
<i>L. suebicus</i> CUPV225	[110]	Phytase and CLA

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<i>L. suebicus</i> CUPV226		
<i>L. sicerae</i> CUPV261 ^T		
<i>L. sicerae</i> CUPV262	[251]	Phytase
<i>L. plantarum</i> CUPV241		
<i>L. plantarum</i> CUPV243	CUPV	CLA

LAB were grown in modified MRS broths in both assays: (i) phy-MRS medium [45] and (ii) LA-MRS medium [254] for phytate-degrading activity and CLA production, respectively. In phy-MRS medium, the inorganic phosphate was replaced by 0.65 g/L sodium phytate (Sigma-Aldrich) and 0.1 M MES buffer (Sigma-Aldrich). The content of yeast and meat extracts was also reduced to 2 g/L and 4 g/L, respectively, in order to create a low-phosphate environment to avoid inactivation of phytate-degrading enzymes [44], [255]–[257]. In LA-MRS medium, free LA was added at a final concentration of 0.5 mg/mL from a stock solution (50 mg/mL) prepared in distilled water containing 2% (w/v) Tween 80. This concentration of LA was reported as not inhibitory for LAB [26], [254], [258].

For the screening of phytate-degrading activity, LAB strains were first activated in MRS-phy medium pH 5.5 at 28 °C and under a 5%-CO₂-atmosphere. Then, they were propagated for two generations under the same conditions, and finally, inoculated into phy-MRS medium at OD_{600nm} of 0.2 and incubated for 20 h.

For the enzymatic assays, whole-cell suspensions were used: for each strain, three samples were collected at OD_{600nm} of 2.0. Cells were harvested by centrifugation (11600 × *g*, 10 min, 4 °C) and washed with 50 mM Tris/HCl (pH 6.5). Finally, they were suspended in 50 mM sodium acetate-acetic acid buffer (pH 5.5) and maintained in an ice bath until use.

4.2.2. Determination of phytase activity

Phytase activity was determined by measuring the release of inorganic phosphate from sodium phytate [259].

First, a standard curve was prepared using KH_2PO_4 (Appendix 1, section 1.4). A stock standard solution at 2 mg P/mL in 50 mM sodium acetate-acetic acid buffer (pH 5.5) was prepared (KH_2PO_4 was first dried for 2 h at 105 °C). It was used to prepare a working standard solution (40 μg P/mL), which served for the preparation of eleven solutions with different concentrations of phosphorus (0, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 $\mu\text{g}/\text{mL}$). Finally, the inorganic phosphorus was determined by the ammonium molybdovanadate method [260], as follows: 2 mL of each solution were mixed with 2 mL of vanadate-molybdate reagent (Sigma), incubated for 10 min at 30 °C and dispensed into a microtiter plate for measuring the absorbance at 415 nm.

The enzyme activity was determined by incubating 400 μL of 0.1 M sodium acetate pH 5.5, containing 1.2 mM sodium phytate and 200 μL of whole-cell suspension for 1 h at 50 °C. Then, the reaction was stopped by adding 100 μL of 20% TCA and the samples were centrifuged (11600 $\times g$, 5 min, 4 °C). Five hundred μL of the supernatants were mixed with 500 μL of vanadate-molybdate reagent (Sigma). The mixture was incubated for 10 min at 30 °C and four aliquots of 200 μL were added to wells of a microtiter plate for absorbance measurement at 415 nm. The concentration of inorganic phosphorus liberated was calculated using the standard curve obtained previously.

Phytase from *Aspergillus oryzae* DSM 14223 (Novozymes) was used as positive control.

One unit of phytase activity (U) was defined as the amount of enzyme that produces 1.0 μmol of inorganic phosphorus (Pi) per hour at 50 °C.

4.2.3. Determination of acid phosphatase activity

Acid phosphatase activity was determined by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate [44].

For quantification, a standard curve was prepared (Appendix 1, section 1.5). First, solutions containing 0, 3, 5, 15, 25, 35, 45, 56 and 60 $\mu\text{g}/\text{mL}$ *p*-nitrophenol were

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prepared. Then, 50 μL of each solution was mixed with 50 μL of 50 mM sodium acetate-acetic acid buffer (pH 5.5) and 100 μL of 1 M NaOH and dispensed in wells of a microtiter plate for measuring the absorbance at 415 nm.

For the activity assay, the reaction mixture was composed of: 250 μL of 0.1 M sodium acetate pH 5.5, containing 5 mM *p*-nitrophenyl phosphate, and 250 μL of whole-cell suspensions. After incubation for 1 h at 50 °C, the reaction was stopped by addition of 500 μL 1 M NaOH. Finally, samples were centrifuged (11600 $\times g$, 5 min, 4 °C) and aliquots of the supernatants (4 \times 200 μL) were dispensed into wells of a microtiter plate for absorbance measurement at 415 nm. The concentration of *p*-nitrophenol liberated was calculated from the absorbance measured through the standard curve. Three positive controls, with reported phosphatase activity [44], were used: *Lactobacillus pentosaceus* CECT4023, *Lactobacillus hilgardii* CECT4786^T, and *Lactobacillus reuteri* CECT925.

One unit of phosphatase activity (U) was defined as the amount of enzyme that produces 1.0 μmol of *p*-nitrophenol per hour at 50 °C.

4.2.4. Detection of a hydratase gene in LAB by PCR

The enzyme responsible for the first step in the biohydrogenation of LA has been described as a hydratase which can act on one of the LA's double bonds, depending on its specificity [242]. The amino acid sequence of a 10-hydratase CLA-HY (BAL42246.1, Appendix 2, section 2.6) from *L. plantarum* AKU1009a [237] was available at the NCBI site. This sequence was used as template for the search of homologies using BLAST with the translated proteins of genomes of two bacteria from our laboratory: *L. collinoides* CUPV237 [253] and *P. parvulus* 2.6 [173]. For each strain a unique putative linoleate double bond hydratase enzyme homologous to CLA-HY was found. The DNA coding sequences of these two putative enzymes (Appendix 2, sections 2.7 and 2.8) were used for the design of a pair of degenerate primers (HY1F: 5' -TTYTGGTAYATGTGGSARACNACN- 3' and HY1R: 5' -NGCCATYTCYTTNCCNGTCA- 3') with the aim of making a first genetic screening for CLA-producing LAB. For the PCR detection, reactions of 25 μL containing 5

pmol/ μ L of each primer, dNTP mix 0.4 mM (TaKaRa), 250 ng DNA of genomic DNA of a LAB strain, 1 U *Taq* DNA Polymerase (TaKaRa), and 1X buffer (TaKaRa), were used. The reactions were maintained at 94 °C for 5 min, and then, 35 cycles of 30 s at 94 °C, 30 s at 51.5 °C and 45 s at 72 °C were performed.

The expected amplicons for lactobacilli and pediococci were 789 bp and 765 bp in size, respectively.

4.2.5. Linoleic acid (LA) effect on LAB's growth

LA can inhibit the growth of some bacteria [247], [261], [262]. Thus, although the concentration of LA chosen (0.5 mg/mL) has been reported as non-toxic for LAB [26], [254], [258], a first assay for testing the influence of LA on LAB's growth was conducted. For that purpose, bacteria were suspended in MRS medium pH 5.5 at 28 °C and grown under a 5% CO₂ atmosphere. Then, the bacteria were inoculated at 1% into MRS broth (10 mL) in the absence of LA or in the presence of 0.5 mg/mL of LA (Sigma, 99% purity) added at 0 h or after 7 h of incubation. Bacteria were incubated for 72 h at 28 °C in a 5% CO₂. The growth of LAB was monitored by measuring the OD at 600 nm at 0 h, 7 h and 72 h. LA-MRS medium alone was used as blank. Also, to detect changes in the turbidity of the medium produced by the LA, the OD was measured before and after the addition of the fatty acid.

4.2.6. UV-based spectrophotometric screening for CLA production

CLA production by LAB was primarily determined using a UV-spectrophotometric method previously described [258], with modifications. Briefly, 5 mL of 72-h cultures grown in LA-MRS medium (0.5 mg/mL LA) were centrifuged (12900 $\times g$, 5 min, 4 °C) and fatty acids were extracted from the culture supernatants. For each strain, 1 mL of each supernatant was mixed with 2 mL of isopropanol and 3.5 mL of n-hexane. Afterwards, the mixture was vortexed vigorously for 15 s and allowed to stand for 3 min. Finally, 2.5 mL of distilled water was added, and the mixture was vortexed and allowed to stand for 6 min. The upper phase (3 mL) was collected in 1 cm-quartz cuvettes and its absorbance was measured in a Helios α

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spectrophotometer (Thermo Spectronic) at 233 nm (characteristic of fatty acids with conjugated double bonds). To determine the concentration of CLA produced by the LAB strains, a standard graph was performed by using the *c9, t11*-CLA isomer (Larodan) in a concentration range from 2.5 µg/mL to 80 µg/mL. The linear regression equation of the standard curve was $A=0.1094C + 0.1093$, where A refers to the absorbance value of CLA at 233 nm and C refers to the CLA concentration (Appendix 1, section 1.6).

4.2.7. Identification of CLA isomers by gas chromatography-mass spectrometry (GC-MS)

Cultures of the strains selected after the evaluation of CLA production (section 4.2.6) were centrifuged ($12900 \times g$, 5 min, 4 °C) and 1 mL of the supernatants was used to extract lipids following the protocol detailed in section 4.2.6, but 0.35 mg of nonadecanoic acid (C19:0) was added before the extraction to calculate the CLA yield. Lipids from the sedimented bacteria were extracted as follows: the pellet from 1 mL culture was resuspended in 2.5 mL of distilled water. Then, cells were mechanically disrupted with a Pellet Pestle Cordless Motor (Kimble Chase Life Science and Research Products LLC). Then, 0.1 mg of C19:0 standard and isopropanol (2 mL) were added and the mixture was vortexed for 15 s and allowed to stand for 3 min. Finally, three washes with n-hexane (3.5 mL each time) were made, vortexing for 15 s and holding the mixture for 3 min between each addition. Then, the lipids extracted from the supernatant and from the pellet were methylated following the protocol of Zabala *et al.* [263] with modifications. The organic layer was evaporated at 35 °C under a N₂ stream. Then, 0.1 mg (0.007 mg for the pellet samples) of heptadecanoic acid (C17:0) was added as internal standard. For methylation, 1 mL (0.5 mL for pellet samples) of chloroform:methanol (1:2) and 0.2 mL (0.1 mL for pellet samples) of trimethylsilyldiazomethane was added. The mixture was vortexed, and allowed to stand for 30 min at RT. To stop the reaction, glacial acetic acid was added drop by drop until the yellow colour disappeared. Finally, organic compounds were evaporated under a stream of nitrogen and the fatty acid methyl esters (FAMES)

were dissolved in 1 mL of n-hexane and maintained at -20 °C in amber glass vials until they were analysed by GC-MS.

One-microliter sample (FAME) was injected into an Agilent Technologies 7890A gas chromatograph in (1:100) split mode using helium (1.8 mL/min) as carrier gas. The injector temperature was 220 °C. The GC was fitted with a mass detector (Agilent Technologies 5975C) working at 70 eV and 230 °C. The column used was an Innowax capillary column (30 m x 0.32 mm I.D., 0.25 µm thickness). Samples were maintained at 150 °C for 1 min. Then, the temperature was raised (10 °C/min) up to 200 °C and maintained for 5 min. Finally, temperature was raised again (6 °C/min) up to 230 °C and maintained for 30 min. The qualitative analysis of CLA was performed by comparison of the R_t with methylated CLA standards (Larodan and Sigma-Aldrich, GC purity): methyl palmitate (C16:0), methyl heptadecanoate (C17:0), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl nonadecanoate (C19:0), 9(*E*), 11(*E*)-octadecadienoic acid methyl ester CLA, 9(*Z*), 11(*E*)-octadecadienoic acid methyl ester CLA, and 10(*E*), 12(*Z*)-octadecadienoic acid methyl ester CLA. The quantitative analysis was made calculating the compounds' concentration from independent standard curves performed for the aforementioned standards from 0.02 mg/mL to 0.5 mg/mL. To generate a calibration curve, all standards except C17:0 were added at 1 mg/mL concentration in a final volume of 5 mL hexane. This was the stock solution to prepare different dilutions in a final volume of 1.5 mL hexane. Internal standard was added to each final tube at 0.1 mg/mL.

As the rumenic acid was the only detectable CLA produced by the LAB, we only worked with its regression curve (Appendix 1, section 1.7). An increase in the c_9 , t_{11} -CLA concentration (from 20 µg/mL to 500 µg/mL) coincided with a linear increase in response ($R^2=0.996$; $y=0.051x$) for the C18:2 c_9 , t_{11} -CLA isomer.

4.2.8. Purification of the unidentified compound by Ag⁺-thin layer chromatography (Ag⁺-TLC)

Production of intermediates in the LA detoxification pathway as hydroxy fatty acids in a O₂-limited condition has been reported [264]. Thus, we approached growth of *L. sicerae* CUPV261^T in microaerophilic conditions in order to produce a greater amount of the undefined compound. Two 30-mL tubes completely full of MRS broth containing 1.5 mg/mL LA were inoculated with CUPV261^T strain and incubated for 72 h under a 5% CO₂ atmosphere. Aliquots of 1 mL of the culture supernatants were recovered and fatty acids were extracted as detailed in section 4.2.3. The organic layers containing the FA were gathered together and the solvent was evaporated with a N₂ stream. Then, 60 mL of chloroform:methanol (1:2) and 20 mL of trimethylsilyldiazomethane were added for methylation of the samples for 30 min at RT. The reaction was stopped with some drops of glacial acetic acid and the solvent was evaporated under a N₂ stream. Finally, the extracted and methylated fatty acids (126.33 mg) were dissolved in 1 mL of n-hexane and loaded into a TLC plate. Three standards were loaded as well: LA (5 mg), methylated LA (5 mg) and methylated *c*9, *t*11-CLA (1.5 mg). The TLC analysis was performed following previously reported protocols [26], [265] in a 20 x 20 cm silica gel plate (Merck). It was poured with a 5% AgNO₃ solution in acetonitrile and kept covered overnight to avoid evaporation of the solvent. Then, the plate was activated by maintaining it for one hour at 100 °C. The plate was eluted with chloroform-acetone (96:4 v/v) twice, and spots were visualised with an UV-lamp by spraying with a solution of 2,7-dichlorofluorescein in 2-propanol (0.2%). The spots that did not fit with any of the standards were removed with a scraper and added to a tube containing 1 mL of n-hexane, vortexed for 1 min and samples were transferred to a second tube by filtering through glass wool. This process was repeated three times to assure extracting the compound. Finally, the sample in the second tube was filtered through a 0.22 µm filter, the solvent was evaporated under N₂ stream and the sample was analysed by NMR.

A 1 µL sample was subjected to GC-MS to confirm that the intermediate had been recovered.

4.2.9. Identification of the hydroxyl-octadecaenoic acid by NMR

The sample purified by Ag⁺-TLC was dissolved in deuterated chloroform (CDCl₃) and subjected to ¹H-NMR spectroscopy at 298 K on a Bruker Avance NEO spectrometer operating at 500.13 MHz.

4.3. Results and Discussion

4.3.1. Screening for phytase and acid phosphatase activities among CUPV LAB

The CUPV contains various *P. parvulus* strains isolated from ropy ciders. As a specific phytate-degrading activity was reported in two *P. pentosaceus* strains isolated from chicken intestine [252], we screened for a phytate-degrading activity among the *Pediococcus* strains and some other bacteria within the CUPV collection.

Phytase activity has been reported to be bound to the cell-walls [44], [45], [252], [255], [259], [266], [267], to be cytoplasmic [266]–[269] or to be present in the culture supernatants [46], [228], [269] of different microorganisms. A rapid preliminary search for phytase activity in *P. parvulus* 2.6 [142] was performed, using cell suspensions, culture supernatants and cellular extracts disrupted by sonication, vortexing or glass-beads. However, no phytase activity was observed in any of the samples assayed. Thus, since the cell wall is the most frequent location for phytases among bacteria, the cell suspensions of the bacteria tested were screened in more depth, and the acid phosphatase activity was also evaluated. The procedures reported for the detection of these activities do not specify a consensus reaction and we first performed a second rapid assay incubating for 30, 60, 90 and 900 (overnight) min for the detection of phytases and 15, 30, 60 and 900 min for the acid phosphatase activity. Phytase activity was not observed at any of the incubation times assayed. However, acid phosphatase activity was detected (Figure 37) with a maximum value after 60 min, which decreased at longer incubation time. In addition, the 30-min incubation proposed by Palacios *et al.* [44] seemed insufficient for the detection of activity in *P. parvulus* 2.6. Consequently, the screening experiments were carried out using a reaction time of sixty min for both activities and measuring immediately after this time.

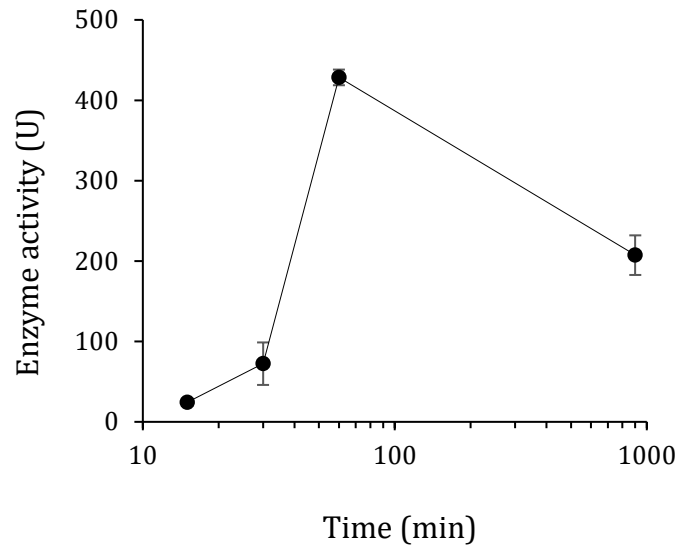


Figure 37. Representation of the phosphatase activity detected in cell suspensions of *P. parvulus* 2.6 at different times. One unit (U) is defined as the amount of enzyme that produces 1.0 nmol of *p*-nitrophenol per time assayed at 50 °C.

Once the assays were clearly defined, *Pediococcus* strains and some lactobacilli from the CUPV were screened for phytase and phosphatase activities. No phytase activity was detected under the conditions assayed in any of the strains tested (Table 11). On the contrary, the majority of them showed an acid phosphatase activity, although it was not very high (Table 11).

Table 11. Acid phosphatase activity of several CUPV LAB strains.

Strains	Phosphatase activity (U)
<i>P. parvulus</i> CUPV1	ND
<i>P. parvulus</i> P2	0.85 ± 0.17
<i>P. parvulus</i> P3	ND
<i>P. parvulus</i> P4	ND
<i>P. parvulus</i> P5	0.24 ± 0.05
<i>P. parvulus</i> P5.1A	1.07 ± 0.04
<i>P. parvulus</i> P5.1B	0.48 ± 0.04
<i>P. parvulus</i> P6	ND

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<i>P. parvulus</i> P7	ND
<i>P. parvulus</i> P8	ND
<i>P. parvulus</i> P9	0.20 ± 0.05
<i>P. parvulus</i> P10	0.20 ± 0.01
<i>P. parvulus</i> P11	0.19 ± 0.01
<i>P. parvulus</i> P12	0.33 ± 0.02
<i>P. parvulus</i> P13	0.33 ± 0.01
<i>P. parvulus</i> P14	0.39 ± 0.06
<i>P. parvulus</i> P16	0.36 ± 0.02
<i>P. parvulus</i> P18	0.26 ± 0.01
<i>P. parvulus</i> G3B1	0.40 ± 0.01
<i>P. parvulus</i> G3B2	0.30 ± 0.05
<i>P. parvulus</i> G3B3	0.40 ± 0.01
<i>P. parvulus</i> G3B4	0.41 ± 0.03
<i>P. parvulus</i> G5B1A	0.16 ± 0.01
<i>P. parvulus</i> G5B1B	0.22 ± 0.01
<i>P. parvulus</i> G5B2	ND
<i>P. parvulus</i> G6B1	0.20 ± 0.01
<i>P. parvulus</i> G6B2	ND
<i>P. parvulus</i> G6B3	ND
<i>P. parvulus</i> G6B5	ND
<i>P. parvulus</i> G6B7	0.21 ± 0.08
<i>P. parvulus</i> Zal1C1	0.38 ± 0.02
<i>P. parvulus</i> Zal2C1	0.17 ± 0.01
<i>P. parvulus</i> Zal2C2	0.16 ± 0.02
<i>P. parvulus</i> Zal2C3	0.14 ± 0.01
<i>P. parvulus</i> Zal2C4A	0.17 ± 0.05
<i>P. parvulus</i> Zal2C4B	0.16 ± 0.02
<i>P. parvulus</i> 2.6	0.67 ± 0.05
<i>P. ethanolidurans</i> CUPV141	0.23 ± 0.04
<i>L. collinoides</i> CUPV237	ND
<i>L. collinoides</i> CUPV2322	0.17 ± 0.02

<i>L. diolivorans</i> CUPV2110	ND
<i>L. suebicus</i> CUPV225	0.39 ± 0.04
<i>L. suebicus</i> CUPV226	0.29 ± 0.04
<i>L. sicerae</i> CUPV261 ^T	0.64 ± 0.05
<i>L. sicerae</i> CUPV262	0.17 ± 0.01
<i>L. hilgardii</i> CECT4786 ^T	1.46 ± 0.06
<i>L. reuteri</i> CECT925	1.52 ± 0.05
<i>L. pentosus</i> CECT4023	0.43 ± 0.02

Data presented as mean ± standard deviation.

ND: no detected activity

These results were to be expected, as some authors have reported acid phosphatases presenting a non-specific phytate-degrading activity in *L. plantarum*, *L. acidophilus* and other LAB [44], [45], [228], which confers to the producing-LAB the capacity to rapidly hydrolyse the monophosphorylated compounds while having a low activity rate against phytate. The positive controls *L. reuteri* CECT925 and *L. hilgardii* CECT4786^T presented the highest phosphatase activities, as also expected. However, the values detected were very low compared with those previously reported [44]. *L. pentosus* CECT4023 also showed a very low phosphatase activity. Three pediococci exhibited the highest phosphatase activities among the tested CUPV strains: P19 (1.07 ± 0.04 U), P2 (0.85 ± 0.17 U) and 2.6 (0.67 ± 0.05 U). In addition, *L. sicerae* CUPV261^T presented the highest phosphatase activity (0.64 ± 0.05 U) among the lactobacilli assayed.

4.3.2. Screening for CLA production among CUPV LAB

4.3.2.1. Detection of *cla-hy* gene by PCR

The first step in CLA production is carried out by a 10- or 13-hydratase, depending upon which double bond of the LA it includes the OH group. As the nucleotide sequence of the *cla-hy* gene, encoding a 10-hydratase, has already been reported [237], a first screening was performed based on the PCR technique looking for homologues to the *cla-hy* gene among the CUPV LAB strains. The bacteria whose

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genomes were analysed are summarised in Table 10 in the Materials and Methods section. In addition, *L. plantarum* CECT220 was included in this assay because it was previously reported to produce CLA [270]. The DNA sequences of the genes that codify for putative hydratases from *L. collinoides* CUPV237 and *P. parvulus* 2.6 strains were used to design degenerate primers, and these strains were used as the positive controls. Figure 38 shows the amplicons obtained by PCR amplification for each strain. All the strains showed a band corresponding to the hydratase gene, sizing 789 bp for lactobacilli (Figure 38A) and 765 bp for pediococcal strains (Figure 38B). Thus, if the gene happened to be functional, the LAB tested would be able to carry out the hydration of LA in a first step of the biohydrogenation pathway. Despite the fact that LAB isolated from cider were not previously reported as CLA-producing bacteria, these results showed that all cider-isolated strains tested are potential producers of CLA isomers. Consequently, all strains were incubated in the presence of free LA to test the ability to produce CLA, and if applicable, to identify the specific isomers.

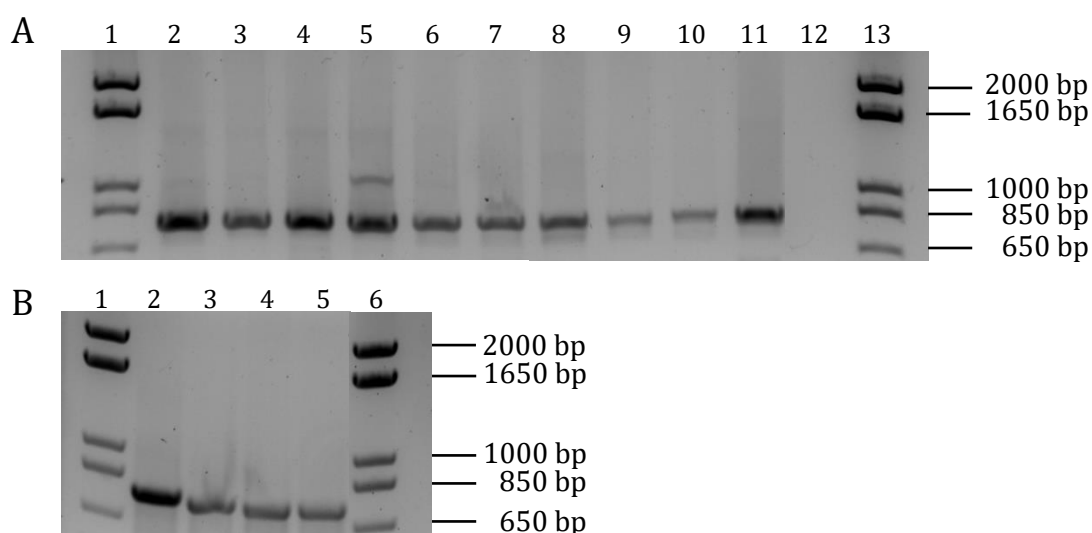


Figure 38. Amplicons obtained with the degenerated primers HY1F and HY1R. (A) 789-bp amplicons of lactobacilli. From left to right: lane 1, size standard; lane 2, *L. collinoides* CUPV237 (positive control); lane 3, *L. plantarum* CECT220; lane 4, *L. plantarum* CUPV241; lane 5, *L. plantarum* CUPV243; lane 6, *L. collinoides* CUPV231; lane 7, *L. sicerae* CUPV261^T; lane 8, *L. suebicus* CUPV225; lane 9, *L. suebicus* CUPV226; lane 10, *L. diolivorans* CUPV2110; lane 11, *L. diolivorans* CUPV218; lane 12, negative control; lane 13, size standard. (B) 765-bp amplicons of pediococci. From left to right: lane 1, size standard; lane 2, *L. collinoides* CUPV237 (positive control); lane 3, *P. parvulus* 2.6 (positive control); lane 4, *P. parvulus* CUPV1; lane 5, *P. parvulus* P22; lane 6, *P. ethanolidurans* CUPV141; lane 7, size standard.

4.3.2.2. Effect of LA on bacterial growth

LA is a toxic compound for bacteria, which convert it into less harmful components through biohydrogenation. Consequently, it is a logical expectation that the addition of LA would inhibit bacterial growth, unless it is metabolised to form CLA. Therefore, to test the hypothesis, a rapid assay was first performed based on the literature [254], in which 0.5 mg/mL of LA was added to analyse if the compound affected the growth of the bacteria tested. Figure 39 shows the data of the OD_{600nm} of bacterial cultures of 0 h (Figure 39A), 7 h (Figure 39B) and 72 h (Figure 39C), when LA was added at the beginning of the assay, after 7 h of growth, or without LA supplementation. Before starting the experiment, we confirmed that the addition of LA did not affect the turbidity of the culture medium, measuring OD_{600nm} before and after the addition of the fatty acid.

The OD of each LAB culture at 0 h was virtually the same under the three conditions tested (Figure 39A). Cultures where LA was added at the beginning of the incubation presented lower values of OD at 7 h of growth than the control cultures where LA had not been added (Figure 39B), suggesting that, under these conditions, a longer lag phase occurred during the first 7 h of incubation with LA, and consequently, there was some growth inhibition due to the LA added. However, at the end of the exponential phase, after 72 h of incubation (Figure 39C), all the strains had reached the same values of OD with quantitatively no relevant differences. In conclusion, as reported by other authors, it seemed that LA at 0.5 mg/mL was not inhibitory for CUPV LAB strains regardless of the time of the addition of the fatty acid.

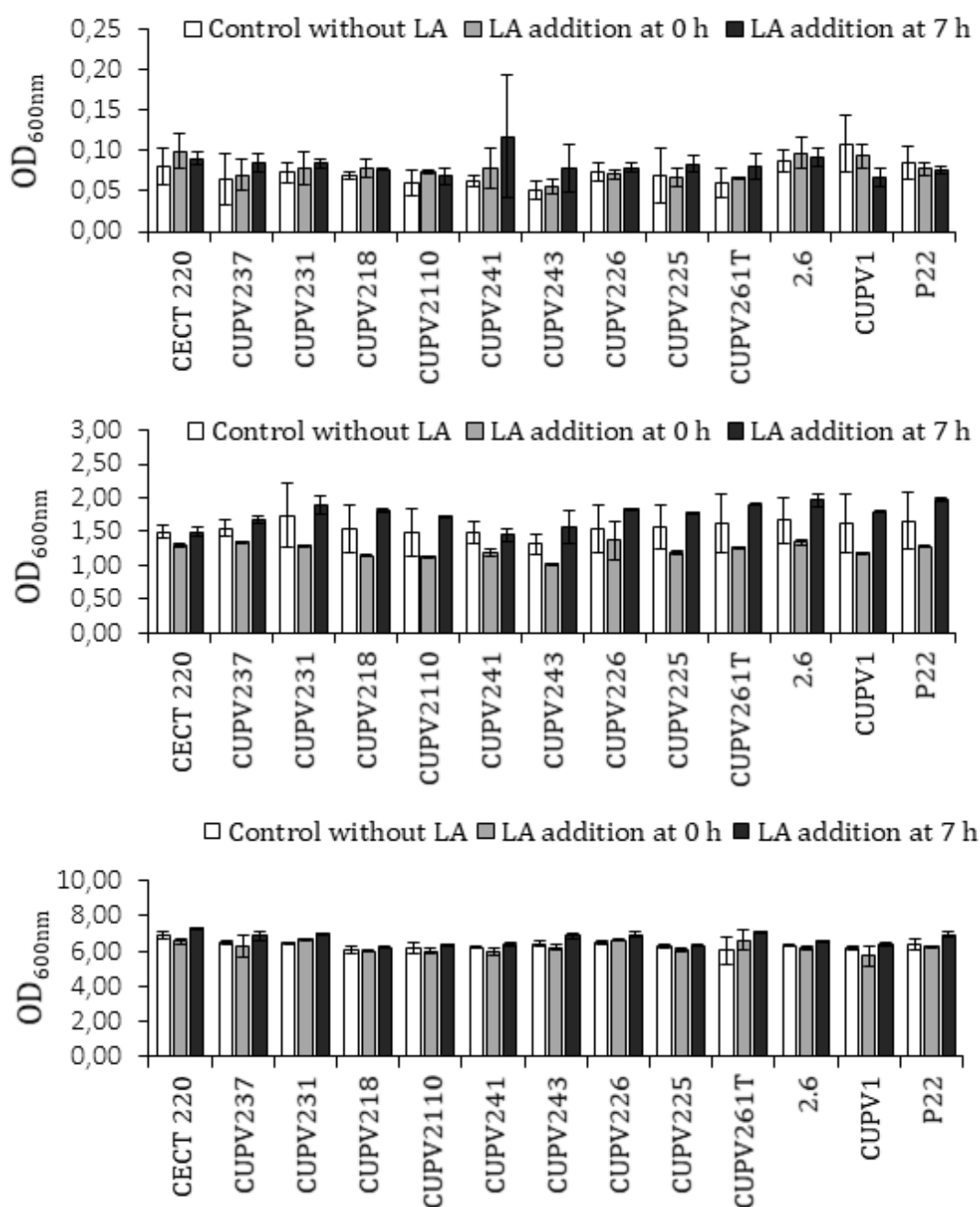


Figure 39. Growth of LAB detected by measurement of OD_{600nm} at 0 h (A), 7 h (B) and 72 h (C) in the presence of LA added at 0 h or after 7 h of incubation as well as without LA addition.

4.3.2.3. UV-based spectrophotometric screening for CLA production

The experiments depicted in Figure 39 showed that the exposure of LAB isolated from cider to 0.5 mg/mL of LA does not impair their growth. Thus, a first

experiment to confirm CLA production was performed by adding the fatty acid at the beginning of the incubation.

A standard curve was performed (Appendix 1, section 1.6) in which an increase in the CLA concentration (from 2.5 µg/mL to 20 µg/mL) coincided with a linear increase in absorbance ($R^2=0.999$; $y=0.1094x + 0.1093$) for the C18:2 *c*9, *t*11-CLA isomer up to an absorbance of 2.3. Thus, CLA concentration was estimated in the organic layer extracted from the cultures by measuring its absorbance at 233 nm. The results of LAB screening for CLA production are presented in Table 12.

Table 12. CLA content in the cultures supernatants calculated with the standard curve of *c*9, *t*11-CLA isomer.

Strains	[CLA] ± SD (µg/mL)
<i>L. plantarum</i> CECT220	6.16 ± 0.41
<i>L. collinoides</i> CUPV237	4.98 ± 0.32
<i>L. collinoides</i> CUPV231	5.92 ± 0.35
<i>L. diolivorans</i> CUPV218	ND
<i>L. diolivorans</i> CUPV2110	ND
<i>L. suebicus</i> CUPV226	3.16 ± 0.03
<i>L. suebicus</i> CUPV225	ND
<i>L. sicerae</i> CUPV261 ^T	5.66 ± 1.02
<i>L. plantarum</i> CUPV241	ND
<i>L. plantarum</i> CUPV243	ND
<i>P. parvulus</i> 2.6	2.30 ± 0.23
<i>P. parvulus</i> CUPV1	ND
<i>P. parvulus</i> P22	ND

From the thirteen bacteria analysed, only six produced CLA. The positive control *L. plantarum* CECT220 [270], was the one with the highest CLA production (6.16

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± 0.41 $\mu\text{g/mL}$), followed by *L. collinoides* CUV231, *L. sicerae* CUPV261^T and *L. collinoides* CUPV237 strains (5.92 $\mu\text{g/mL}$, 5.66 $\mu\text{g/mL}$ and 4.98 $\mu\text{g/mL}$, respectively). Finally, *L. suebicus* CUPV226 produced a lower quantity of CLA and *P. parvulus* 2.6 produced a detectable amount, but which was outside of the limit of quantification. The concentrations of CLA determined were in accordance with those reported previously for other LAB. For instance, *Lactobacillus acidophilus* LAC1 produced 3.89 $\mu\text{g/mL}$ in MRS medium after incubation for 24 h [271]. Also, in the same medium and after 48 h of growth, *L. plantarum* and *L. casei* strains produced between 6 and 7 $\mu\text{g/mL}$ of CLA [272], and a similar values have been reported for *Lc. mesenteroides* L3A21M4 (4.21 $\mu\text{g/mL}$) and L2B21E3 (2.52 $\mu\text{g/mL}$) [273].

4.3.2.4. Gas chromatographic analysis

Most of the LAB screened for the production of CLA are also EPS producers, and all of them have been isolated from cider. According to the previous spectrophotometric assay (Table 12), six LAB strains were able to produce CLA extracellularly, thus, they were subjected to a more accurate analysis of the CLA-isomers produced by GC-MS, due to their potential technological interest.

First, the internal CLA production of the six strains was analysed. The chromatograms obtained by GC-MS analysis from the biomasses of all the LAB studied showed the same pattern of peaks, therefore, the chromatographic profile for the biomass of *L. sicerae* CUPV261^T was presented in Figure 40 as reference. In the chromatogram, a peak corresponding to LA was present, as well as the two peaks corresponding to the internal and external patrons C17:0 and C19:0. The signals of C16:0, C18:0 and C18:1 were also detectable in the LA-MRS medium chromatogram (data not shown), thus, these were recorded as components of the medium. Furthermore, the peaks corresponding to two CLA isomers were observed (*c9*, *t11*-CLA (rumenic acid) and *t9*, *t11*-CLA) at 18 min and 19.5 min, respectively. Finally, other two peaks were detectable at 32 min and 33 min, but they did not fit with any of the standards used for the calibration.

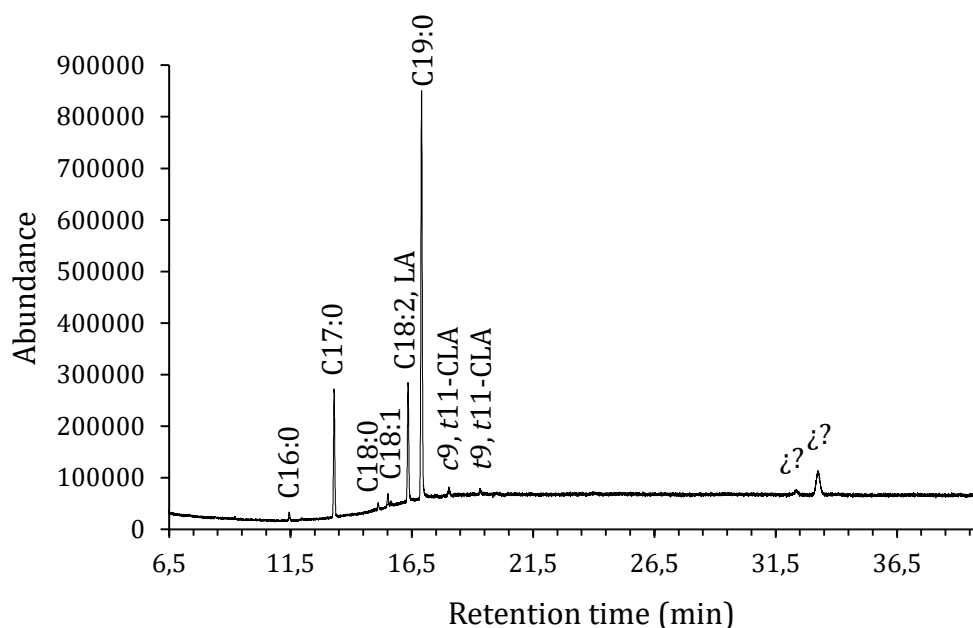


Figure 40. Chromatogram of fatty acids extracted from the supernatant of *L. sicerae* CUPV261^T.

The quantification of the ruminic acid isomer produced intracellularly by the LAB strains, by means of the peak area integration is depicted in Table 13. Despite the detection of the bioactive isomer, its production by all the LAB strains tested was very low, thus a deviation of the values was difficult to obtain because in the duplicates this production was under the limit of detection. However, the intracellular production of this *c9, t11*-CLA isomer by CUPV LAB strains was in accordance to the internal production by other LAB. For instance, *L. delbrueckii* subsp. *bulgaricus* CCRC 14009 was reported to yield 30.9 μg [47] of the bioactive *c9, t11*-CLA, or the 63.9 $\mu\text{g/mL}$ and 41.2 $\mu\text{g/mL}$ of the isomer produced by *L. gasserii* LM21 and *L. salivarius* HN6, respectively [48].

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Table 13: Detection and quantification by GC-MS of the *c9, t11*-CLA bioactive isomer extracted from the biomass of LAB

Strains	[<i>c9, t11</i> -CLA] from the biomass ($\mu\text{g/mL}$)
<i>L. plantarum</i> CECT220	62
<i>L. collinoides</i> CUPV237	45
<i>L. collinoides</i> CUPV231	ND
<i>L. suebicus</i> CUPV226	43
<i>L. sicerae</i> CUPV261 ^T	57
<i>P. parvulus</i> 2.6	ND

Some authors have described the production of the *t9, t11*-CLA isomer along with the *c9, t11*-CLA isomer by LAB, but in less concentration. These are the cases of *L. acidophilus* CCRC14079 and *Propionibacterium freundenreichii* subsp. *shermani* which produced 174 and 32 $\mu\text{g/mL}$ of the *t9, t11*-CLA isomer, respectively, whereas the production of the *c9, t11*-CLA isomer was 379 and 70 $\mu\text{g/mL}$, respectively [274], or *L. delbrueckii* subsp. *bulgaricus* CCRC14009 which produced 30.9 μg and 16.6 μg of the *c9, t11*-CLA and *t9, t11*-CLA isomers, respectively [47].

In the chromatograms obtained from the biomass for the six bacteria tested, the signals detected for the *t9, t11*-CLA isomer were in all cases very small and were out of the range of quantification for the standard curve used for these cider-isolated LAB.

The chromatograms obtained by GC-MS analysis from the supernatants of the six LAB studied showed the same pattern of peaks as the one obtained from the biomasses of the LAB. However, the signals corresponding to the CLA isomers were out of the limit of detection and quantification for the calibration curve used. Moreover, in the spectrophotometric analysis, CLA external production yielded around 5 $\mu\text{g/mL}$ for these LAB strains, thus this result obtained from analysis of the supernatants by GC-MS was expected. Therefore, the concentration of CLA in

the biomass of the strains is 10-fold higher than that detected in the culture supernatants, suggesting a membrane localisation of the responsible enzyme, as reported by some authors [237], [246], [261].

The chromatograms obtained either from the culture supernatants or from the biomasses of the LAB showed two quantitatively relevant peaks around 32 and 33 min that were not identified. Numerous authors have reported the production of intermediates with hydroxyl or carbonyl groups in the metabolic pathway to produce CLA isomers [49], [235], [237], [238], [244], [245], [248], [275]–[277]. Thus, a characterisation of the two compounds was desirable in order to get a deeper knowledge about the pathway followed by the strains studied. First, a bibliographic search was performed and the compound eluting at 33 min was identified by its mass spectrum (Figure 41A). Two significant MS fragments at 169 m/z and 201 m/z are characteristic of a hydroxyl group in the carbon 10 of the fatty acid chain [244], which identified the compound as 10, 13- dihydroxy-octadecanoic acid (Figure 41B). In addition, it has already been reported to be produced by LAB species in the process of biohydrogenation of LA. This is also the case for *L. acidophilus* NTV001 [278], *Pediococcus* sp. AKU1080 [49], *Streptococcus pyogenes* M49 [245] and *L. acidophilus* NBRC13951 [242], [279]. Moreover, hydroxyl acids have also been reported to have antifungal activity [51], immunomodulation properties [239] and other biological activities [240].

The mass spectrum of the compound eluting at 32 min (Figure 42) showed a molecular ion of 294, which corresponds to the molecular mass of a CLA isomer. However, the R_t did not fit with those of the CLA isomers used in the calibration. Thus, we tried to isolate it for an NMR analysis in order to elucidate the exact position of the double bonds.

Results and Discussion

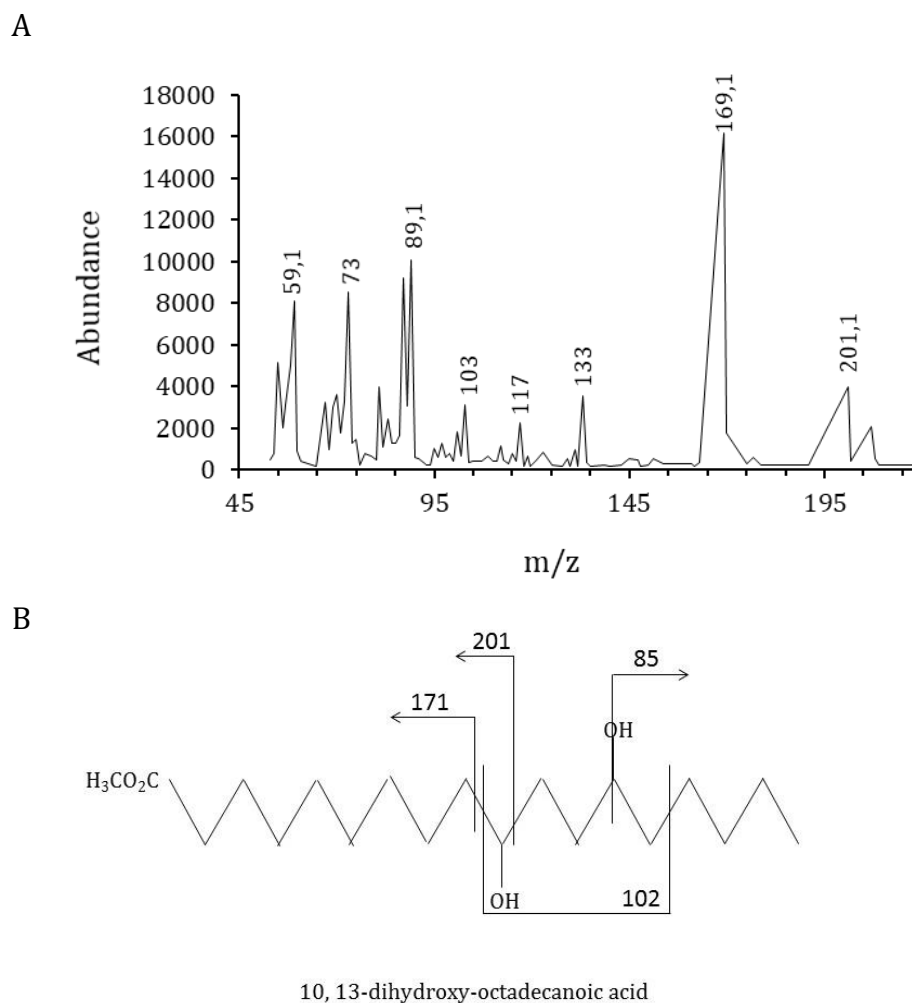


Figure 41. (A) Mass spectrum of the compound eluting at 33 min. (B) Structure of 10, 13-dihydroxy-octadecanoic acid and its fragmentation pattern.

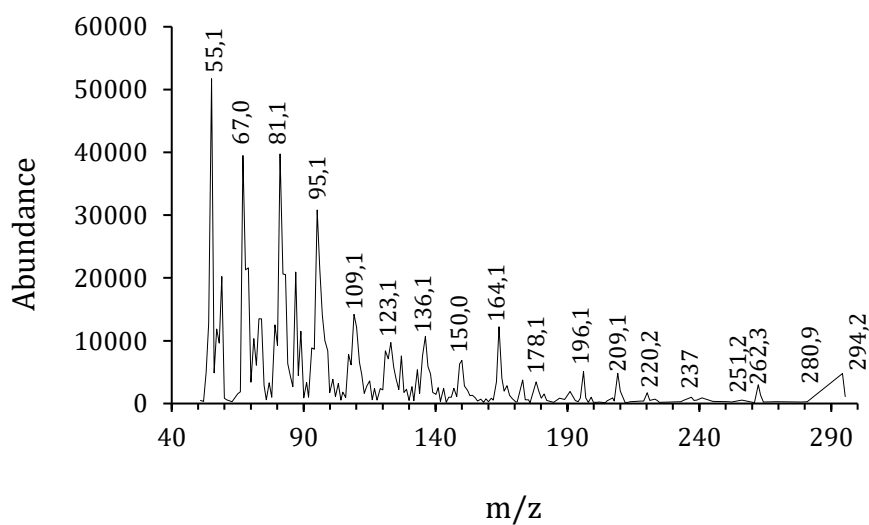


Figure 42. Mass spectrum of the CLA-isomer eluting at 32 min.

4.3.2.5. Identification of the unknown intermediate by Ag⁺-TLC

The unknown CLA-isomer that eluted at 32 min, could not be characterised by its mass spectrum. Thus, *L. sicerae* CUPV261^T was incubated in O₂-limited conditions to try to enhance the production of this intermediate. This strain was chosen due to its potential technological interest as well as because of the availability of a draft of its genome (unpublished). The GC chromatogram (Figure 43) not only confirmed that the unknown compound had been produced under microaerophilic conditions, but also that it was the only CLA isomer produced. This was an advantage for the isolation procedure through Ag⁺-TLC by its ability to bind silver.

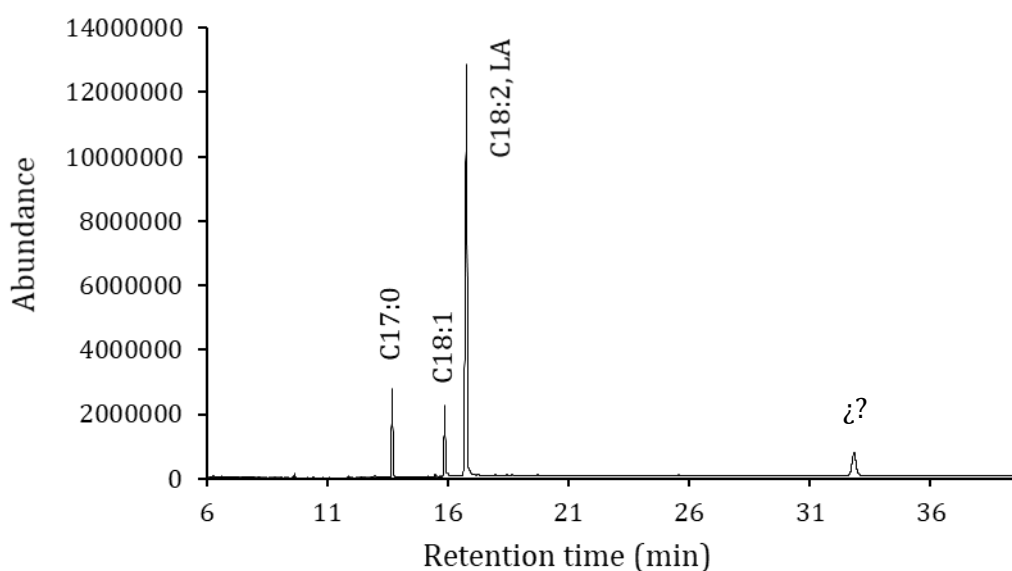


Figure 43. Chromatogram of the supernatant-extracted lipids from *L. sicerae* CUPV261^T grown under microaerophilic conditions.

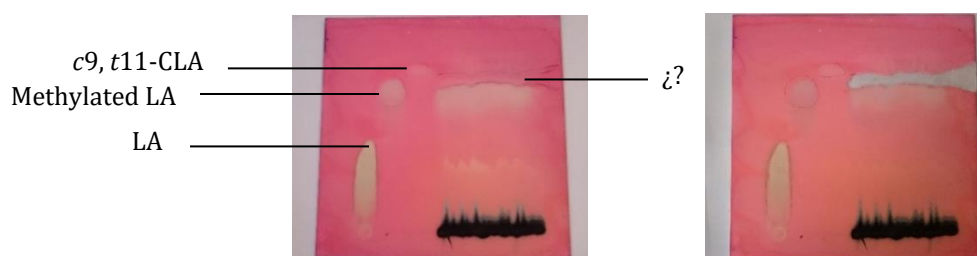


Figure 44. Ag⁺-TLC plate.

Left, different fatty acids from the supernatant of *L. sicerae* CUPV261^T separated. Right, plate after scraping the spots corresponding to the unknown CLA-isomer.

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The plate is shown in Figure 44. As it can be observed, a high amount of LA has not been methylated, so the protocol used can lead to errors in quantification by GC-MS. Finally, the CLA-isomer, which does not fit with any of the standards used, was scraped from the plate to be analysed by NMR, but the quantity isolated for the characterisation of the CLA-isomer was not sufficient for NMR analysis. Therefore, the location of the double bonds could not be elucidated.

4.3.2.6. *In silico* analysis of CLA-producing enzymes

The drafts of the genomes of two of the strains screened, *L. collinoides* CUPV237 [253] and *P. parvulus* 2.6 [173], were available. In addition, the draft of the genome of *L. sicerae* CUPV261^T [251] was performed in our laboratory (unpublished). Thus, an *in silico* analysis of the enzymes responsible for the metabolic pathway of LA biohydrogenation was undertaken. To do so, the four amino acid sequences (Appendix 2, section 2.6) reported for the multi-enzymatic LAI of *L. plantarum* AKU1009a [237], [238] were compared to the three genomes available in the RAST platform in search of homologies using BLAST. The three strains showed homologies for hydratase CLA-HY and dehydrogenase CLA-DH enzymes, responsible for the hydration of LA and the conversion of 10-hydroxy-*cis*-12-octadecenoic acid into 10-oxo-*cis*-12-octadecenoic acid, respectively. In the case of *L. sicerae* CUPV261^T a putative oleate hydratase (42% identity and 51% similarity) and a putative 3-oxoacyl reductase (26% identity and 49% similarity) were found. For *L. collinoides* CUPV237 a putative antigen, ortholog to the MCRA (79% identity and 87% similarity) and a putative short-chain dehydrogenase/oxidoreductase (60% identity and 75% similarity) were obtained. Finally, for *P. parvulus* 2.6 a predicted oxidoreductase and MCRA ortholog (54% identity and 71% similarity) and a putative oxidoreductase, short-chain dehydrogenase/reductase family (31% identity and 55% similarity) were found. In addition, for *L. collinoides* CUPV237 and *P. parvulus* 2.6 strains a homologue to the CLA-ER enzyme was also obtained: a putative NADH dehydrogenase (53% identity and 68% similarity) and a predicted oxygen insensitive NADPH nitroreductase (21% identity and 41% similarity), respectively for each strain. All sequences obtained for *L. collinoides* CUPV237, *L.*

sicerae CUPV261^T and *P. parvulus* 2.6 are depicted in the Appendix 2, sections 2.7, 2.8 and 2.9, respectively.

In addition, amino acidic sequences of a 10-hydratase and a 13-hydratase (AHW98240.1 and AHW98239.1, respectively, Appendix 2, section 2.10) from *L. acidophilus* NBRC 13591 [242] were available at the NCBI site, thus a multiple sequence alignment with all the hydratases was performed (data not shown). The amino acidic sequence of *L. sicerae* CUPV261^T hydratase was not complete; part of the sequence was missing upstream. Thus, a new alignment without that sequence was performed (Appendix 3, section 3.5) and it was concluded that sequences of the hydratases from *L. plantarum* AKU2009a and our strains are more evolutionary related than with the ones obtained from *L. acidophilus* NBRC 13591.

The presence of these enzymes in the genome of the screened LAB would be necessary for the production of the intermediate identified as 10, 13-dihydroxy-octadecanoic acid. However, for the elucidation of the complete metabolic pathway, further studies will be required.

4.4. Conclusions

In this chapter, the production of bioactive compounds by LAB has been evaluated. Specifically, phytate-degrading activity was screened mainly in pediococcal strains from the CUPV with negative results, but a non-specific acid-phosphatase activity was found in all the LAB tested. This activity confers to the producing-LAB the ability to degrade low phosphate forms and also, at a low rate, phytates. Moreover, the potential of CLA bioactive isomers production was also evaluated in CUPV strains mainly belonging to the lactobacilli group. All of them possessed a gene homologous to *cla-hy*, which encodes a 10-hydratase responsible for the first step in the biohydrogenation of LA to produce CLA. Six of the strains assayed produced intracellular concentrations of *c9, t11*-CLA bioactive isomer. In addition, two more compounds were produced by these LAB. One of them was characterised as the 10, 13-dihydroxy-octadecanoic acid, a possible intermediate on the metabolic pathway for the CLA production. However, the other compound produced could not be characterised with the quantity available. Therefore, the production of bioactive isomers as well as other functional compounds makes the six LAB strains studied potentially interesting for the development of fermented food with an added value.

GENERAL DISCUSSION

In this thesis, bifidobacteria and LAB strains belonging to *Pediococcus*, *Lactobacillus* and *Leuconostoc* genera isolated from different habitats have been characterised. *B. longum* INIA P132 and *B. infantis* INIA P731 were isolated from faeces of a breast-fed infant. *P. ethanolidurans* CUPV141 and *L. mali* CUPV271 were isolated respectively, from a ropy cider and an apple must from Spanish producers in the Basque Country. Finally, *Lc. carnosum* CUPV411 was isolated from the ropy slime on the surface of a vacuum-packed sliced cooked ham.

All the isolated strains produced EPS. The bifidobacteria synthesised HePS composed of glucose, galactose and rhamnose in different ratios. *L. mali* and *Lc. carnosum* strains produced a HoPS, characterised as a dextran with 84.8% and 81.1% of α -(1 \rightarrow 6) linkages, respectively. *P. ethanolidurans* CUPV141 was able to synthesise both, a HoPS identified as a 2-substituted (1,3)- β -D-glucan and a HePS composed of glucose, galactose, glucosamine and glycerol-3-phosphate. Strains belonging to the *Oenococcus* genus have already been described as HoPS and HePS producers, such as *O. oeni* I4 [280] and *O. oeni* IOEB0205 [39]. Moreover, some LAB and bifidobacteria have also been reported to produce more than one HoPS or HePS. This is the case with *Lactobacillus diolivorans* G77 (renamed CUPV218) [38], which produced a (1 \rightarrow 3)(1 \rightarrow 2)- β -D-glucan and a dextran with branches in α -(1 \rightarrow 2), and with *L. suebicus* CUPV225 and CUPV226, which each synthesised two different HePS [110]. In addition, *B. longum* NB667, *B. animalis* A1 and *B. animalis* subsp. *lactis* IPLA-R1 (A1dOx) were also able to synthesise more than one complex HePS [123]. Furthermore, different gene clusters encoding the proteins involved in the synthesis and secretion of more than one HePS have been found in *O. oeni* [39], *L. plantarum* [145] and *Bifidobacterium* [53] strains. However, as far as we know, this is the first report of a *Pediococcus* producing a HoPS and a HePS simultaneously. Only combinations of high and low MW EPS simultaneously produced by a bifidobacteria strain have been described until now. This is the case for the EPS of *B. animalis* subsp. *lactis* A1 (2.8 \times 10⁴ Da and 5.0 \times 10³ Da fractions) [123], *B. animalis* subsp. *lactis* IPLA-R1 (3.5 \times 10⁶ Da, 3.0 \times 10⁴ Da and 4.9 \times 10³ Da fractions) [123], *B. longum* NB667 (4.4 \times 10⁶ Da and 4.3 \times 10³ Da fractions) [122], [123] or *B. longum* IPLA B667dCo (5.4 \times 10⁶ Da and 4.5 \times 10³ Da fractions) [122]. The HePS fractions obtained from *B. longum* INIA P132 and *B. infantis* INIA P731,

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which were characterised in this work, were also composed of two fractions each, one of high MW (1.0×10^6 Da and 1.9×10^5 Da, respectively) and another of low MW (1.3×10^5 Da and 1.2×10^4 Da, respectively). Furthermore, LAB have also been reported to synthesise various EPS with different molecular weights [281]. For instance, *L. suebicus* CUPV225 and CUPV226 produce a high MW fraction ($1.5\text{-}7.8 \times 10^6$ g/mol) and a low MW fraction ($1.90\text{-}2.63 \times 10^4$ g/mol) [110]. In addition to the molecular mass, the sugar composition and the amount of the polymers produced can be affected by the composition of the medium (carbon and nitrogen sources, phosphate, minerals, vitamins, aminoacids, growth factors etc.) and the growth conditions (temperature, pH, oxygen tension, incubation time, etc.) [282], [283]. For instance, an improvement in the yield of EPS (from 60 to 107 mg/L) was obtained for *O. oeni* I4 when grown in the presence of a mixture of glucose and fructose rather than when glucose was used as the sole carbohydrate source [280]. Similarly, *L. suebicus* strains improved their EPS production when grown in the presence of ribose instead of glucose, maintained the production when xylose was the sole carbon source and the yield decreased when arabinose-fed cultures were used [110]. In addition, *L. plantarum* CIDCA8327 was reported to synthesise EPS with a different monosaccharide composition in MRS medium and in milk [284] and, as expected, *L. sakei* MN1 and different *Lc. mesenteroides* strains lost their capacity of dextran production when grown in glucose instead of sucrose [107], [185], which is the substrate of dextransucrases. In agreement, in this work, we have shown that *L. mali* CUPV271 and *Lc. carnosum* CUPV411 also lost their ability to synthesise EPS when growing in glucose rather than in sucrose. Changes in the growth temperature led to the appearance of a high molecular mass fraction and an increment in the total amount of EPS produced in *L. paracasei* strains [285] and modification of the pH of the culture medium also affected the EPS production of the two *L. suebicus* strains [110]. Similarly, a pH change from pH 5.5 to pH 4.8, slightly decreased EPS production (this work and [37]) by the isolate *P. ethanolidurans* CUPV141, characterised in this work.

The synthesis of EPS has been linked to the protection of the cell [18], [19], [21], therefore it is natural to think that EPS production takes place when cells are exposed to different stresses, and some studies report changes of EPS

concentration in the presence/absence of a particular stress. For instance, the survival of *L. lactis* and *P. parvulus gtf⁺* strains was around 1 to 2 log units higher in stressful conditions appearing in wines, such as acidic pH, alcohol and SO₂ compared to *gtf* deficient strains [10]. In addition, a recombinant *L. paracasei* NFBC338, carrying the *gtf* gene of *P. parvulus* 2.6 in a plasmid, showed higher viability than the parental strain, lacking the plasmid, when exposed to different stresses like heat, acid or bile salts [154]. In this context, the synthesis of the 2-substituted (1→3)-β-D-glucan and the dextran by the two cider isolates *P. ethanolidurans* CUPV141 and *L. mali* CUPV271, respectively, could confer a major tolerance to the ethanol present in cider, and thus, it would mean a survival advantage to the producing-strains. Moreover, *B. animalis* subsp. *lactis* was reported to start producing EPS in the presence of bile, maybe in order to coat its surface with a protective layer against the action of bile salts [286]. Therefore, the strains INIA P132 and INIA P731 isolated from infants' faecal samples could also be producing their HePS in order to protect them from the harsh conditions of the intestinal tract. However, this hypothesis would need further experiments for confirmation.

The tolerance to the highly acidic conditions inside the stomach and to the high concentrations of bile salts found in the small intestine is the most important trait that a probiotic has to possess in order to survive the passage through the GIT. Probiotics are living, health-promoting microorganisms that are incorporated into various types of food. It is thus important that the probiotic strain can reach alive and survive in the location where it is supposed to be active, and for a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonise at this specific location. Thus, these properties have become important selection criteria for new probiotic functionality [78]. Moreover, probiotic bacteria must also exhibit resistance to technological processes and not all strains can be easily produced industrially. The low yields in the growth media and a poor survival to freezing or freeze-drying are the main factors for which otherwise promising strains cannot be marketed [103]. Moreover, the selection of probiotics includes: phenotypic and genotypic stability (including plasmid stability); carbohydrates and protein utilisation patterns; intestinal epithelial adhesion properties;

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production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens, spoilage organisms, or both; and immunogenicity [78]. Up to now, the main bacterial strains with identified beneficial properties include bifidobacteria, along with *Lactobacillus* strains. In this work, some preliminary technological properties of *B. longum* INIA P132 and *B. infantis* P731 were assessed. Both survived fairly well the simulated conditions of the GIT. Moreover, regarding the technological properties, both strains showed promise. *B. longum* INIA P132 endured freezing and freeze-drying processes better, and was able to grow in milk, whereas INIA P731 had a better tolerance to storage at 4 °C. In addition to withstanding the harsh physicochemical environment of the GIT, the ability to adhere to the intestinal mucosa cells would be necessary for probiotics selection, because it is considered a prerequisite for colonisation and interactions between probiotic and host cells [78]. In this work, the adhesion of *B. longum* INIA P132 and *B. infantis* INIA P731 to Caco-2 cells was evaluated. *B. infantis* showed superior adhesion levels to those of the probiotic *B. animalis* BB12, which could be expected considering that INIA P731 was isolated from an intestinal environment. However, the INIA P132 strain, isolated from the same environment, showed very low adhesion to the enterocytes. Moreover, *B. infantis* INIA P731 was able to form a biofilm layer, whereas INIA P132 was not. The mechanism by which the adhesion to the intestinal cells or mucus is established is not yet well understood. Nevertheless, many studies have focused on the role that the EPS produced by probiotic strains may play in these interactions. The adhesion to HT29 cells of the EPS-producing *B. animalis* subsp. *lactis* Balat_1410^{S89L} was higher than that of the parental non-mucoid Balat_1410, indicating a favourable tendency of the cells with mucoid phenotypes to better colonise the gut [287]. Contrarily, it seems that high MW EPS could physically shield some bacterial adhesins, and thus, block the interaction of the bacteria with the eukaryotic receptor, making it difficult to adhere to the intestinal epithelium [106], [133], [288], [289]. Therefore, the EPS produced by *B. longum* INIA P132, with higher MW than that of INIA P731, might be hindering the adhesion of the producing-bacteria to the enterocytes. Moreover, we have also presented in this work that the adhesion of *L. mali* CUPV271 to Caco-2 cells decreased when the assay was performed in the presence of sucrose, condition necessary for the production of the EPS. This behaviour has also been

reported for *L. sakei* MN1 [107], [134], whose dextran was demonstrated to interfere in the self-aggregation of the bacterium [134]. Moreover, the results presented here revealed that the levels of adhesion of *Lc. carnosum* CUPV411 seemed not to be affected by the presence/absence of the dextran produced by the strain, as previously reported for *Lc. mesenteroides* strains [107]. Apart from this inhibition or decrease of the bacterial adhesion in the presence of EPS, a role in the increment of adhesion of pathogens has also been reported for the EPS produced by probiotic bacteria. This is the case with the EPS fractions of *B. animalis* IPLA-R1, *B. longum* NB667 and *Lactobacillus rhamnosus* GG, and the effect showed an EPS-dose dependent trend [102]. Moreover, a positive influence of a 2-substituted (1,3)- β -D-glucan (β -glucan) on adhesion and biofilm formation of the producing LAB has been demonstrated. Thus, the β -glucan-producing *P. parvulus* 2.6 showed a considerably higher level of adherence than that of the non-EPS-producing strain 2.6NR [177]. In addition, when the β -glucan was removed from *P. parvulus* 2.6 and CUPV22 producing bacteria by washing, a sharp decrease in the adhesion capability of the strains to Caco-2 cells was observed [12], [177]. Conversely, when the β -glucan was added to the 2.6NR strain, the adhesion of this bacterium to the enterocytes considerably increased, confirming the direct contribution of this EPS in the adherence to enterocytes [43]. *L. lactis*, *P. parvulus* IOEB8801 and *O. oeni* IOEB0205 harbouring the *gtf* gene exhibited significant adhesion while assaying the ability of biofilm formation, compared to their *gtf* deficient mutant variants [10]. However, the results from the current work have shown that the β -glucan-producing *P. ethanolidurans* CUPV141 was not able to adhere to Caco-2 cells to the same level as 2.6. This could be explained as commented above for other EPS, by an adhesin-mediated adhesion of the strain to the intestinal epithelium, where the EPS would be physically inhibiting the interaction. By contrast, CUPV141NR strain, with significantly lower production of the β -glucan than CUPV141 strain, showed higher levels of adhesion than its parental strain (this work and [37]). The two bacteria also produced a HePS, which could be exposed in the CUPV141NR strain and be directly mediating its adhesion to the eukaryotic cells. In addition, a wider accessibility of the adhesins is also hypothesised when only the HePS is present. Moreover, an aggregation assay has been conducted with the two strains, where the ropy cells producing the β -glucan were able to form huge aggregates compared

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to those of the CUPV141NR strain. Thus, taken together, these results would suggest a role of the 2-substituted (1,3)- β -D-glucan in the interaction between CUPV141 cells, while the HePS would mediate the interaction bacteria-eukaryotic cells.

Some LAB and bifidobacteria may have interest not as probiotics but for the EPS they produce. Some of these EPS have been reported to exert beneficial properties for health, for instance for their immunomodulatory activity [42], [107], [290], [291]. Among the EPS investigated in this work, the HePS produced by the two bifidobacteria also showed an anti-inflammatory tendency on THP-1-macrophages co-treated with the LPS from *E. coli* O111:B4. Furthermore, the EPS isolated from the culture supernatants of *L. mali* CUPV271, *Lc. carnosum* CUPV411, *P. parvulus* 2.6 and *P. ethanolidurans* CUPV141 also showed to produce an anti-inflammatory trend, although these results have not been presented, since they were preliminary, and their statistical significance is not yet established. Nevertheless, other works from our group have shown the functionality of some of the above-mentioned EPS *in vivo*. The two HePS isolated from INIA P132 and INIA P731 were observed to decrease the mortality of zebrafish larvae in an *in vivo* DSS-induced enterocolitis model in a dose-dependent manner (experiments detailed in the PhD thesis of Ana Isabel Puertas). However, although an anti-inflammatory effect or a physical protection barrier of the HePS were hypothesised, the mechanisms by which the mortality was reduced have not been ascertained. In addition, the 2-substituted (1,3)- β -D-glucan produced by *P. parvulus* 2.6 was demonstrated to have a positive effect on the colonisation of the zebrafish gut by the producing strain, as well as in competition of the bacterium with the pathogen *Vibrio anguillarum*. Moreover, the treatment with the purified β -glucan decreased the expression of genes encoding pro-inflammatory cytokines [43]. Finally, the dextrans produced by *L. sakei* MN1 and *Lc. mesenteroides* RTF10 were reported to possess an antiviral activity against salmonid viruses in rainbow trout [185]. Therefore, by homology of the EPS produced, *P. ethanolidurans* CUPV141, *L. mali* CUPV271 and *Lc. carnosum* CUPV411 may also exert an immunomodulatory effect *in vivo*, although this hypothesis would need further confirmation.

Production of HePS by LAB and bifidobacteria only yields mg amounts per L, thus they are not suitable for industrial productions. By contrast, LAB produce HoPS on a g per L scale, making them attractive for the food and beverage industries to improve the texture and flavour of fermented products. Among them, dextran addition to ice-creams has been reported to effectively increase their T_g , indicating their value as cryoprotectants, and they have also been shown to be able to significantly reduce the 'iciness intensity' in heat-shocked ice-cream samples, due to their ability to slow ice crystal growth during storage and thermal abuse [292]. These HoPS have also been described to improve the volume and texture of wheat bread [293], to contribute in obtaining softer breads with longer shelf-life [294] or to influence the rheological properties of doughs [295]. In addition, network structures of dextran-protein showed an improvement of the texture of quinoa-based yoghurt fermented with the dextran producer *Weissella cibaria* MG1 [296], and modification on the gelation properties and the rheology of milk gels have also been attributed to dextrans [297], [298]. The dextrans produced by *L. mali* CUPV271 and *Lc. carnosum* CUPV411 have been characterised in this work, and the analysis of their rheological properties revealed that they have a pseudoplastic behaviour upon mechanical stress, which is a good feature for their use in the food industry as additives such as thickeners or texture improvers, as well as cryoprotectants. Furthermore, we have demonstrated that their thermal degradation starts around 220 °C in aerobic conditions, making them suitable for withstanding the temperatures reached in the industrial processes.

Nowadays, new trends are arising in society, which demand more non-dairy fermented products. In this context, studies focused on fermented cereal-based products are producing promising results. For instance, *P. parvulus* 2.6 (*P. damnosus* 2.6 in the reference) has been reported to be able to ferment and produce *in situ* the O-2 substituted β -(1→3)-glucan in an oat-based non-dairy medium, where changes in the viscosity and ropiness were observed [299]. In addition, the intake of an oat-based product fermented by this strain in which its β -glucan was also synthesised *in vivo*, reduced the levels of total cholesterol [300]. Moreover, this bacterial β -glucan was successfully used for the *in situ* bio-fortification of cereal flours, leading to the possible development of carriers to

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enhance the beneficial properties of probiotic bacteria [301]. Oat-based products were also shown to be suitable for intestinal bacteria species such as *L. reuteri* ATCC55730, *L. acidophilus* DSM20079 and *B. bifidum* DSM20456 [302]. Therefore, it should be informative to analyse the performance of the β -glucan-producing *P. ethanolidurans* CUPV141 and of the isogenic CUPV141NR strain in different cereal-based matrices for the *in situ* production of the EPS and to evaluate its influence in the final viscosity and ropiness of the matrices. This type of experiment should be also performed with the dextran producers *L. mali* CUPV271 and *Lc. carnosum* CUPV411, with which we have already performed a first study on their rheological properties.

Nevertheless, EPS are not the only bioactive molecules secreted by bifidobacteria and LAB, and some of them can be helpful to counteract undesirable compounds present in cereal-based products. For example, phytates are considered as anti-nutrient factors because they can chelate minerals such as iron or calcium, decreasing their bioavailability for the consumers. Therefore, strategies to overcome this inconvenience include the search for microorganisms containing phytases or non-specific enzymes with phytate-degrading activity. The improvement on the iron absorption from an oat-based beverage has been demonstrated by the addition of phytases, citric acid and iron supplementation [303]. It has also been reported that mineral concentrations of iron, zinc and manganese increased during dough fermentation by phytate-degrading LAB strains [46]. Addition of bifidobacterial phytases to amaranth and wheat flours resulted in high phytate degradation without affecting the bread quality [230] and the use of *Bifidobacterium pseudocatenulatum* ATCC27919 as starter in sourdough for the production of whole rye-wheat mixed bread, also contributed to phytate hydrolysis, yielding breads with higher mineral availability [304]. In cereal-based infant formulae, phytases from bifidobacteria have also been used to reduce the phytate content [231]. Moreover, *L. casei* BL23 expressing phytases from *B. pseudocatenulatum* ATCC27919 and *B. longum* subsp. *infantis* ATCC15697 reduced the phytate content of a soy drink during fermentation [93]. Consequently, in this work, pediococci and lactobacilli belonging to the CUPV were screened for phytase activity. None of them contained phytases, although a non-specific phosphatase

activity was detected in many of them. Since this phosphatase activity, reported for other LAB, has been said to be useful in the degradation of lower forms of phytate [44], [45], [228], the capacity of these strains to ferment cereal matrices can be evaluated in a further study in order to test their potential as starters of non-dairy products.

On the other hand, the use of fermented products as vectors of beneficial properties is a common trend among food industries. For instance, fermentation of milks with different CLA-producing LAB and bifidobacteria is being studied in order to obtain the beneficial effects of the CLA isomers [30], [247], [271], [272], [305], [306]. Thus, we also screened bacteria from the CUPV for CLA production. Unfortunately, none of these bacteria showed a high CLA production, although an intermediate of the metabolic pathway was detected and characterised as 10, 13-dihydroxy-octadecanoic acid. This is interesting because hydroxy fatty acid intermediaries have been reported to have functional properties. In addition, a very small amount of an unidentified CLA isomer was also detected. Therefore, the elucidation of its structure as well as the potential functionality of the hydroxy fatty acid found as intermediate of CLA production require further investigation, with the aim to develop new non-dairy fermented beverages with functional properties.

OVERALL CONCLUSIONS

The main general conclusions deduced from this work are listed below:

1. Diverse EPS synthesised by different strains of LAB and bifidobacteria have been isolated and characterised.
2. The HePS produced by *B. longum* INIA P132 and *B. infantis* INIA P731 produced an anti-inflammatory tendency in macrophages. Moreover, the EPS synthesised by INIA P731 enhanced the adhesion of the producing bacteria to the enterocytes Caco-2.
3. *B. infantis* INIA P731 and *B. longum* INIA P132 above all, have good technological properties for their utilisation in the food industry.
4. *P. ethanolidurans* CUPV141, isolated and identified in the current work, produces simultaneously a HoPS and a HePS, which had never before been reported among pediococci.
5. The HoPS produced by this strain has been characterised as the (1→3)(1→2)- β -D-glucan. This β -glucan is synthesised by a GTF-glycosyltransferase and the encoding *gtf* gene is located in the 34.4 kbp pPE3 plasmid.
6. *P. ethanolidurans* CUPV141, ropy, and CUPV141NR non-ropy isogenic strains produce the HePS. This polymer exposed in the CUPV141NR strain could be directly mediating its adhesion to the eukaryotic Caco-2 cells, while the β -glucan produced by the CUPV141 strain would be acting in bacteria-bacteria interactions promoting the formation of huge aggregates.
7. *L. mali* CUPV271 and *Lc. carnosum* CUPV411 synthesise dextrans of similar structure, with a linear backbone of α -(1→6)-glucopyranose residues, slightly branched at O-3 and O-4 positions.
8. The presence of the dextran decreases the adhesion of the producing *L. mali* CUPV271 strain to Caco-2 cells. However, the presence/absence of the dextran

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produced by *Lc. carnosum* CUPV411 does not have any effect on the adhesion properties of the bacterium. This differential pattern could be due to differences in the supramolecular structure of the polymers.

9. Both dextrans would possess pseudoplastic behaviour and they only degrade at temperatures over 220 °C. These features make them very suitable for their application in the food industry.

10. A non-specific phytate-degrading activity is present in pediococci and lactobacilli strains from the CUPV. Incorporation of these strains to cereal-based products could enhance their nutritive values.

11. Six strains of pediococci and lactobacilli from the CUPV produced rumenic acid, the *c*9, *t*11-CLA isomer, as the only CLA and a hydroxyl-fatty acid characterised as 10,13-dihydroxy-octadecanoic acid.

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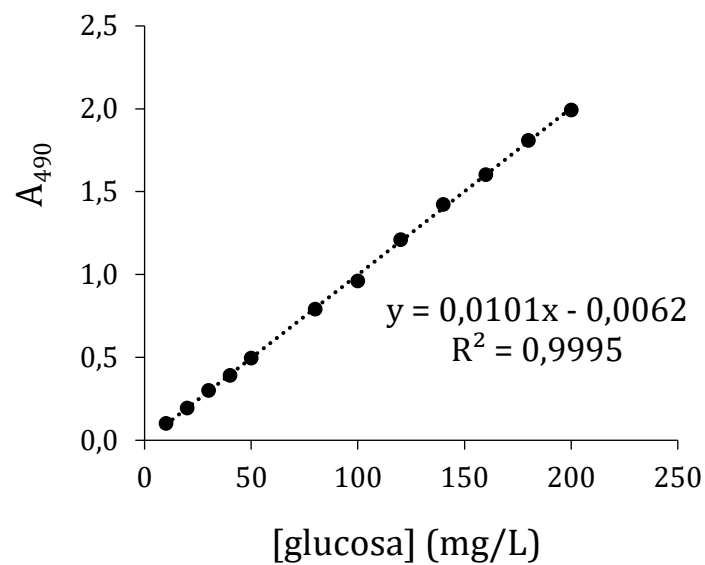
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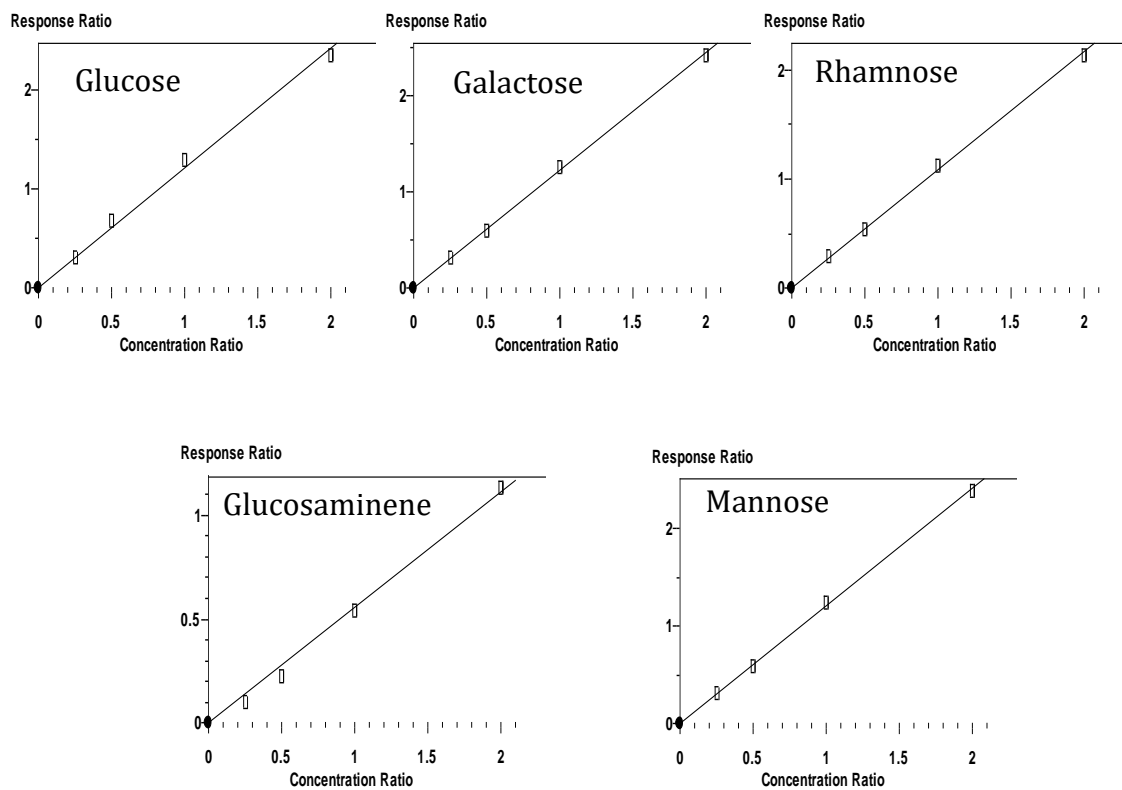
APPENDICES

APPENDIX 1. CALIBRATES

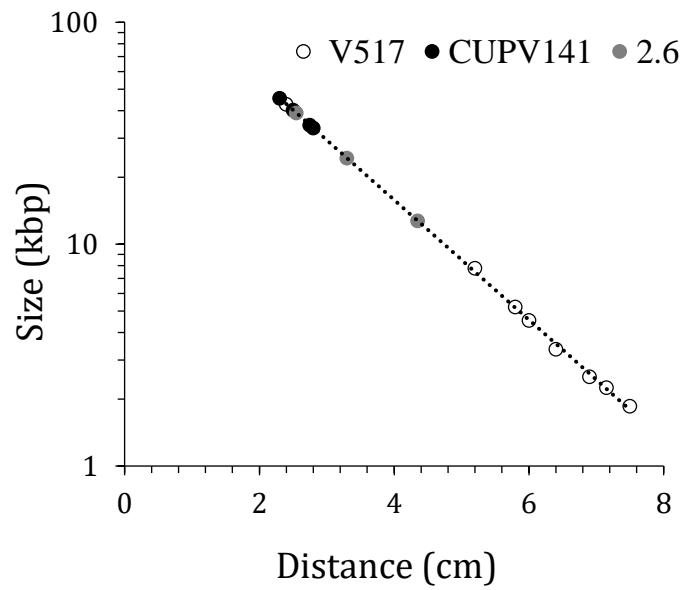
1.1. Standard curve for the quantification of neutral sugars in EPS



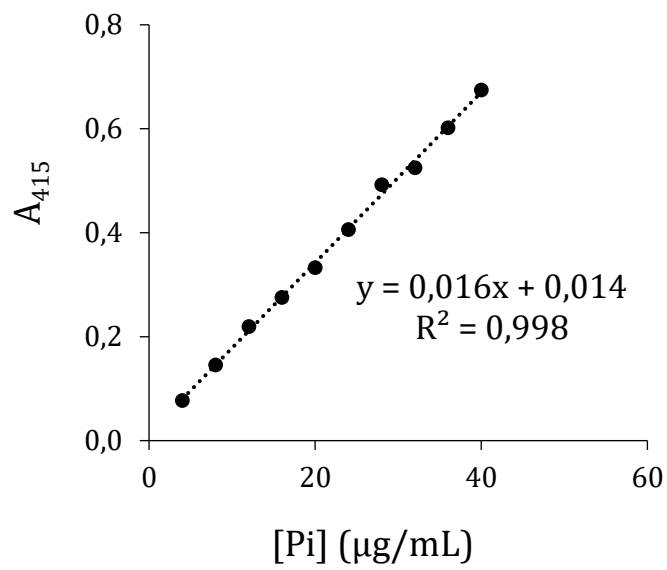
1.2. Calibration curves for monosaccharide determination by gas chromatography



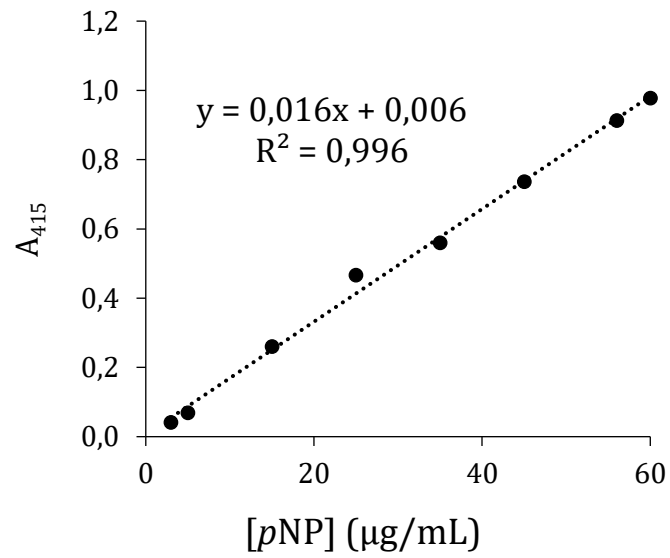
1.3. Calibration curve for plasmid size determination



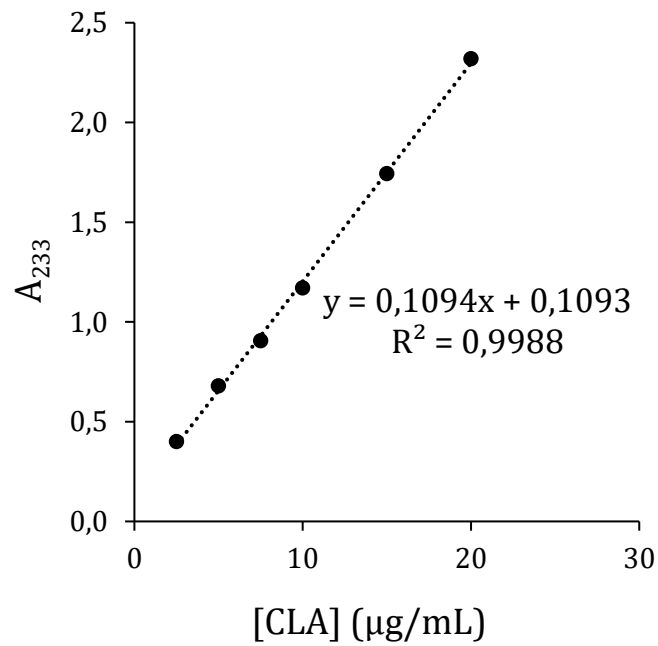
1.4. Standard curve for inorganic phosphorus determination



1.5. Standard curve for *p*-nitrophenol determination

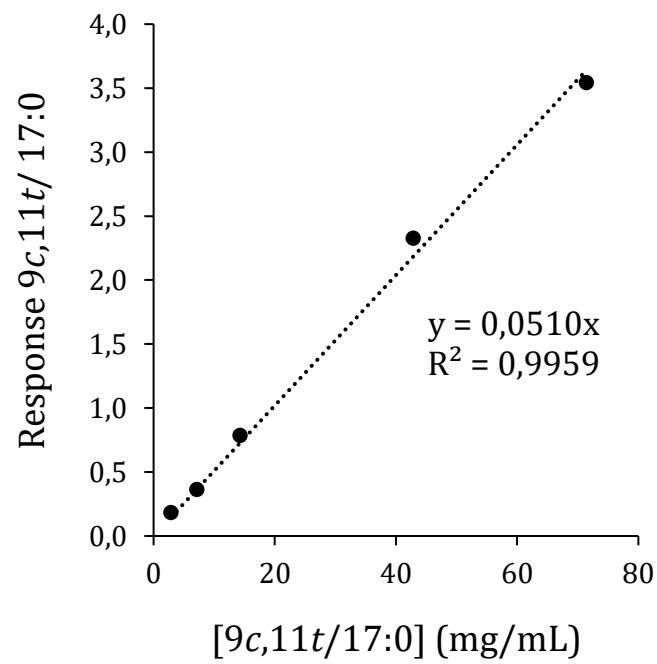


1.6. Standard curve for the quantification of CLA content in cultures supernatants



Calibrates

1.7. Regression curve for quantification of *c9, t11*-CLA from biomass 72-h-LA-cultures.



APPENDIX 2. SEQUENCES

2.1. *P. ethanolidurans* CUPV141 16S rRNA partial coding sequence

GAGATGCTTGCATCGAAGATGATTTTAACTATAAAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC
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 GGCAAGCGTTATCCGGATTTATTTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAAGTCTAATGTGAAAGCC
 TTCGGCTTAACCGAAGAAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAACTCCAT
 GTGTAGCGGTGAAATGCGTAGATATATGGAAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTG
 ACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGA
 ATGCTAAGTGTGGAGGGTTTCGCCCTTCAGTGTGCAGCTAACGCATTAAGCATTCGCCCTGGGGAGT
 ACGACCGCAAGGTTGAAACTCAAAGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
 CGAAGCTACGCGAAGAACCCTTACCAGGTCTTGACATCTTCTGCTAACCTAAGAGATTAGGCGTTCCCTTC
 GGGGACGGAATGACAGGTGGTGATGGTTGTCTGCTCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCC
 ACGAGCGCAACCCCTTATTATTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAAC
 CGGAGGAAGGTGGGGACGACGTCAAAATCATCATGCCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
 ACGGTACAACGAGTTGCGAGACCGCGAGGTTTAGCTAATCTCTTAAAACCGTCTCAGTTCGGACTGCAG
 GCTGCAACTCGCCTGCACGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTT
 CCGGGCCTTGACACACCGCCCGTACACCATGAGAGTTTGTAAACACCCAAAGCCGGTGGAGTAACCTTC
 GGGAGCTAGCCGTCTAAGGTGGGACAGATGA

2.2. Nucleotide sequence of *P. ethanolidurans* CUPV141 *gtf* gene

ATGTTAAATGATAATGATTCAGAACTAAAAAATTTCACTTGTTCATTCTAAACCAGTCTTTGTACCAG
 TTATTTAATTAATTTTGTGTTTATTATGTGCTTATATGAATATTTAACATACACAGATAGCATACTTCC
 TATTTTAGCTAAAAAGCAACCACTAGAAGTAATTTTACCTATATCAATCAATTAATTTGTGGCACTTTTC
 TTTTGTGTTGGAATAACTAATATTTATTCGCTATCCGCTATGCAATGATTAAGACAAAGCAAAGAAT
 CCGAACTAGCAATACTCGCAAAGAAACGCCTGCAGACTGGCACCCATAAGTTGAGTTATTGTATACGAC
 CTATAATGATTTTATACCTTATGCACTAGTCAATGTTTAAAACAGACATATGATAACACGCAGGGCGTT
 ATTTTGGATAACTCTACAGACCCCCAAATACATCAAGATGATTGATGATTTTGTGATAGCCCATCCTAATG
 TAAAGTTAGTCAGAGATTCTCAAAAACAGCATGCTAAAGCTGGAAACTTAAACAATTAATTTGTGTAATGG
 CACTCATGACTACGATTACTTTGTTATCCTAGATAGCGATGAATTAATAGAAAATAGATTTGTAGAAAAA
 TGTTTAAAGATGTTTATTAACAATGATATTGGCATTCTTCAGTGTAAATCACATTAGTGGACAAAACCACA
 ATTCGTTTATGCGTACTTTCTCTAGTTCGGCAATATTTTTGGCCAGTGCAAAACGTTGTACGAAGCGT
 TGAAGGTGGCTGGTTAAATAAACTGTGTCTGGCGTTTCTGTAGGCCAAACTGGAGGTGCATTAATGTATT
 GAATTAGGTCATGGCGTCATGATTTACGTGAATGCTTTGAAGATATTGGACAAATACCCATTCGGGTGG
 CAGAAGACCTTTGTACTTCTATTGAAGCTACACTAAAAGGCTGGAACATTAATTTGCTTACAAAATTTA
 CGGTAATGAAGCGTTTCTGTTAATATGGCAGCATTAAATGATTAGATCTAGTAAGTTTGTCTGCAAAAT
 TTTGAATTTTTTAAAAAATATTCGGCGAGAATCATCAAGTCAAAGACCATAAGTCTCTATCAAAAAATCG
 ACTTGTTTTGTTTTACCCTATCAGTTCCAAATAAGTGCTTTTCAATATATTAGCTTAGTTATTACTAGTAT
 AATTTGTCCAGTGTGACATTTCCACTAGTAACACAATTAATTTATGTTATTACCAACGTTAGTCTGTTAC
 TTTAGTCAAAGTTTGGTTCGATACTGTCTTTTATTTGACAAACGGTATGAAATTCCTTAGATTTATTGATTT
 ATGAAGTAGAATCAATGTTGTTATATGGGTCTTTTTATTTTATTACAATCAAGTCTACCGTACTAGCTTT
 AATGAACAAACCTGCTAAATTCATAGTTACACCGAAGGTTAATGAGCATATAACTTTTCTGCATGCAATA
 AGAAATCATTATCAAGGAATCTTATTTTCAATATTTACAATAATTGCATGTATTGCAATTTCTGGAAGTT
 ATTGGGTATTATTATCATTTATTCGGGTTGTTTTGGGTTTTTGTTCGAAATGCAAGCTAATCATCGGAC
 ATCAGAAGAACAAATAAAAAGCGGATAAATTACAGAGTTACAACAATAA

2.3. Partial nucleotide sequence of *P. ethanolidurans* CUPV141 *p-gtf* gene

TTTCATTTTATTCGTAAAACCTCAATTGACGAGCTGCCACAGTTAGTTAATGTATTAAGGGTGATATGA
 GTCTTGTGGGCCGACCGTCACTACCCTCTGAAGTAGAACAATATACAGATTATGATAAGCAACGATT
 GTATGTTGTTCCGGGATATACAGGCCCTTTGGCAAATCACAGGTTCGTAATAATATTA

2.4. *Lc. carnosum* CUPV411 16S rRNA partial coding sequence

CAGTCGACGCACAGCGAAAAGGtGCTTGCACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACA
 ACCTGCCTCAAGGCTGGGGATAACATTTGGAAACAGATGCTAATACCGAATAAAACCTTAGTATCGCATGA
 TACCAGGTTAAAAGGCGCTACGGCGTCACTAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTGGGGTA

Sequences

AAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGG
CCCAAACCTCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCG
CGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTTGTATGGGAAGAAAAGTTAAGGTAGGGAATGAC
CTTAGCCTGACGGTACCATAACCAGAAAGGGACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGT
CCCGAGCGTTATCCGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTGGTTAAGTCTGATGTGAAAGCC
CGGAGCTCAACTCCGAAAGGCATTGAAAACCTGGTTAACTTGTAGTGCAGTAGAGGTAAGTGGAACTCCAT
GTGTAGCGGTGGAATGCGTAGATATATGGAAGAACCAGCGGCGAAGGCGGCTTACTGGACTGTAAGTGA
ACGtTGAGGCTCGAAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGA
ACACTAGGTGT

2.5. *L. mali* CUPV271 16S rRNA partial coding sequence

CGGAAGTTTTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCAGAAAGAGGGGGATAACACTTG
GAAACAGGTGCTAATACCGCATAACAATAAAAAACCGCATGGTTTTTTATTTAAAAGATGGTTTTTGCATCA
CTTCTGGATGGACCCGCGGCTATTAGCTAGTTGGTAAGGTAAAGCCTTACCAAGGCAATGATACGTAGC
CGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACCTTACGGGAGGCAGCAGTAG
GGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAAGTGAAGAAGGTTTTTCGGATCGTA
AAACTCTGTTGTTAGAGAAGAACGTGTGTGAGAGTAACTGCTCATGCAGTGACGGTATCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCCTTGTCCGGATTTATTGGGCG
TAAAGGGAACGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGTCATGCATTGGA
AACTGAAAGACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATG
GAAGAACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAACGACGCTGAGGTTTCGAAAGTGTGGGTAGCA
AACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAATGCTAAGTGTGGAGGGTTTTCCGCCCT
TCGGTGTGCTGACGCTAACGCATTAAGCATTCCGCCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGGCCCGACAAGCGGTGGAGCATGTGGTTTTAATTCGAAGCAACGCCAAGAACCTTACCAGGT
CTTGACATCTTCTGACAACCTAAGAGATTAGGTGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATG

2.6. Amino acid sequences of the *L. plantarum* AKU1009a LAI multi-component enzyme

>BAL42246.1 hydratase/dehydratase CLA-HY

MGALFMVKSKAIMIGAGLSNMAAAVYLIQDGHWDGKDI TFYGVDMHGANDGGATTDFNEYNKNHPMAN
TTGYVARGGRRLNRYTYVDLMDLLDRIPSVTEPGMTAAEDTRDFDAKHRTYDIARLMQGGKGI INAGKLG
FNNKDRLLTKLIMMPDSEETKLDNVSIAEYFKDDPHMFQTNFWYMWETTFAFRTQSSAQELRRYMHQMI
YEFTQIEHLVGVNRYNQFESMILPLIKYLQGGVTFIDNKIVKDWQFKDTPMQDEITVTGLVIEDAQT
GETEEVEVDEDTAVIF TNGSITDSATMGDYNTPAPENMDYGVSAASLWKKATERFYNLGTPDKFFNDRNAS
EWVSFTLTTKNHLFLNEIVRIT TQE PGNALNSFLSTTPI TPLNQKDVNMSIVVHHQPHFTTQQPNETVLW
GYFLYPRRQGEFVNKPYIKMTGKEMAQELIGQLSKVDPGPGNIKDKKEDNLDIVNNIPVYMPYASALFN
NRAKSDRPEVLPKHSTNLAFTGEFAEQPYQMI FTEQSAVRSGEIAAYHFAGVPMNLVKTTRYDKDPKTL
LKATKKMFD

>BAL42247.1 oxidoreductase CLA-DH

MKDFKDKVMFITGAAHGFGQVIAEGAADRGMKLTIVDIDEPALKKTYQHILDKGAEVLMVTADVTKEASV
DDAVEQAMEKFRIDLINNAGIALPGRIWELPTRDWEWIMHINLMSQVYAMKRVIPI MIQQKTHADILN
VASIAGLVDTPGMPSYHASKFASVGMTEATAYDLQRANIDIDMHVMCPGFVQTDLYHTENHRPAQYS DPT
DPYYQSEAYLKGQQFAKYVITNGKPIDT IADTVFKALEDNRFYILTHPEYNPLIEDRVKRIVTDGAPDVH
IMDGIM

>BAL42248.1 isomerase CLA-DC

MASFIASDQDVKNFLTAPSMNDQEGIGFAYATDEAVLKALIPAPLKLMAVVCVYVVMHGKPTFSAPYLE
ESLFFALVSYKDKMMGAYPINLLLHGPAGAESGVIAGREGAGIPKKLADDIELRRNDNSATATVERHGKTL
NVSWTAGELNDPSIMKQFAGQLALGKEAEMNSFFYKYDIDQHEGDTNHFSNVQLVATQLRSLADQVEPGN
LSIQLESTDDDFGELKVLKPLGAAWFHFDTSVMFN TLKLEVDAAATTMPKLLTGRYDRSFFNPKAATYI
I

>BAO04454.1 enone reductase CLA-ER

MSEAVKNLVNNDLADVMFNHRHSVRQFDPNVKIGRDELQKMI AEAATAPSACNLQSWHFVVVDTP EAKAKF
KQAVMKFNYPQVDSASAI VFIAGDTQSHYVYRDVWNKVYEDGNITKERLDQILGTF LPLYENATPDFLKF
DATIDCSVVMQQLLVARAHGYDANAFSGIDFEKMIPTLGLDPKRYVPVMGIAIGKAAQEPLHTTRYDAK

TQTDFLA

2.7. *L. collinoides* CUPV237 CLA production. Sequences of putative genes and the corresponding enzymes.

>FIG|33960.3.peg.3172 ANTIGEN, PUTATIVE

ATGGTAAAACGTAAGCAATCATGATTGGGGCCGGCCTGGCAAACATGGCCGGAGCCGTTTACTTAATTC
 AAGAGGCACACTGGAAGGGTGAACAGATCACCTTTTATTCAATCGATGACCACGGCTCAAACGACGGGTC
 TACAGCAACGCTCTGCTAAGGGCGAGTATTGGAACACCGACTACCCAGTTGAAAACCATAACCGGTTTCGTC
 GCTCGTGGCGGTTCGGATGCTGAACTACCGACATACGTTGACCTCATGGATCTCATGGGTGCAATCCCAT
 CCGCCACTGAAAAAACAATGACCCGACGCCGAAGACACCCACGATTTTGACGCCACCACCGGACCTTTGA
 TAAGCCCCGACTGCTGCAGGGCGGCGTTGGTATCGTTAACGGCAGTCACTTAGGCTTAAATAATAAGGAC
 CGCTCCCTGCTCACAAAATTGATCTTGATGCCGGATAAGGAAGAACTCGGCTGGATAATGTCACCATTG
 CGGAATACTTCAAGGATGATCCGCACATGTTTCAAACGAATTTCTGGTACATGTGGGAAACCACCTTCGC
 CTTACAGGGTGCAGAGTTCTGCGCAGGAACTGCGGCGTTACATGCACCAAATGATCTATGAATTTACCCAA
 ATTGAACACTTGGTCGGCGTTAACCGGACACGTTATAACCAGTATGAAAGTATGATGCTGCCTCTGATCA
 ACTACTTGAAGGAACAGGGCTGCAAAAATCGTTTTGAATCGCCGGGTCACCGACTTCACCTTTAAGGACAC
 CGCGCTGCAAGACGAAATCACTGTCACCGGCTTAGAGATGGAAAACACAGAGACCAACGAAACGGAAACG
 GTCGCTGTGGATGATGATACCGCCGCTTCTTTTACCAACGGGTCGATCACCAGTCCGCTACTTTGGGCG
 ACTTCAACACCCCGCATCCGAAAACATGGACTACGGTGTGCTGCCAGCTTGTGGAAGAAAGCAACGGA
 AACTTCTACAACCTTGGGAACCCCGACAAGTTCTTTGCTGACCGAAACGCCAGTGAATGGTCAAGCTTT
 ACCTTAACGACGAAAAACCACTTACTGTTAAACGAGATTACCCGGATCACCACGCAGTTGCCAGGTAACG
 CACTGAACTCGTTTCTGTCTACAAAGCCGATTACACCGCTGGGCCAAAAAGATGTCAATATGTCCATTGT
 GGTCCATCATCAACCGCACTTTACCACGCAAAGACCAAACGAGTCGGTTATCTGGGGCTACTTCCTTTAC
 CCGCGTCGAAAGGGTGAATTTATTTAGAAAACCTTACATCGAAATGACCGGAAAGAAATGGCTGAGGAAC
 TGATTGGTCAACTGTGCAAGGTCGATCCAGGGCCAAACGAACATTATGGACAAGAAAGACGAGATCATGGA
 CAGTATCATCAACAACGTGCCCGTTTACATGCCATACGCTTACAGCATTTGTTCAACAACCGGGCAAAGACG
 GACCGGCTGAAGTGTATCCCGCACATTTCTACTAATTTGGCCCTTACCGGTGAGTTTGGCGAAACACCTT
 ACCAAAATGGTCTTACCGAACAGAGTGCCACCCGATCAGGTGAAATCGCCGCTTACCACTTACCGGCAT
 TTCGATGAACAATTTGGTCGACACCCCGGGTATGATAAGGATATGAAGACGCTTGTCAAGGCGACAAAG
 AAGCTCTTCTCTTAG

>FIG|33960.3.peg.3172 ANTIGEN, PUTATIVE

MVKRKAIMIGAGLANMAGAVYLIQEAHWKGEQITFYSIDDHGSNDGSTATSAKGEYWNNTDYPVENHTGFV
 ARGGRMLNRYTYVDLMDLMGRIPSAATEKNMTAAEDTHDFDAHRTFDKARLLQGGVGI VNGSHLGLNND
 RSLLTKLILMPDKEETRLDNVTIAEYFKDDPHMFQTNFWYMWETTFAFRVQSSAQELRRYMHQMIYEFTQ
 IEHLVGVNRTRYNQYESMMLPLINYLKEQGCKIVLNRRTDFTFKDTALQDEITVTGLEMENTETNETET
 VAVDDDTAVFFFTNGSITDSATLGDFTNPASENMDYGAAASLWKKATEHFYNLGNPKFFADRNASSEWSSF
 TLTTKNHLLLEITRIITTLQLPGNALNSFLSTKPIITPLGQKDVNMSIVVHHQPHFTTQRPNESVIWGYFLY
 PRRKGEFIEKPYIEMTGKEMAEELIGQLSKVDPGPTNIMDKKDEIMDSIINNVPVYMPYASALFNNRAKT
 DRPEVIPAHSTNLAFTGEFAEQPYQMVFTEQSATRSGEIAAYHFTGISMNNLVDTPRYDKDMKTLAKATK
 KLFS

>FIG|33960.3.peg.2303 SHORT-CHAIN DEHYDROGENASE/OXIDOREDUCTASSE

ATGAAGGATTTTAAAGATAAGGTTGCGCTGATTACAGGTTTACGCCATGGCTTTGGTAAAGTTTTAGCAA
 TCGAAAAGCCAAAGCGGGCATGAAACTTGCTCTGGCTGATGTTGATGAGTCCGCTCTAACCGAAACAGT
 TAAGCAAGCATCTGACTTAGGCGCCGAAGTCAATTTCCATTCCGACAGACGTGACTGAAGAAACAGCAGTA
 GATCATATGGTACAAAACAACCAAGGATAAATACGGTCAATCGATCTACTAATCAATTCAGCGGGAGTCG
 CCGTTTCTGGACCAATCTGGGAGCTCCCAACACGAGACTGGGACTGGATCTTACATGCCGACCTATTAAG
 CCAAGTCTACGCGCTCAAACGGGTCATTTCCAATCATGCGTCAACAGGAGGGGCACGCCGATATTTCTCAAT
 GTTGCCCTCCATGGCTGGTTTGGTTTCTAGTCCGCTTATGCTGCATACTACGCTACTAAATTTGCGGTAG
 TTGGCATGACTGAAGCTGTTGAATATGATTTGCAAGGACGCAAGCAAATGTGGGTATGCACGTTTTCTG
 TCCAGCATTTGTTTACACAGACTTGCAGCATTTCTGAGAATCATCGACCGGCTGCTTATACAAATACTGAC
 GACCCGTATTACAGCAGCGATACCTTCAAACAGGGACAAGCATTGCGGAGAAAGATATTACGACTGGTA
 TGCCGCTTGATAAGATTCCAGATATCGTTTTTGGGGCGTTAGAACGTGATGACTTCTATATTCTGTCACA

Sequences

CCCAGGCATGAACCCTGCGATCGTGGCCCGTGCCCAAAGTATTCCTACTGGCAAAGGACCCGACTTGGCC
GCTCTTGCCCCATTCTTGAAAAACACACGATGCTTAA

>FIG|33960.3.peg.2303 SHORT-CHAIN DEHYDROGENASE/OXIDOREDUCTASSE
MKDFKDKVALITGSAHGFVKVLAIESAKRGMKLALADVDESALTETVKQASDLGAEVISIPTDVTEETAV
DHMVQTTKDKYGRIDLINSAGVAVPGPIWELPTRDWDWILHADLLSQVYALKRVIPIMRQQEGHADILN
VASMAGLVSSPLMPAYYATKFAVVMTEAVEYDLQAAKANVGMHVFCPAFVHTDLQHSENHRPAAYTNTD
DPYYSSDTFKQGQAFKEDITTMPLDKIPDIVFGALERDDFYILSHPGMNP AIVARAQSIPTGKGPLDA
ALAPFLEKTHDA

>FIG|33960.3.peg.2302 NADH DEHYDROGENASE (EC 1.6.99.3)
ATGATTACCATCAGTGAAACACAAGACAAAAGTATTTACCAACAATGATTTCCAAAACGTAATGTTAGGTC
GGCAGTCCATTCGAAATTTTGACAAGTCAGTTAAGATTCCCCACGATGAAATGGTCAAGATGATTGAAGA
AGCCACTACGGCACCTTCTGCATGCAATCTGCAGGCATGGCGGTTTCGTTGTTGTGGACACCCCTGAAGGC
AAAGAGAAAATTGAGCACCTACTTCATGAAGTTCAATACTCCACAAGTGCAAACAGCTTCAGCGATGGTCC
TGCTGTTTGGCGACACCCAAAGCCTTTAAATCCTACCGCGCACTGTGGAACAAAGCTTATGAGAACAAACA
AATCACTAAGGAAAAGCTTGAAGAAGTCTTCAACACCTTCTGCCACTATACGAGCATGCTTCCAGAGAT
ATGCTGGTTGCCGATTCAACGGTTGACACTGCACTGGTTCGCTATGCAATTCATGCTGGCAGCTCGTGGTC
ATGGTTATGAAAATAACCCGATTGCCGGCTACGACCCAAAGAAGGCTGCAACAACCTGGGGGCTTGATCC
GGAACGTTACGTCCAGTGATGGCTATTGCAGTTGGTAAGCCCGCACCCGATGACGAAGGAGAGGTTTCG
TCAGTTCGTTATCCAGTTGAACAGGTTTACGAATTTTCATTAG

>FIG|33960.3.peg.2302 NADH DEHYDROGENASE (EC 1.6.99.3)
MITISETQDKVFTNNDFFQNVMGRQSI RNFDKSVKIPHEMVKMIEEATTAPSACNLQAWRFVVVDTPEG
KEKLSTYFMKFNT PQVQTASAMVLLFGDTQAFKSYRALWNKAYENKQITKEKLEEVFNTFLPLYEHASRD
MLVADSTVDTALVAMQFMLAARGHGYETNPIAGYDPKKAATTWGLDPERYVPVMAIAVGKPA PDDEGEVR
SVRYPVEQVYEFH

2.8. *L. sicerae* CUPV261^T CLA production. Sequences of putative genes and corresponding enzymes

>FIG|1578.57.PEG.824 OLEATE HYDRATASE (EC 4.2.1.53)
TCCAAC TGACAAATCGCTGAGATTGCTCAAGCTGCCAATTCGATTCCAATTCATATGCCGTACATTACT
TCCTACTTCATGCCACGCCAAGCTGGTGATCGACCATTAGTTGTCCCAAATGGCTCTCAGAATTTAGCAT
TCATCGGCAATTTTGTGAGACGCAGCGTGATACAGTCTTTACCACCGAGTATTCAGTCCGGACAGCGAT
GGAAGCTGTCTACCAATATTTGAATATCGACCGTGGGGTGCCGGAAGTCTTTGATTCTGCTTTTGACCTG
CGAACGATTTTAAATGCATATATATTTGAATGATCAAAAAGCTCTGAGCGAGTTGCCGTTAAGTCAA
TCGAAAAAGTTCGGTTTAAAAGCTTGCTTGAAAAAGATCCATGGTACTTATTTAGAAGAGATCCTACAAGC
TGCGAAGTTGCTTTAA

>FIG|1578.57.PEG.824 OLEATE HYDRATASE (EC 4.2.1.53)
PTDQIAEIAQAANSIPIHMPYITSYFMPRQAGDRPLVVPNGSQNLAFIGNFAETQRDVTFTTEYSVRTAM
EAVYQLLNIDRGVPEVFD SADFRLRTILNALYYLNDQKALSELPLSQIEKVGLKACLKIKHGTYLEEILQA
AKLL

>FIG|1578.57.PEG.515 3-OXOACYL-[ACYL-CARRIER PROTEIN] REDUCTASE (EC
1.1.1.100)
ATGGATTTAACAGTAAAACAGTTTTTATAACTGGTAGTTCGCGGGGATTTGGTGCCTCAGATTGCCTTAG
CTTTTGCTCGACATGGCAGTGACATCATTTTAAATGCGCGTCATGAAATATCTGAGGATTTGACTGAACA
AATAAAAAAGACCGGTGTAAATGTCAAATAATCTTAGGAGATATTGCCAAATCAGCTGATGTAGCACGA
ATCTTGACGGAAATCTATCAGTCAAATCAGCAACTTGATATTTAGTTAATAATGCAGGTATTACTAAAG
ACAAGTTAATCATTTGGTATGTCAACCGATGATTTTGAACAGGTTCTTAAGACCAACTTGATTGGAGCGTT
TGATTTAACACGCCAAGTTTTAAAAAAAATGTATCGGCAAAGAAGTGGTGTTCATTAATTTAGCCAGT
GTGGTTGGATTGCATGGCAATCTGGGACAGGCAAAC TACGCTGCTAGCAAAGCCGGATTGATTGGGCTAA
CTAAGACAATTGCCAAAGAGGGCGCTTTACGAAATATTCGTTGTAATGCGCTTGCTCCAGGTATGATCAA

AAGTAGTATGACTGAAGTTTTAAGTGAGAAGGTTTCAGCAAAAAATGCTGGAAGAAATTCCTTAGGACGT
 TTTGGTCAACCTGCTGAGGTTGCTGATGCAGCAGTCTTTTTAGCGGAAAATGATTATATTACTGGGGAAG
 TTTTAAACAATTGATGGCGGTATGACGATTTAG

>FIG|1578.57.PEG.515 3-OXOACYL-[ACYL-CARRIER PROTEIN] REDUCTASE (EC
 1.1.1.100)

MDLTGKTVFITGSSRGIGAQIALAFARHGSDIILNARHEISEDLTEQIKKTGVKQCQIILGDIAKSADVAR
 ILTEIYQSNQQLDILVNNAGITKDKLIIGMSTDDFEQVLKTNLIGAFDLTRQVLKMKYRQRSGVIINLAS
 VVGLHGNLQANYAASKAGLIGLTKTIAKEGALRNIRCNALAPGMIKSSMTEVLSEKVQQKMLEEIPLGR
 FGQPAEVADAAVFLAENDYITGEVLTIDGGMTI

2.9. *P. parvulus* 2.6 CLA production. Sequences of putative genes and corresponding enzymes

>FIG|54062.3.PEG.2005 PREDICTED OXIDOREDUCTASE; MYOSIN-CROSSREACTIVE
 ANTIGEN ORTHOLOG

ATGGCAAAAAGCATATATGATCGGATCAGGAATTGGTAATCTGGCAGCTGGCATTATTTAATACGTGACG
 GTGGCTGGAGTGGTGATCAAATTACCATGTTTGGTCTTGAAAAACACGGTGCCAATGATGGTGCTAAGGT
 AGCTGATTATGAGTCCGAATACGGTAACCCAGAATTAAGTAACAATAAAGGATTCTTGGCGAAGGGTGGC
 CGAATGCTGAACGAAGAACTTATGAAACTTATGGGATGTTTTACGTTACGTACCATCGCTGGATAATC
 CAGGACAATCAGTTACGGACGATATTTTGAACTTTGACCATGCACATCCTACACATGATGTTGCGCGATT
 AATGGATCGGACGGATGGCATTTCGTAATAAGGGTGATCAAAAAGATTATAATCATATGCAATTTAATAAT
 CAGGATCGCTTCCTATTAACCAAGTTAATGATGATGCCTGAATCTAAGGAACCACAATTAATGATGTCA
 GCATTGAACAGTGGTTTGCTAAGAGTCCCATATTTTCACAACATAATTTCTGGTACATGTGGCAAACAAC
 TTTTCGCGTTTTAAGAAAAGAAAGCTCTGCAATGGAGCTACGGCGTTACATGAATCGAATGATCTTAGAATTC
 AGTCGAATCAATACTTTGGCCGGCGTGACGCGAACACCCATATAATCAGTATGAAAGCATCATTTTACCAA
 TGCGTAAAATTTGACGGATCATGGTGTGAACTTTGTAAATAATCGTAAAATCACTGAATTTGTATTTAA
 AGATACACCGTTACGCGATGACATCATTTGTAACAGGCTTGAAGTATGAAGAAGTGGATAACGATAACAAA
 CCCGGTGAGATTGAAATTTGGCGAAAATGATTTTGTCTTTGATACGAACGGTGCAATTACGGATAGTTCTT
 CCATTGGCGATTTAAACACCCCAATTAAGAAAACATGGAGTATGCGCCAAGTGCAGCTCTTTGGAAACA
 GGCACCGAAGACTTGTACAATTTGGGTAATCCAGACAAAATTTCTTGGCGATCGTAAACAAAGTGGTGG
 TTAAGTTTCACTGTGACAACCAATAATCATTTCTTATTAATGAGATTTCTCGGATTACACAACAGGAGC
 CAGGAAATGCGCTGAATACTTTGGGTTGACAGATAACAATTTGATGTCAATCGTTGTCCATACCAACCTCA
 TTTTTCACGTTCAAAAAGGAAAATGAAACTGTTTTCTGGGGCTACGTCATGTATCCACGTCGCAAAGGAGAT
 TATGTTGACAAAACATTTATCGAAATGACTGGTAAAGAGATGTTAGAGGAGCTGTTAGGCCATCTAGCGG
 CTGTGGATCCAGCGCTGACAACATTTGCTGATCATACCGAAGAGATTATGGACAGCATTTGTAACGTCAT
 TCCAGCCTACATGCCGTATGCTAGCGGTTGTTTAAATCGTCGTGCTGTGGGGGACCGTCCAGCTGTTGTG
 CCAAAGAACTCTAAGAATTTGGCTTTTATCAGTCAGTTTGGCGAAATGCCATTTGACATGGTCTTCACGG
 AACAATATTCATTTAGATGTGCCAAGTGGCTGTATATCACTTCATGGGAATTCCTGATAGTGAGTTAAC
 ACCACTCCATCATTTATGAAAAACAACCTAAAGTTTTGGCAAGAGCAACAAAGACAATGTTTATAGATAA

>FIG|54062.3.PEG.2005 PREDICTED OXIDOREDUCTASE; MYOSIN-CROSSREACTIVE
 ANTIGEN ORTHOLOG

MAKAYMIGSGIGNLAAGIYLIRDGGWSGDQITMFGLEKHGANDGAKVADYESEYGNPELSNNKGFLLAKGG
 RMLNEETYENLWDVLRVPSLDNPGQSVTDDILNFDHAHPHVARLMDRTDGI RNKGDQKDYNHMQFNN
 QDRFLLTKLMMPEPESKEPQLNDVSIQWFAKSPHIFTTNFWYMWQTTFAFKKESAMELRRYMNRMILEF
 SRINTLAGVTRTPYNQYESIILPMRKYLTDHGVNFVNNRKITEFVFKDTPLRDDIIVTGLKYEEVDNDNK
 PGEIEIGENDFVFDNNGAITDSSSIGDLNTPIKENMEYAPSAALWKQATEHFYNLGNPDKFFADRKQSEW
 LSFTVTNNHFLLEISRITQQEPGNALNTWVDSNNLMSIVVHHQPHFHVQKENETVFWGYVMYPRKGD
 YVDKPFIEMTGKEMLEELLGHLAAVDPARDNIADHTEEMDSIVNVI PAYMPYASALFNRRRAVGDRAV
 PKNSKNLAFISQFAEMPFDMVFTEQYSFRCAQVAVYHFMGIPDSELTPLHHYEKQPKVLARATKTMFR

>FIG|54062.3.PEG.36 OXIDOREDUCTASE, SHORT-CHAIN
 DEHYDROGENASE/REDUCTASE FAMILY

ATGTCAAAAAGTAAAGTTATTTGTAATTACGGGTGCCACATCAGGTATTGGCGAGGCTAGTGCAAAATTTAT
 TAGCAAAAAGATGGTACAAGCTAGTTTTGGGTGCTCGTCTGTAACAAAATTTGCAAGAAGTCACCAAGGC
 GGTGGAAGAGAATGGTGGTCAAGCAATATACGGAATAACTGATGTCACAAAAGACGAAGATGTTGAAGCC
 TTGGCAAAGTTAGCTGTGCAAAAAGTTTGGCAGAATTGATGTTTGGTTGAATAATGCTGGAATCATGCCCG
 AATCTTTATTAAGTCAAAAAAGATTTCTTGACTGGGATAATACAATTGATATTAATATTAAGGCGTCTCT

Sequences

TTATGGGATTGGCGCTGCACTTCCTTACATGGAAGCACAGAAAAGTGGTCAATTTATTAATATTTCTTCA
GTTGCCGGACATGTTGCTGGTCTGGAAAGTGC GGTTTATTCGGCAAGTAAGTATGCTGTTCCGGCAATTA
GTGAGTCGTTAAGACAAGAAATGGCACAAAGATAAAAAGTAATGTTTCGCGTGACGGTGGTTTTACCTGGTGC
AATTTTCGACAAAATTTATTGAATTTCTGTTACAGATAATAAAGTCAAAGCCGGGATGGAGCAGTTCTATGAT
GCTTTTGTATTCCAGTAGATCGTGTGCTTAACATATTAAGCAGGCAATTGATATACCGGAAGATGCTG
CTTGAATGAAGTAATTTATCCGACCAACAGCACAAACAAATGTAG

>FIG|54062.3.PEG.36 OXIDOREDUCTASE, SHORT-CHAIN
DEHYDROGENASE/REDUCTASE FAMILY
MSKSKVIVITGATSGIGEASAKLLAKDGHKLVLGARRENKLQEVTKAVEENGGQAIYGITDVTKDEDVEA
LAKLAVEKFRIDVWLNAGIMPQSLLSQKILDWDNTIDINIKGVLYGIGAALPYMEAQKSGQFINISS
VAGHVAGPGSAVYSASKYAVRAISESLRQEMAQDKSNVRVTVVSPGAISTNLLNSVTDNKVKAGMEQFYD
AFAIPVDRVALTIKQAIDIPEDAANNEVIIRPTAQQM

>FIG|54062.3.PEG.907 OXYGEN-INSENSITIVE NADPH NITROREDUCTASE
TTGACAAC TAACGAAACCTTACATTTATTAACCCACCATCGGTCAATTCGCCGTTTTAAAAACCAGTCTT
TAGATCGTGACACCATTCAAACCTTGTTAACTGCTGCTCAACGAACATCTTCAAGTATGTTTCATGCAACA
ATTCAGCGTGATTAGCGTACCGATCCTCAATTAACAGCAATTTGCCGAAATTACTACCTATCCATTT
ACACAAAGTAACGCCATTTATTCATTATGATTGCCGATCAACATCGCAATGAATTTATTGGAGAAGAGG
CACATGAAAATACCAAATTACTCCATGATGGTGATCGCAGTTTGGCCGCTTTTGACGACGCTGCGTTGGC
TGGCCAAAAGCATTGTAACCTGCTGCCGAAAGTATGGGACTTGGAGCCGTAATCCTTGGCAGCATATTAAT
GACGCCCAACAAGTAATTGATCTTCTTCACTTACCCAACTAACTTTTCCCGTCTTTGGAATCGCTGTTG
GCTACCCTGATGAGAAAGCCAAAATTTGAAACCCCGCTTGCCACTTGAAGTGTCCATTTTGAACCATTA
TCAAGAACCAGATAAACAAACATTAGATGCCTATGATCAAACATTCAGTCTTATTATACACATCGCGAT
TCAAACACTCGTAATGAGACCTTCAGACATCATATCATTGATGACATGCAAGTAAGTCCTAAGAAACGTT
CCGAAATCTATCTATTTTGAACCAAGTTTTTTTGAACCAATTA

>FIG|54062.3.PEG.907 OXYGEN-INSENSITIVE NADPH NITROREDUCTASE
MTTNETLHLLTHRSIRRFKNQSLDRDTIQTLTAAQRTSSSMFMQQFSVISVTDQPQLKQFAEITTYPF
TQSNGLHFIMIADQHRNEFIGEEAHENTKLLHDGDRSLAAFDDAALAGQSI VTAAESMGLGAVILGSI LN
DAQQVIDLHLPLKLTFFVFGI AVGY PDEKPNLKPRLPLESVHFENHYQEPDKQTLDAYDQTIQSYTHRD
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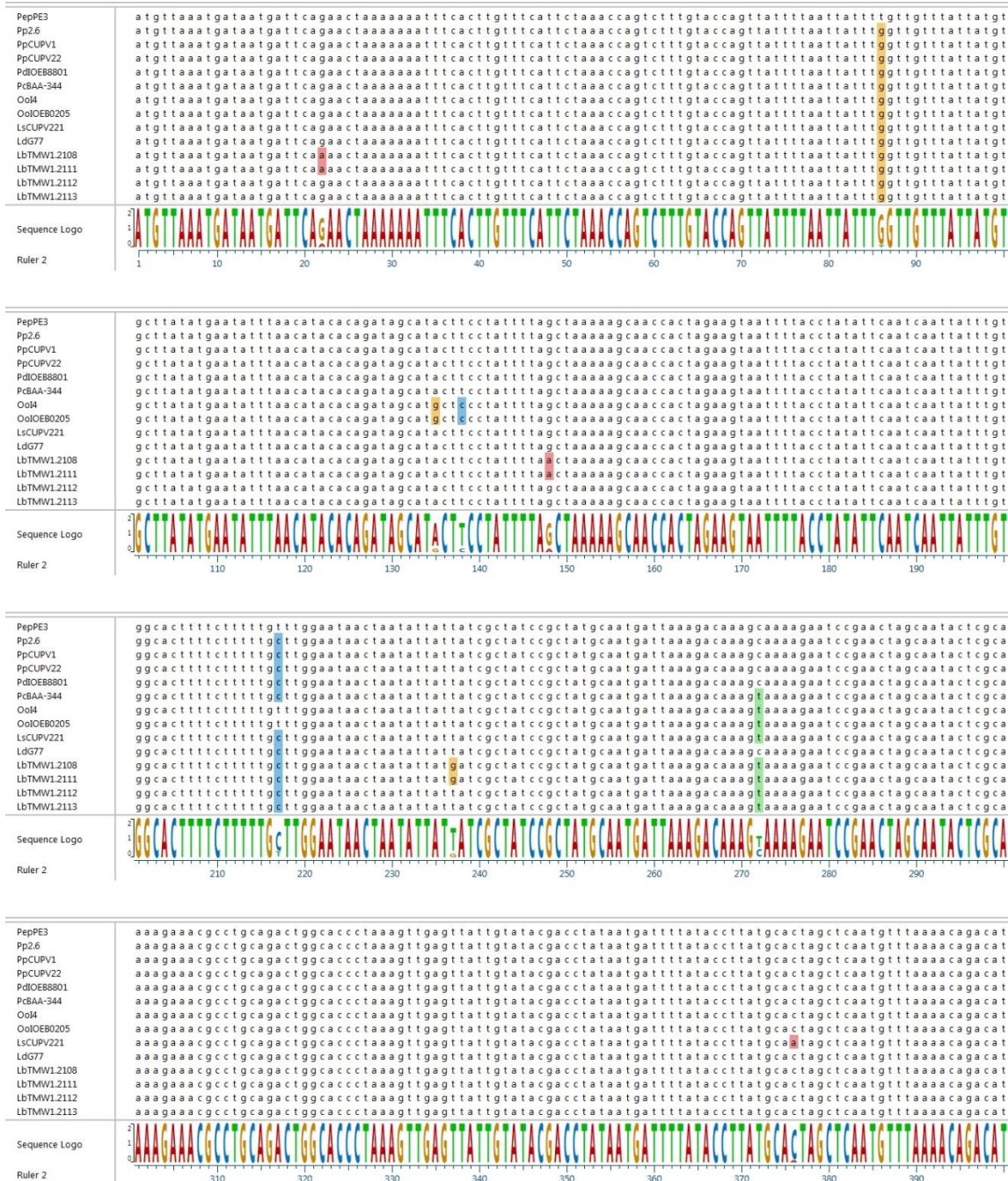
2.10. Amino acid sequences of *L. acidophilus* NBRC 13591 10- and 13-hydratase

>AHW98239.1 LINOLEATE 13-HYDRATASE
MHYSSGNYEAFVNASPKDQKSAIYLVGSLASLASAVFLIRDGHMKGDRIHILEELSLPGGSMGDIYN
KQKESYIIRGGREMEAHFECLWDLFRSIPSAENKDESVLDEFYRLNRKDP SFAKTRVIVNRGHELPTDGQ
LLLTTPKAVKEIIDLCLTPEKDLQNKINEVFSKEFFESNFWLYWSTMFAFEPWASAMEMRRYLMRFVQHV
STLKNLSSLRF TKYNQYESLILPMVKYLKDRGVQFHYNTVVDNIFVNRSNGEKIAKQIILLTENGEKSID
LTENDLVFVTNGSITESTTYGDNLHPASEEHKLGATWKLWQNLAAQDDDFGHPDVFC KDIPKANWVMSAT
ITFKNNDIVPFIEAVNKKDPHSGSIVTSGPTTIKDSNWLLGYSISRQPHFEAQKPNELIVWLYGLFSDTK
GNYVEKTMPCNGIELCEEWLYHMGVPEERIPEMASAATTI PAHMPYITSYFMPRALGDRPKVVPDHSKN
LAFIGNFAETPRDVTFTTEYSVRTAMEAVYTLNIDRGVPEVFASAFDVRMLMNAMYLLNDQKKLEDL DL
PIAEKLAIKGMLKVKGTYIEELLKKYKLV

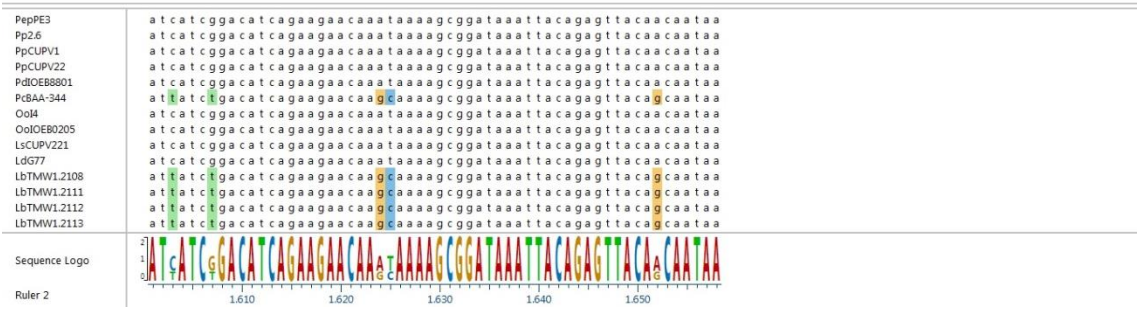
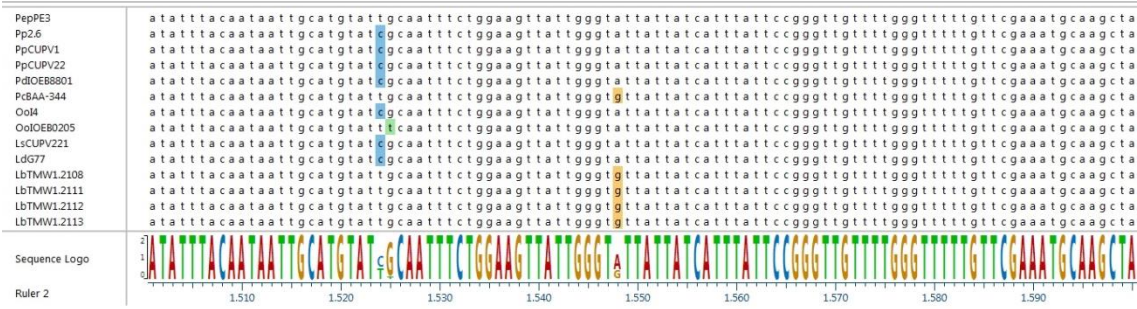
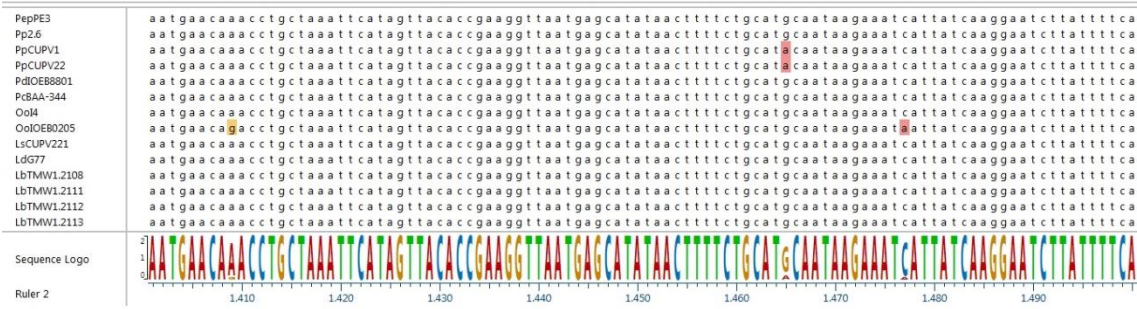
>AHW98240.1 10-HYDRATASE
MYSSNGNYEAFADPKKPAAGVDKKSAYIIGSGLAGLSTAVFLVRDAQMKGENIHILEELPVAGGSLDGADR
PNAGFVVRGGREMEAHFECLWDMYRSIPSEVPGASYLDEYYWLDKEDPNSSNCR LIYNRGDRLP SDGQY
GLGKCANEIVKLIMTPEKEIEGQTIIEEFFSDEFFKTNFWTYWSTMFAFEKWHS LAEMRRYAMRFIHHIDG
LPDFTALKFNKYNQYESMVKPLLAYLKD HGVQFEYDCHVKNVEVDHEGDSKIAKKIVMTQNGKDK EIDL T
HNDIVFVTNGSITESSTYGDQNT PAPITNAKGDSWKLWENLAKQDPAFGHPDVFCENLPERSWVVSATAT
LENKKLAPYFERLTKRSLYDGKVTGGIITIVDSNWELSFTHRQPHFKSQNPDI VVWIYALYS DTEGN
YIKKRIVDCTGKEIAEELLYHLGVPESQISELASEENMNTVPVYMPYITSYFMPRRDGRPDVVPEGSIN
LAFIGNFAESPTRDVTFTTEYSVRTAMEAVYTLNVDRGVPEVFD SIYDIRQLLRAMYMSDKKKLADQD
MPLPEKLAVKTGMRKIKKTWVEELLKEANLV

APPENDIX 3. ALIGNMENTS

3.1. Multiple alignment of the nucleotide sequences of the *gtf* genes from LAB. Coloured residues are those differing from the equivalents in the reference *P. ethanolidurans* CUPV141 *gtf* gene.

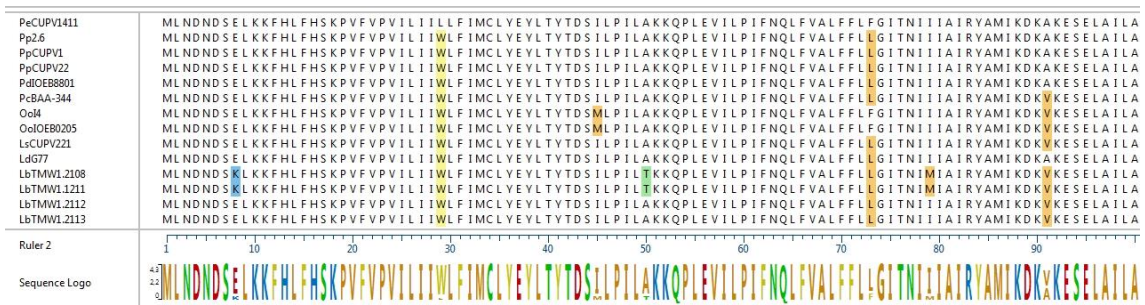


Alignments



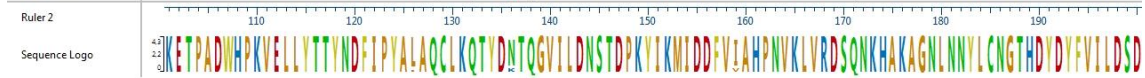
3.2. Multiple alignment of the amino acids sequences of the GTF glycosyltransferase from LAB.

Coloured residues are those differing from the equivalents in the reference P. ethanolidurans CUPV141 GTF enzyme.

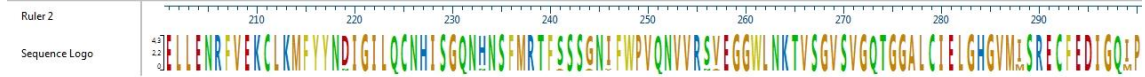


Alignments

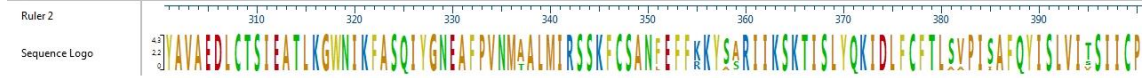
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Pp2.6	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
PpCUPV1	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
PpCUPV22	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
PdIOE88801	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
PcBAA-344	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
Oo4	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
OoIE080205	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LsCUPV221	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LdG77	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LbTMW1.2108	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LbTMW1.1211	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LbTMW1.2112	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS
LbTMW1.2113	KETPADWHPKVELLYTYYNDFIPYALACLKQTYDNTQGVILDNSTDPKYIKMIDDFVIAHPNVKLVDRSQNKHAKAGLNLLYLCNGTHDYDVFVILDS



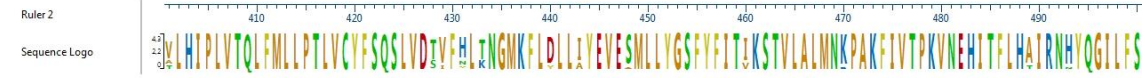
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PpCUPV1	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
PpCUPV22	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
PdIOE88801	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
PcBAA-344	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
Oo4	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
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LsCUPV221	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
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LbTMW1.2108	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
LbTMW1.1211	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
LbTMW1.2112	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP
LbTMW1.2113	ELLENRFVEKCLKMFYYNDIGILQCNIHISGQNHNSFMRTFS5SGNIFWVQNVVRSVEGGWLNKTVSGVSVGQTGGALCIELGHGVMISRECFEDIQIP



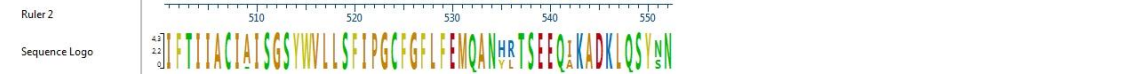
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Pp2.6	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
PpCUPV1	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
PpCUPV22	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
PdIOE88801	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
PcBAA-344	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
Oo4	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
OoIE080205	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
LsCUPV221	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
LdG77	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
LbTMW1.2108	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
LbTMW1.1211	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
LbTMW1.2112	YVAEEDLCTSIEATLKGWNIKFASQIYGENEAFVNNMAALMIRSSKFCANFEFFKYSARIKSKTISLYQKIDLFCFTLSVPISAFQYISLVTISIIICP
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Oo4	VLIHPLVTQLFMLPRTLVCYFSQSLVDTVFHLTNGMKFLDLLIYVESMLLYGFSFYFITIKSTVLALMKNKPAKIVTPKVNEHITFLHAIIRNHYQGILFS
OoIE080205	VLIHPLVTQLFMLPRTLVCYFSQSLVDTVFHLTNGMKFLDLLIYVESMLLYGFSFYFITIKSTVLALMKNKPAKIVTPKVNEHITFLHAIIRNHYQGILFS
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LbTMW1.1211	VLIHPLVTQLFMLPRTLVCYFSQSLVDTVFHLTNGMKFLDLLIYVESMLLYGFSFYFITIKSTVLALMKNKPAKIVTPKVNEHITFLHAIIRNHYQGILFS
LbTMW1.2112	VLIHPLVTQLFMLPRTLVCYFSQSLVDTVFHLTNGMKFLDLLIYVESMLLYGFSFYFITIKSTVLALMKNKPAKIVTPKVNEHITFLHAIIRNHYQGILFS
LbTMW1.2113	VLIHPLVTQLFMLPRTLVCYFSQSLVDTVFHLTNGMKFLDLLIYVESMLLYGFSFYFITIKSTVLALMKNKPAKIVTPKVNEHITFLHAIIRNHYQGILFS



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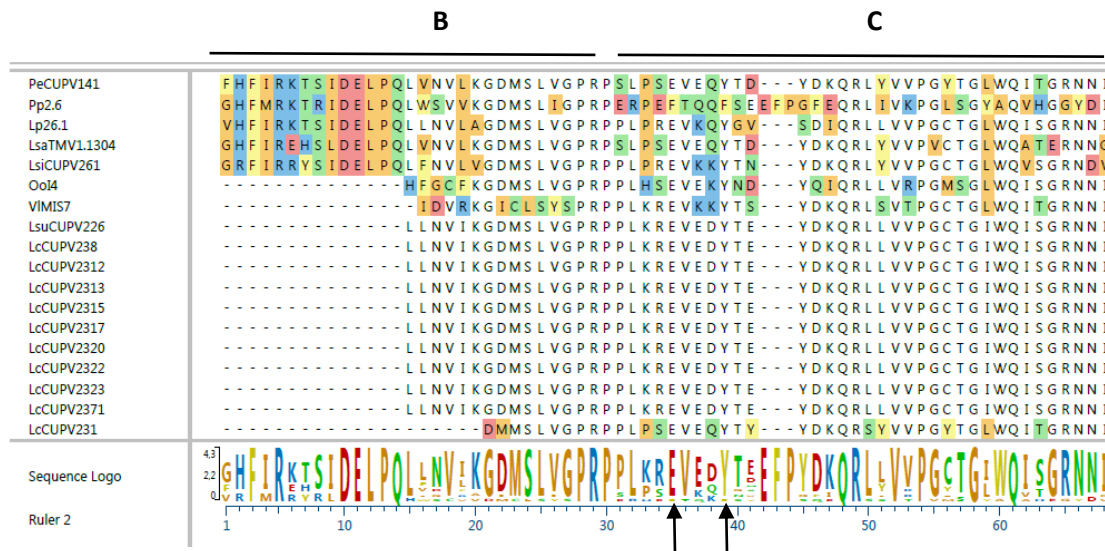
Alignments

3.3. Multiple alignment of the nucleotide sequences of the *p-gtf* gene from LAB.

Coloured residues are those differing from the consensus nucleotidic sequence, which was generated with the nucleotides present in at least five *p-gtf* genes.



3.4. Multiple alignment of the amino acids sequences of the p-GTF from LAB. The consensus sequence corresponds to the amino acids present in at least five polypeptides. Coloured residues are those differing from the consensus amino acid sequence. Black arrows indicate the glutamate (E) and tyrosine (Y) residues, which could be involved in catalysis.



3.5. Multiple sequence alignment of the amino acid sequences of hydratases from LAB lacking *L. sicerae* CUPV261^T strain.

Asterisks (*) indicate positions which have a single, fully conserved residue; Colons (:) indicate conservation between groups of strongly similar properties; periods (.) indicate conservation between groups of weakly similar properties.

```

AHW98239.1  MHYSSGNYEAFVNASPKDVKDQKSAYLVGSGLASLASAVFLIRDGHMKGDRIHILEELSL 60
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2.6        -----MAKAYMIGSGIGINLAAGIYLIRDGGWSGDQITMFGLEK- 38
BAL42246.1  -----MGALFMVKSKAIMIGAGLSNMAAAVYLIQDGHWDGKDIITFYGVDM- 45
CUPV237     -----MVKRKAIMIGAGLANMAGAVYLIQEAHWKGEQITFYSIDD- 40
          . *  : : * : * : . . . : . : * : . . . . . * . * :

AHW98239.1  PGGSMDCI-----YKQKESYIIRGGREME-AHFCELDWDLFRSIPSAENKD 105
AHW98240.1  AGGSLDGA-----D-RPNAGFVVRGGREME-NHFCELDWDMYRSIPSEVPG 104
2.6        -HGANDGAKVADYSEYGNP-ELSNKGFSLAKGGGRMLNEETYENLWDVLRVSLDNP 95
BAL42246.1  -HGANDGGATTDFTEYWNKNHPMANTTGYVARGGRMLNRYTYVDLMDLLDRIPSVTEPG 104
CUPV237     -HGSNDGSTATSAKGEYWNTPVENHTGFVARGGRMLNRYTYVDLMDLMGRIPSATEKN 99
          * : **          . : : * * * : : : * * : : * * :

AHW98239.1  ESVLDEFYRLNRKDPFAKTRVIVNRGHELPTDG-----QLLLTPKAVKEIIDLCLTPE 159
AHW98240.1  ASYLDEYYWLDKEDPNSSNCRILIYNRGDRLPDSDG-----QYGLG-KCANEIVKLIMTPE 157
2.6        QSVDDILNFDHAHPTHDVARLMD-RTDGI RNKGDQKDYNHMQFNQDRFLLTKLMMMP 154
BAL42246.1  MTAEDTRDFDAKHRTYDIARLMQ-GGKGI INAG-----KLGFNKDRFTLLTKLIMPD 157
CUPV237     MTAEDTHDFDAHRTFDKARLLQ-GGVGIVNGS-----HLGLNKNDRSLLTKLILMPD 152
          : : : : : . . : : : : : : : : : : : : : : : : * :

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Alignments

```
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AHW98240.1 K---EIEGQTIIEFFSD-EFFKTNFWTYWSTMFAFEKWHSLAEMRRYAMRFIHHIDGLP 212
2.6 SKPEQLNDVSIQWFAXSPHIFTNFWYMWQTTFAFKKESAMELRRYMNRMILEFSRIN 214
BAL42246.1 SEETKLDNVISIAEYFKDDPHMFQTNFWYMWETTFAFRVQSSAQELRRYMHQMIYEFTQIE 217
CUPV237 KEETRLDNVTIAEYFKDDPHMFQTNFWYMWETTFAFRVQSSAQELRRYMHQMIYEFTQIE 212
. :. : * : * . : * : * * * . * * * . * * * * : : . . :

AHW98239.1 NLSSLRFTKYNQYESLILPMVKYLKDRGVQFHYNTVVDNIFVNR-SNGEKIAK-QIL--- 269
AHW98240.1 DFTALKFNKYNQYESMVKPLLAYLKDGHVQFEYDCHVKNVEVDH-EGDSKIAK-KIV--- 267
2.6 TLAGVTRTPYNQYESIILPMRKYLTDHGVNFVNNRKITFVFKDTPLRDDIIVTGLKYEE 274
BAL42246.1 HLVGVNRTRYNQFESMILPLIKYLQGGVTFIDNKIVKDWQFKDTPMQDEITVTGLVIED 277
CUPV237 HLVGVNRTRYNQYESMMLPLINYLKEQGCKIVLNRRTDFTFKDTALQDEITVTGLEMEN 272
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AHW98239.1 LTENGEKKSIDL TENDLVFVTNGSITESTYGDNLHPASEEHKLGATWKLWQNLAQDDD 329
AHW98240.1 MTQNGKDKIEDLTHNDIVFVTNGSITESSTYGDQNTPAPITNAKGDSWKLWENLAKQDPA 327
2.6 VDNDNKPGEIEIGENDFVFDNGAITDSSSIGDLNTPIKENMEYAPSAAALWKQATEHFYN 334
BAL42246.1 AQT-GETEEVEVDEDTAVIFVTNGSITDSATMGDYNTPAPENMDYGVSAALWKKATERFYN 336
CUPV237 TET-NETETVAVDDDTAVFVTNGSITDSATLGDFTNTPASENMDYGAASLWKKATEHFYN 331
: : : : : * : * * * * * : * * * * : : : : . . . * :

AHW98239.1 FGHDPVFCCKDIPKANWMSATITFKNNDIVPFIEAVNKKDPHSG---SIVTSGPTTIKDS 386
AHW98240.1 FGHDPVFCENLPERSWFVSATATLENKKLABYFERLTKRSLYDG---KVNTGGIITIVDS 384
2.6 LGNPDKFFADRKQSEWL-SFTVTTNNHFLNEISRITQQEPGNALNTWVDSN----- 385
BAL42246.1 LGTDPKFFNDRNASEWV-SFTLTTKNHFLNEIVRITTTQEPGNALNSFLSTPITPLNQK 395
CUPV237 LGNPDKFFADRNASEWS-SFTLTTKNHLLNEITRITTTQLPGNALNSFLSTKPIITPLGQK 390
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AHW98239.1 NWLLGYSISRQPHFEAQKPNELIVWLYGLFSDTKGNYVEKTMPCNGIELCEEWLYHMGV 446
AHW98240.1 NWELSFTIHRQPHFKSQNDQIVVWVIALYSDTEGNYIKKRIVDCTGKEIAEELLYHLGV 444
2.6 -NLMSIVVHHQPHFHVQKENETVFWGYVMYPRRKGDYVDKPFIEMTGKEMLEELLGHLAA 444
BAL42246.1 DVNMSIVVHHQPHFTTQQPNETVLWGYFLYPRRQGEFVNKPKYIKMTGKEMAQELIGQLSK 455
CUPV237 DVNMSIVVHHQPHFTTQRPNESVIWGYFLYPRRQGEFIEKPYIEMTGKEMAELIGQLSK 450
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AHW98239.1 PEERIPEMAS-----A-ATTIPAHMPYITSYFMPRALGDRPKVVPDHSKNLAFIGNFA 498
AHW98240.1 PESQISELAS-----EENMNTVPVYMPYITSYFMPRRDGRPDVPEGSINLAFIGNFA 498
2.6 VDPARDNIADHTEEMDSIVNVI PAYMPYASALFNRRRAVGDRAVVPKNSKNLAFITQFA 504
BAL42246.1 VDPGPGNIKDKKEKENLDSIVNNI PVYMPYASALFNRAKSDRPEVLPKHSNLAFTGEFA 515
CUPV237 VDPGPTNIMDKKDEIMDSIINNVPVYMPYASALFNRAKTDREVI PAHSTNLAFTGEFA 510
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AHW98239.1 ETP-RDVTFTTEYSVRTAMEAVYTLNIDRGVPEVFASAFDVRMLMNAMYLLNDQKKLED 557
AHW98240.1 ESPTRDVTFTTEYSVRTAMEAVYTLNIDRGVPEVFDSIYDIRQLLRAMYMSDKKLLAD 558
2.6 EMP-FDMVFTEQYSFRCAQVAVYHFMGIPDSE-----LTPLHHYEKQPKVLAR 551
BAL42246.1 EQP-YQMI FTEQS AVRSGEIAAYHFAGVPMDN-----LVKTPRYDKDKPKTLK 562
CUPV237 EQP-YQMV FTEQS ATRSGEIAAYHFTGISMNN-----LVDTPRYDKDKMKT LAK 557
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AHW98239.1 LDLPIAEKLAIKGMLKKVKGYIEELLKKYKLV 590
AHW98240.1 QDMPLPEKLAVKTGMRKIKKTWVEELLKEANLV 591
2.6 A-----TKTMFR----- 558
BAL42246.1 A-----TKKMF----- 569
CUPV237 A-----TKKLF----- 564
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APPENDIX 4. PUBLISHED WORK

Title of the book: 'Emerging trends and developments in beverage science'; Volume XII: The science of beverages; Chapter X: Functional beverages produced by lactic acid bacteria. In press.

Editor: Mihai Grumezescu, Alexandru

Editorial: Elsevier

Year: 2018

CHAPTER: Functional beverages produced by lactic acid bacteria

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Abstract

Currently, in developed countries there is a concern about leading a healthy life style including eating 'organic' and 'functional' food. Moreover, in recent years, the percentage of the population with food allergies or intolerances has increased. Within this framework, lactic acid bacteria (LAB), which are able to ferment a variety of foodstuffs and also colonize the human digestive tract, can play a beneficial role. Many LAB strains have the Generally Regarded As Safe (GRAS) status and are therefore being used in the food industry, especially to produce different types of fermented beverages, because they confer 'functionality' to drinks due to the production of bioactive compounds.

Keywords: beverages, CLA, exopolysaccharides, functional food, lactic acid bacteria, phytate, vitamins.

Heteropolysaccharide-producing bifidobacteria for the development of functional dairy products (Submitted to LWT-Food Science and Technology)

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Abstract

Bifidobacterium longum INIA P132 and *Bifidobacterium infantis* INIA P731, isolated from infant-faeces, were investigated in this work. Their probiotic and technological potential was evaluated, and both were resistant to gastrointestinal tract-simulated conditions. *B. longum* showed high survival upon lyophilisation, after freezing and thawing, and was able to grow in milk. *B. infantis* had higher adhesion capacity to human Caco-2 cells than the commercial probiotic *B. animalis* BB12 strain. Moreover, both bacteria secreted heteropolysaccharides (HePS) composed of rhamnose, galactose and glucose. The treatment with each of these HePS of zebrafish larvae with induced enterocolitis caused a decrease in mortality. In addition, HePS from *B. longum* immunomodulated *in vitro* human macrophages treated with the inflammatory *Escherichia coli* O111:B4 lipopolysaccharide. Thus, both bifidobacteria and their HePS have potential beneficial effects on health and thus, could be applied in functional foods.

Keywords: Bifidobacteria; exopolysaccharide; immunomodulation; zebrafish; technological properties.

Characterization of dextrans produced by *Lactobacillus mali* CUPV271 and *Leuconostoc carnosum* CUPV411 (Submitted to Food Hydrocolloids)

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Abstract

The exopolysaccharide (EPS)-producing *L. mali* CUPV271 and *Lc. carnosum* CUPV411 were isolated from Spanish rosy apple must and slimy ham, respectively. The polymers were purified from bacterial cultures' supernatants and subjected to physicochemical and rheological characterization with the aim to evaluate their potential for future industrial utilization. Methylation analysis, Fourier-Transform Infrared Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) revealed that both polymers were dextrans, partially branched at *O*-3 and *O*-4 positions of the main α -(1 \rightarrow 6)-D-glucopyranose backbone. The molar masses of the EPS of *L. mali* and *Lc. carnosum*, were of 1.23×10^8 g/mol and 3.58×10^8 g/mol, respectively. The bacterial strains were tested for binding to the human Caco-2 cell line in the presence and absence of their respective dextran, revealing that the EPS production by *L. mali* decreased the binding capacity of the bacterium while the adhesiveness of *Lc. carnosum* did not change. As the structure and molecular mass of both dextrans were comparable, other characteristics of the dextrans were studied to explain this behaviour. Atomic force micrographs showed some differences at the supramolecular level, suggesting that the different spatial distribution of the dextrans might be on the basis of the results of the adhesion studies. Both polysaccharides resulted to be amorphous materials with T_g around 226 °C, and showed slightly different thermal degradation patterns. Rheologically, they showed to have a pseudoplastic behaviour, but very different critical concentrations: 3.8% for the EPS of *L. mali* and 0.4% for that of *Lc. carnosum*.

Keywords: dextran, *Lactobacillus*, *Leuconostoc*, characterization, adhesion.



Characterization of *Pediococcus ethanolidurans* CUPV141: A β -D-glucan- and Heteropolysaccharide-Producing Bacterium

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OPEN ACCESS

Edited by:

Vittorio Capozzi,
University of Foggia, Italy

Reviewed by:

Maria Dimopoulou,
Aristotle University of Thessaloniki,
Greece

Claudio Hidalgo Cantabrana,
North Carolina State University,
United States

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 16 June 2018

Accepted: 13 August 2018

Published: 04 September 2018

Citation:

Llamas-Arriba MG, Pérez-Ramos A,
Puertas AI, López P, Dueñas MT and
Prieto A (2018) Characterization
of *Pediococcus ethanolidurans*
CUPV141: A β -D-glucan-
and Heteropolysaccharide-Producing
Bacterium. *Front. Microbiol.* 9:2041.
doi: 10.3389/fmicb.2018.02041

Pediococcus ethanolidurans CUPV141 is an exopolysaccharide (EPS)-producing lactic acid bacterium, first isolated from Basque Country cider (Spain). Physicochemical analysis of the EPS synthesized by the bacterium revealed that CUPV141 produces mostly a homopolysaccharide (HoPS), characterized as a 2-substituted (1,3)- β -D-glucan, together with a small quantity of a heteropolysaccharide (HePS) composed of glucose, galactose, glucosamine, and glycerol-3-phosphate, this being the first *Pediococcus* strain described to produce this kind of polymer. On the contrary, an isogenic strain CUPV141NR, generated by chemical mutagenesis of CUPV141, produced the HePS as the main extracellular polysaccharide and a barely detectable amount of 2-substituted (1,3)- β -D-glucan. This HoPS is synthesized by the transmembrane GTF glycosyltransferase (GTF), encoded by the *gtf* gene, which has been previously reported to be located in the pPP2 plasmid of the *Pediococcus parvulus* 2.6 strain. Southern blot hybridization revealed that in CUPV141 the *gtf* gene is located in a plasmid designated as pPE3, whose molecular mass (34.4 kbp) is different from that of pPP2 (24.5 kbp). Analysis of the influence of the EPS on the ability of the producing bacteria to adhere to the eukaryotic Caco-2 cells revealed higher affinity for the human enterocytes of CUPV141NR compared to that of CUPV141. This result indicates that, in contrast to the 2.6 strain, the presence of the HoPS does not potentiate the binding ability of *P. ethanolidurans*. Moreover, it supports that the phosphate-containing bacterial HePS improved the interaction between *P. ethanolidurans* and the eukaryotic cells.

Keywords: *Pediococcus ethanolidurans*, β -glucan, *gtf*, heteropolysaccharides, priming-glycosyltransferase, plasmid, adhesion

INTRODUCTION

Some lactic acid bacteria (LAB) produce exopolysaccharides (EPS), extracellular polymers that may remain tightly attached to the bacteria, constituting a capsule, or may be released to the environment (De Vuyst et al., 2001). These polymers often possess useful properties, such as improvement of the rheological properties of food and even beneficial effects for health as



Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Probiotic properties and stress response of thermotolerant lactic acid bacteria isolated from cooked meat products

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ARTICLE INFO

Keywords:

Lactic acid bacteria
Thermotolerant
Probiotic properties
Adhesion

ABSTRACT

The aim of this study was to evaluate the probiotic properties of six thermotolerant lactic acid bacteria isolated from cooked meat products. The bacteria were typed, by determination of the DNA sequence of their 16S rRNA coding genes, as one *Enterococcus faecium* (UAM1 strain) and five *Pediococcus pentosaceus* (UAM2–UAM6 strains). Under gastric stress conditions the viability of the *Pediococci* decreased more than five-fold, whereas *E. faecium* showed a high resistance (61% survival). Exposure to small intestine stress did not drastically affect the survival of any of the strains (less than one-fold decrease), which were able to grow in the presence of 0.3% bile. A hydrophilic surface profile was observed, with higher affinity for chloroform than for xylene. Strains showed high levels of auto-aggregation as well as co-aggregation with Gram-positive and Gram-negative bacterial pathogens. The adherence of *E. faecium* UAM1 to human Caco-2 cells (around 20%) was significantly higher than that obtained with the *P. pentosaceus* strains (2%–5%) and *Lactobacillus acidophilus* LA-5 (6%). The overall results indicate that *E. faecium* UAM1, has probiotic properties that predict its capability to colonize in competition with pathogens in the intestinal tract. This bacterium deserves further investigation for its potential as a component of functional food.

1. Introduction

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO & WHO, 2001). The majority of probiotics are bacteria, with lactic acid bacteria (LAB) being the most representative, and are used for the manufacture of fermented dairy, meat and vegetable-based foods. Probiotic strains include members of the genera *Pediococcus*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Buntin et al., 2008).

Enterococcus is a genus used as a probiotic which may improve the microbial balance of the intestine, and is ubiquitous in nature. Pieniz et al. (2015) studied the probiotic potential and antioxidant properties of *Enterococcus durans* LAB18s, a strain capable of selenium bioaccumulation, concluding that these strains could be used as dietary selenium supplementation. Also, Rao et al. (2013) examined the adhesion of *Enterococcus faecium* *in vitro* and concluded that this strain had an effective barrier function in the small intestinal mucus layer of pigs.

Carasi et al. (2014) isolated and identified a strain of *E. durans* from kefir and their results showed the potential functionality of this bacteria as probiotic. Moreover, they indicated that the presence of *E. durans* in kefir does not represent a threat to consumer health, and shows its potential functionality as a probiotic. Li et al. (2014) identified and evaluated the probiotic properties of five *Enterococcus* strains isolated from silage, and one of those (L2) seems to be a promising candidate for future use as a probiotic in humans.

Strains belonging the genus *Pediococcus* has been tested and already used as a probiotic bacteria. Vidhyasagar and Jeevaratnam (2013) evaluated six strains of *Pediococcus pentosaceus* for probiotic properties *in vitro*. They concluded that the strains exhibited growth inhibition of intestinal Gram positive and Gram negative pathogens and could be used in functional foods as a probiotic strain. Similar results were found with *Pediococcus pentosaceus* strains isolated from fermented vegetables (Savedboworn, Riansa-ngawong, Sinlapacharoen, Pajakang, & Patcharajarukit, 2014). Also, Dubey et al. (2015) reported about

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<https://doi.org/10.1016/j.lwt.2017.12.063>

Received 9 June 2017; Received in revised form 24 October 2017; Accepted 26 December 2017

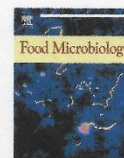
Available online 16 January 2018

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Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Impact of growth temperature on exopolysaccharide production and probiotic properties of *Lactobacillus paracasei* strains isolated from kefir grains



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ARTICLE INFO

Article history:

Received 30 March 2017

Received in revised form

15 August 2017

Accepted 18 August 2017

Available online 19 August 2017

Keywords:

Lactobacillus paracasei

Exopolysaccharide

Kefir

Probiotics

Growth temperature

ABSTRACT

EPS-producing LAB are widely used in the dairy industry since these polymers improve the viscosity and texture of the products. Besides, EPS might be responsible for several health benefits attributed to probiotic strains. However, growth conditions (culture media, temperature, pH) could modify EPS production affecting both technological and probiotic properties. In this work, the influence of growth temperature on EPS production was evaluated, as well as the consequences of these changes in the probiotic properties of the strains. All *Lactobacillus paracasei* strains used in the study showed changes in EPS production caused by growth temperature, evidenced by the appearance of a high molecular weight fraction and an increment in the total amount of produced EPS at lower temperature. Nevertheless, these changes do not affect the probiotic properties of the strains; *L. paracasei* strains grown at 20 °C, 30 °C and 37 °C were able to survive in simulated gastrointestinal conditions, to adhere to Caco-2 cells after that treatment and to modulate the epithelial innate immune response. The results suggest that selected *L. paracasei* strains are new probiotic candidates that can be used in a wide range of functional foods in which temperature could be used as a tool to improve the technological properties of the product.

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1. Introduction

The consumption of some lactic acid bacteria (LAB), in particular from the genera *Lactobacillus*, has proven to be beneficial for human health and as a consequence those strains are usually considered as probiotics that are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO-WHO, 2006). Certain LAB are able to produce extracellular polysaccharides (EPS) that can be either tightly associated with the cell surface forming a capsule, or loosely attached to the outer cell structures or secreted into the environment (Ruas-Madiedo et al., 2008). The major role of EPS is to protect the cells in the environment against toxic metals, host innate immune factors, phage attack and desiccation (Ryan et al., 2015; Zannini et al.,

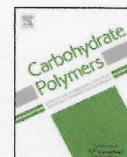
2016). Furthermore, EPS layer is thought to be involved in the protection against adverse environmental conditions of gastrointestinal tract (GIT) including low pH, bile salts, gastric and pancreatic enzymes (Ryan et al., 2015). Additionally, it has been suggested that EPS might play a role in bacterial aggregation, bio-film formation and interaction with intestinal epithelial cells (IEC) (Dertli et al., 2015; Živković et al., 2016); the last being of great importance to improve the persistence of the microorganism in the human gut and their interaction with gastrointestinal immune system and gut microbiota (Pennacchia et al., 2006). Probiotics, which are usually administered orally, must be able to survive the gastrointestinal conditions in order to reach and colonize the human gut to exert their health benefits (Amund, 2016) and the presence of EPS layer around the bacteria could imply an advantage for that purpose. In addition, LAB's EPS might be responsible for several health benefits attributed to probiotic strains. A remarkable feature that certain EPS present is their capacity to modulate the host's immune response either stimulating it in order to improve

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<http://dx.doi.org/10.1016/j.fm.2017.08.012>

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Rheology and bioactivity of high molecular weight dextrans synthesised by lactic acid bacteria



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ARTICLE INFO

Article history:

Received 31 December 2016

Received in revised form 24 June 2017

Accepted 28 June 2017

Available online 29 June 2017

Keywords:

Exopolysaccharides

Dextran

Lactic acid bacteria

Leuconostoc mesenteroides

Rheological properties

Immunomodulation

Adhesion

ABSTRACT

Dextrans synthesised by three *Leuconostoc mesenteroides* strains, isolated from mammalian milks, were studied and compared with dextrans produced by *Lc. mesenteroides* and *Lactobacillus sakei* strains isolated from meat products. Size exclusion chromatography coupled with multiangle laser light scattering detection analysis demonstrated that the dextrans have molecular masses between 1.74×10^8 Da and 4.41×10^8 Da. Rheological analysis of aqueous solutions of the polymer revealed that all had a pseudo-plastic behaviour under shear conditions and a random, and flexible, coil structure. The dextrans showed at shear zero a difference in viscosity, which increased as the concentration increased. Also, the purified dextrans were able to immunomodulate *in vitro* human macrophages, partially counteracting the inflammatory effect of *Escherichia coli* O111:B4 lipopolysaccharide.

During prolonged incubation on a solid medium containing sucrose, dextran-producing bacteria showed two distinct phenotypes not related to the genus or species to which they belonged. Colonies of *Lc. mesenteroides* CM9 from milk and *Lb. sakei* MN1 from meat formed stable and compact mucoid colonies, whereas the colonies of the other three *Leuconostoc* strains became diffuse after 72 h. This differential behaviour was also observed in the ability of the corresponding strains to bind to Caco-2 cells. Strains forming compact mucoid colonies showed a high level of adhesion when grown in the presence of glucose, which decreased in the presence of sucrose (the condition required for dextran synthesis). However no influence of the carbon source was detected for the adhesion ability of the other *Lc. mesenteroides* strains, which showed variable levels of binding to the enterocytes.

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Abbreviations: C*, critical concentration for interaction between dextran molecules; CDMS, CDM define medium lacking glucose and supplemented with 0.8% sucrose; cfu, colony forming unit; CM9, CM30, SM34 and RTF10, *Leuconostoc mesenteroides* strains; DMEM, Dulbecco's Modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; EPS, exopolysaccharide; $\dot{\gamma}$, shear rate; HePS, heteropolysaccharides; HoPS, homopolysaccharides; HPLC, high-performance liquid chromatography; LAB, lactic acid bacteria; LPS, lipopolysaccharide from *Escherichia coli* O111:B4; MRSG, Man Rogosa Sharpe broth containing 2% glucose; MRSS, MRS medium containing 2% sucrose instead of glucose; MSE, Mayeux Sandine Elliker medium; η_0 , viscosity at near-zero shear rate; η , apparent viscosity; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SEC-MALLS, size exclusion chromatography coupled to multiangle laser light scattering detection; PMA-THP-1, THP-1 monocytes differentiated to macrophages with PMA; TEM, transmission electron microscopy.

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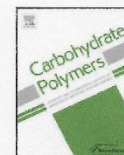
<http://dx.doi.org/10.1016/j.carbpol.2017.06.113>

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Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Lactobacillus plantarum CIDCA 8327: An α -glucan producing-strain isolated from kefir grains



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ARTICLE INFO

Article history:

Received 16 January 2017

Received in revised form 7 April 2017

Accepted 19 April 2017

Available online 23 April 2017

Keywords:

Lactobacillus plantarum

Kefir

Exopolysaccharides

Glucans

ABSTRACT

Lactobacillus plantarum CIDCA 8327 is an exopolysaccharide (EPS)-producer strain isolated from kefir with promising properties for the development of functional foods. The aim of the present study was to characterize the structure of the EPS synthesized by this strain grown in skim milk or semidefined medium (SDM). Additionally, genes involved in EPS synthesis were detected by PCR. *L. plantarum* produces an EPS with a molecular weight of 10^4 Da in both media. When grown in SDM produce an heteropolysaccharide composed mainly of glucose, glucosamine and rhamnose meanwhile the EPS produced in milk was composed exclusively of glucose indicating the influence of the sugar source. FTIR spectra of this EPS showed signals attributable to an α -glucan. Both by ^1H NMR and methylation analysis it was possible to determine that this polysaccharide is a branched α -(1 \rightarrow 4)-D-glucan composed of 80% linear α -(1 \rightarrow 4)-D-glucopyranosyl units and 19% (1 \rightarrow 4)-D-glucopyranosyl units substituted at O-3 by single α -D-glucopyranosil residues.

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1. Introduction

Among the “Food-Grade” biopolymers obtained from natural sources, exopolysaccharides (EPS) synthesized by lactic acid bacteria (LAB) have focused the attention of researchers and manufacturers since these EPS contribute to the rheology of the fermented product and –on account of EPS potential health promoting properties– may also contribute to the development of functional foods (Das, Baruah, & Goyal, 2014; Patten & Laws, 2015).

EPS produced by LAB present a wide range of compositions, structures, molecular masses and conformations depending on the

strain. The EPS can stay attached to the cell surface (capsular) or can be released to the culture media (Patten & Laws, 2015). High molecular weight polysaccharides are widely used in the food industry as stabilizers, emulsifiers, and to improve texture and viscosity. The functionality of these polymers is originated from the structural differences in the sugar subunits, which is also the reason of the great diversity among bacterial EPS and novel EPS structures among LAB (Mozzi et al., 2006; Patten & Laws, 2015). Complex genetic mechanisms of EPS production, carbohydrate source, incubation temperature and time, or pH of the culture medium were reported to affect *in situ* EPS production levels as well as their conformational characteristics, sugar linkages, and molecular mass (Ibarburu et al., 2015).

Many EPS synthesized by LAB have demonstrated to elicit some biological effect (Patten & Laws, 2015). It has been reported that some EPS can have immunomodulatory (Hidalgo-Cantabrana et al., 2012; Medrano, Racedo, Rolny, Abraham, & Pérez, 2011; Notararigo et al., 2014) and antitumoral activity *in vivo* (Wang et al., 2014), as

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<http://dx.doi.org/10.1016/j.carbpol.2017.04.053>

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Draft Genome Sequence of *Lactobacillus collinoides* CUPV237, an Exopolysaccharide and Riboflavin Producer Isolated from Cider

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***Lactobacillus collinoides* CUPV237 is a strain isolated from a Basque cider. *Lactobacillus collinoides* is one of the most frequent species found in cider from Spain, France, or England. A notable feature of the *L. collinoides* CUPV237 strain is its ability to produce exopolysaccharides.**

Received 29 April 2016 Accepted 2 May 2016 Published 9 June 2016

Citation Puertas AI, Capozzi V, Llamas MG, López P, Lamontanara A, Orrù L, Russo P, Spano G, Dueñas MT. 2016. Draft genome sequence of *Lactobacillus collinoides* CUPV237, an exopolysaccharide and riboflavin producer isolated from cider. *Genome Announc* 4(3):e00506-16. doi:10.1128/genomeA.00506-16.

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Lactobacillus collinoides CUPV237 is a strain isolated from a Basque cider at the Department of Applied Chemistry, Faculty of Chemistry (University of the Basque Country UPV/EHU, San Sebastián, Spain). In the Basque Country (Northern Spain), natural cider is produced in small factories using traditional techniques, normally using juices obtained from many varieties of fresh cider apples (bitter, bittersweet, and sweet) to obtain a balanced content of sugars, acids, and polyphenols, without the addition of extra sugar or CO₂. Alcoholic and malolactic fermentations occur spontaneously due to the indigenous yeasts and lactic acid bacteria present in the musts (1–3). The microbial decarboxylation of L-malic acid into L-lactic acid is of great importance from an organoleptic point of view, and most of the lactic acid bacteria isolated from cider have this capacity (1, 4–7). *Lactobacillus collinoides* is one of the most frequent species found in cider from Spain, France, or England (5, 8–10). A notable feature of the *L. collinoides* CUPV237 strain is its ability to produce exopolysaccharides.

Using the TruSeq DNA sample prep kit FC-121-1001 according to the manufacturer's instructions, 2 μg of genomic DNA of *L. collinoides* was subjected to library preparation. Whole-genome sequencing was performed using the Illumina GAIIx platform at the CREA Genomics Research Centre (Fiorenzuola d'Arda). A total of 8,107,203 paired-end reads ranging from 90 to 115 bp in length were *de novo* assembled using CLC Genomics Workbench v7.0. The assembly resulted in 127 contigs with an N₅₀ length of 70,285 bp. The shortest contig was 203 bp and the longest was 243,224 bp. The draft genome consists of 3,707,616 bp. The genome sequence was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok).

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number JYDC00000000. The version described in this paper is version JYDC01000000.

ACKNOWLEDGMENTS

This study was supported by grants from the Spanish Ministry of Science and Innovation (AGL2012-40084-C03 and AGL2015-65010-C3-1-R) and the Basque Government (IT866-13). V.C. was supported by a grant from the Apulian Region in the framework of the "FutureInResearch" program (practice code 9OJ4W81).

FUNDING INFORMATION

This work, including the efforts of Ana Isabel Puertas, was funded by Spanish Ministry of Science and Innovation (AGL2012-40084-C03 and AGL2015-65010-C3-1-R). This work, including the efforts of Ana Isabel Puertas, was funded by Basque Government (IT866-13). This work, including the efforts of Vittorio Capozzi, was funded by the Apulian Region in the framework of "FutureInResearch" program (practice code 9OJ4W81).

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Lactic acid bacteria and bifidobacteria are able to produce different bioactive compounds. They synthesise exopolysaccharides (EPS), which sometimes occur in beverages or cooked ham, spoiling them. However, these EPS are very useful in the food industry, as thickeners or stabilisers. In addition, they have been reported to exert beneficial effects on human health. Therefore, this work focuses mainly on the isolation of EPS from different origins to characterise them structurally and functionally. Moreover, the production of phytases and conjugated linoleic acid *in situ* gives to fermented food an added value. Thus, the screening of a large number of lactic acid bacteria from the culture collection of the University of the Basque Country, for the production of these two components, was also performed in this work.